www.biodicon.com

Biological Diversity and Conservation

e -ISSN 1308-8084, ISSN 1308-5301

Research article
https://doi:10.46309/biodicon.2026.2013391
19/4 (2026) 368-381

Development, Optimization, and Validation of a DNA Barcoding–Based Multiplex PCR System for Molecular Characterization of *Xanthium strumarium* and *Xanthium spinosum*

İrem CEYHAN[1], Nurdan SANDIKÇI[1,2], Tuğba ÜNSAL SAPAN[* 1,2]
ORCID: 0009-0006-0582-5265**;** 0000-0001-5393-2969**;** 0000-0003-4744-2298

[1] Üsküdar University, Institute of Addiction and Forensic Sciences, 34662, İstanbul, Türkiye
[2] Üsküdar University, Faculty of Engineering and Natural Sciences, Department of Forensic Sciences 34662, İstanbul, Türkiye

Abstract

Purpose: Accurate species identification is essential for the assessment of plant diversity, taxonomic classification, and the reliable evaluation of plant-derived materials. Molecular approaches have become increasingly important when morphological identification is limited by interspecific similarity, environmental variation, or the loss of diagnostic characteristics. The genus *Xanthium*, which is widely distributed throughout the flora of Türkiye, represents a suitable model taxon for molecular and taxonomic investigations due to its spiny, easily transferable fruits and pronounced phenotypic plasticity influenced by environmental conditions. These characteristics often limit the reliability of morphological identification and highlight the need for molecular-based approaches. The present study aimed to develop and validate a DNA barcoding based molecular identification method supported by multiplex PCR for the molecular characterization of *Xanthium strumarium* and *Xanthium spinosum*, with potential application in forensic botanical examinations.

Method: A total of 50 Xanthium samples were analyzed using an optimized multiplex PCR system targeting five barcode loci: D35, psbA–trnH, trnL–trnF, ITS-2, and trnQ–rps16. The amplified products were evaluated by capillary electrophoresis. The method was validated in terms of analytical threshold, dynamic range, sensitivity, stochastic threshold, and repeatability.

Findings: Capillary electrophoresis results revealed consistent and reproducible electrophoretic profiles among the analyzed samples, with allele size ranges spanning 66–218 bp. Method validation established an analytical threshold of 93.39 RFU, a dynamic range of 0.1–3 ng/µL, and a minimum DNA concentration of 0.5 ng/µL for full-profile recovery. Specificity testing demonstrated no amplification in the taxonomically distant species Salvia officinalis and Ephedra major.

Conclusion: The validated multiplex system provides reproducible molecular characterization of *Xanthium* samples and may contribute to DNA barcoding–based species-level assessment; however, based on the current fragment-size data, it does not permit reliable discrimination between *X. strumarium* and *X. spinosum*. Additional discriminatory markers or sequence-level analyses are required for robust species assignment.

Keywords: DNA barcoding; molecular identification; multiplex PCR; plant taxonomy; *Xanthium strumarium*; *Xanthium spinosum*

---------- * ----------

Xanthium strumarium ve *Xanthium spinosum*'un moleküler tanımlanması ve taksonomik ayrımına yönelik DNA barkodlama temelli multipleks PCR sisteminin geliştirilmesi, optimizasyonu ve validasyonu

Özet

Amaç: Doğru tür tanımlaması; bitki çeşitliliğinin değerlendirilmesi, taksonomik sınıflandırma ve bitki kökenli materyallerin güvenilir biçimde incelenmesi açısından temel öneme sahiptir. Morfolojik tanımlamanın türler arası

[*] *Corresponding author*: Tel.: +90216-400 2222/ 2436; Fax.: +90 216 4741256; E-mail: tugba.unsalsapan@uskudar.edu.tr
All published content is licensed under **CC BY 4.0**; Received: 07.08.2026; Published: 15.12.2026; BioDiCon.ID: 1247-070826

benzerlik, çevresel değişkenlik veya ayırt edici özelliklerin kaybı nedeniyle sınırlı kaldığı durumlarda moleküler yaklaşımlar giderek daha fazla önem kazanmaktadır. Türkiye florasında yaygın olarak bulunan *Xanthium* cinsi; dikenli ve kolay taşınabilir meyveleri ile çevresel koşullardan etkilenen belirgin fenotipik plastisitesi nedeniyle moleküler ve taksonomik araştırmalar açısından uygun bir model taksondur. Bu özellikler, morfolojik tanımlamanın güvenilirliğini sıklıkla sınırlandırmakta ve moleküler temelli yaklaşımlara duyulan gereksinimi ortaya koymaktadır. Bu çalışmada, *Xanthium strumarium* ve *Xanthium spinosum* türlerinin moleküler karakterizasyonuna yönelik, adli botanik incelemelerde potansiyel uygulama alanı bulunan multipleks PCR destekli DNA barkodlama temelli bir moleküler tanımlama yönteminin geliştirilmesi ve valide edilmesi amaçlanmıştır.

Metod: Toplam 50 *Xanthium* örneği, beş barkod lokusunu hedefleyen optimize edilmiş bir multipleks PCR sistemi kullanılarak analiz edilmiştir. Hedeflenen lokuslar D35, psbA–trnH, trnL–trnF, ITS-2 ve trnQ–rps16'dır. Amplifikasyon ürünleri kapiller elektroforez ile değerlendirilmiştir. Yöntem; analitik eşik, dinamik aralık, duyarlılık, tam profil elde edilmesi, tekrarlanabilirlik ve özgüllük açısından valide edilmiştir.

Bulgular: Kapiller elektroforez sonuçları, incelenen örnekler arasında tutarlı ve tekrarlanabilir elektroforetik profiller elde edildiğini ve alel büyüklük aralıklarının 66–218 bp arasında değiştiğini göstermiştir. Yöntem validasyonu sonucunda analitik eşik 93,39 RFU, dinamik aralık 0,1–3 ng/µL ve tam profil elde edilmesi için gerekli minimum DNA konsantrasyonu 0,5 ng/µL olarak belirlenmiştir. Özgüllük testlerinde, taksonomik olarak uzak türler olan *Salvia officinalis* ve *Ephedra major* örneklerinde amplifikasyon gözlenmemiştir.

Sonuç: Bulgular, önerilen DNA barkodlama ve multipleks PCR temelli yaklaşımın *Xanthium strumarium* ve *Xanthium spinosum* türlerinin karakterizasyonu için güvenilir, duyarlı ve uygulanabilir bir moleküler araç olduğunu göstermektedir. Bu yöntem, morfolojik değerlendirmeyi tamamlayabilir ve adli incelemelerde karşılaşılan bitki kökenli deliller de dahil olmak üzere morfolojik olarak benzer bitki taksonlarını konu alan moleküler tanımlama çalışmalarına katkı sağlayabilir.

Anahtar kelimeler: DNA barkodlama; moleküler tanımlama, multipleks PCR; bitki taksonomisi; *Xanthium strumarium*; *Xanthium spinosum*

1. Introduction

Accurate identification of plant species is fundamental to biodiversity assessment, taxonomic classification, ecological research, and the reliable evaluation of plant-derived materials. Traditional identification methods are largely based on morphological characteristics; however, these methods may be insufficient when diagnostic traits are absent, environmentally modified, or shared among closely related taxa. Molecular techniques have therefore become increasingly important as complementary tools for plant identification and taxonomic discrimination. Among these techniques, polymerase chain reaction (PCR)-based approaches enable genetic analyses to be performed even on limited, fragmented, or partially degraded biological materials [1,2].

The molecular analysis of non-human biological materials has also gained importance in applied fields, including forensic biology, environmental monitoring, conservation, and the investigation of illegal biological trade. In accordance with Locard's Exchange Principle, forensic biology and forensic genetics focus on determining the source of biological materials and are no longer restricted solely to human DNA. DNA derived from animals and plants is increasingly regarded as complementary evidence in a wide range of cases, including homicide, environmental crimes, and illegal trade [3]. However, in plant-derived materials, interspecific morphological similarities, environmentally driven phenotypic variation, and characteristics such as clonal reproduction may limit the reliability of classical morphological identification methods. Consequently, the application of molecular-based approaches has become essential not only in forensic botany but also in plant systematics and biodiversity-related investigations.

In this context, DNA barcoding has emerged as an effective molecular method for species-level identification using short and standardized DNA regions. Although the mitochondrial COI gene has been successfully applied as a universal barcode in animals, no single universal barcode region has been established for plants due to lower mutation rates and the complexity of plant genomes [4,5]. Therefore, plastid regions such as *rbcL*, *matK*, *trnH–psbA*, as well as the nuclear ITS region, are widely used for plant species discrimination. The CBOL Plant Working Group has recommended the *matK* + *rbcL* combination as a standard plant DNA barcode [6,7].

DNA barcoding offers significant advantages for the molecular identification and taxonomic evaluation of plant materials, particularly when specimens have lost their diagnostic morphological features, are fragmented, processed, environmentally exposed, or present only in small quantities. Moreover, this approach enables the assessment of both interspecific and intraspecific genetic variation, thereby enhancing the reliability of species identification and the interpretation of plant-derived biological materials. These advantages are particularly relevant in forensic botany, where the evidentiary value of a plant trace depends on the accurate determination of its taxonomic origin. In recent years, the evaluation of the complete plastid genome as a "super-barcode" and the use of taxon-specific plastid regions have further improved the resolution of DNA barcoding for the discrimination of closely related species [7].

The genus *Xanthium* comprises morphologically variable taxa that are widely distributed in the flora of Türkiye. Environmental conditions may substantially influence their phenotypic characteristics, complicating reliable identification based solely on morphology. In addition, the spiny and adhesive fruits of *Xanthium* species can readily attach to animals, humans, clothing, and other surfaces, facilitating their dispersal and transfer between locations. These characteristics make the genus relevant to studies of plant diversity, molecular taxonomy, and the examination of transferred botanical materials, including those encountered in forensic contexts [8].

The present study aims to develop, optimize, and validate a DNA barcoding–based multiplex PCR system for the molecular identification and taxonomic characterization of *Xanthium strumarium* and *Xanthium spinosum*. By integrating morphological identification with multilocus molecular data, the study seeks to contribute to the reliable evaluation of morphologically similar *Xanthium* taxa and to assess the potential applicability of the developed method to plant-derived materials examined in forensic botanical investigations.

2. Material and method

The experimental workflow consisted of sequential steps including sample collection and morphological identification, sample preparation, DNA extraction and quantification, primer design and multiplex panel organization, multiplex PCR amplification, capillary electrophoresis, electropherogram-based data analysis, and method validation. A schematic overview of the complete experimental design is provided in Figure 1 to summarize the analytical workflow and facilitate reproducibility of the procedure.

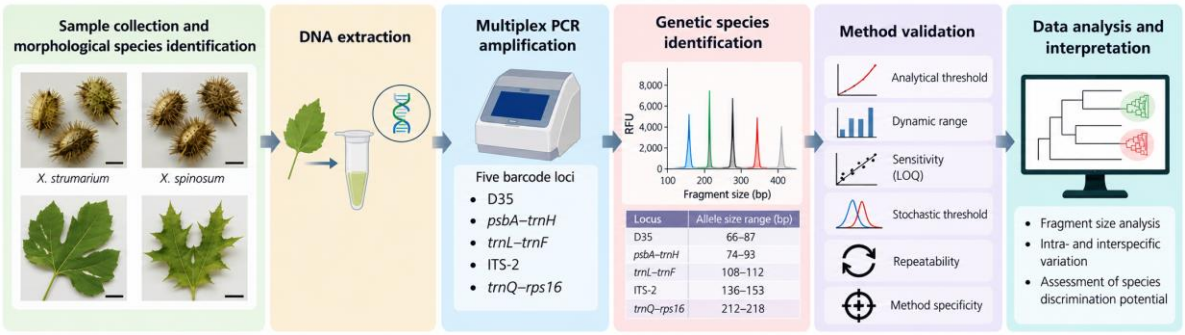

Figure 1. Flowchart of the study

2.1. Plant collection and storage

In this study, a total of 50 plant samples belonging to *Xanthium strumarium* and *Xanthium spinosum* were analyzed. Samples were collected from İzmir (Figure 2), Türkiye (Kınık–Cumalı), İstanbul, Türkiye (Arnavutköy–Dursunköy and Zeytinburnu), and Sakarya, Türkiye. Each plant sample was obtained from a separate root system to ensure that all specimens originated from distinct individuals. Morphological species identification was performed at the Department of Pharmaceutical Botany, Faculty of Pharmacy, Istanbul University, by comparison with authenticated herbarium materials. The collected samples were numbered, air-dried for approximately four months, and stored under appropriate conditions until prepared for experimental analyses.

2.2. Preparation for DNA extraction

Prior to DNA extraction, a standardized pre-treatment protocol was applied to all samples. Dried fruit samples were initially coarsely fragmented using a sterilized blender (Sarex) and subsequently mechanically ground using a mortar and pestle to further reduce particle size [9].

To minimize the risk of contamination, the blender and mortar used during sample preparation were thoroughly cleaned before each use and sterilized under ultraviolet (UV) light for approximately 3 h. Mechanically fragmented samples were transferred into sterile 1.5 mL microcentrifuge tubes, each containing three beads (Qiagen, Germany). Homogenization was performed using a TissueLyser LT (Qiagen, Germany) at a frequency of 50 Hz for 5 min.

Following homogenization, approximately 100 mg of each sample was weighed using a precision balance and prepared for DNA extraction. All pre-treatment procedures were applied under identical conditions for all 50 samples included in the study.

Figure 2. Natural appearance of *Xanthium strumarium* samples in the field (İzmir, 2024)

2.3. DNA extraction and DNA quantification

Genomic DNA was extracted from all 50 prepared samples using the i-genomic Plant DNA Extraction Mini Kit (Intron), following Protocol E (for fruit tissues) according to the manufacturer's instructions [10]. DNA quantification was performed using a Qubit® Fluorometer (Invitrogen™) in combination with the Quant-iT™ dsDNA High Sensitivity Assay Kit (Invitrogen™), in accordance with the recommended protocol [11].

2.4. Primer design and optimization

Primer design was conducted using sequences retrieved from the National Center for Biotechnology Information [NCBI] database, targeting five different gene regions specific to *Xanthium strumarium* and *Xanthium spinosum* [12]. One primer pair was designed for each selected region. Fluorescent dyes were assigned based on primer length compatibility, and primers were synthesized accordingly (Table 1).

Table1. Characteristics of primer sequences used in the multiplex PCR system

Locus name	Repeat motif	NCBI accession number	Expected product length (bp)	Primer length (bp)	Forward / Reverse primer sequence (5′–3′)	Fluorescent dye
D35	TAA–TTA–AAA	KY215673.1	70	F: 20	F: TCAGGTTGCTAGTTTGGGCA	JOE
				R: 20	R: CACAGTCCCAGCTTTCGGAT	
psbA–trnH	TTTT–TATT	KY215741.1	81	F: 25	F: TGAAGCTCCATCTACAAATGGATAA	JOE
				R: 21	R: AGGGCGTTATTGCTCCTTTTT	
trnL–trnF	TAT–TTT–CTT	MH985486	111	F: 21	F: TCTTTGAGCAAGGAATCCCCA	JOE
				R: 20	R: ACAAAGCCTCATCCAGGTCC	
ITS-2	GAA	MG548808.1	140	F: 20	F: ATTGATCGAGATGCGACCCC	JOE
				R: 20	R: CGCTCGATTTTCAAGCTGGG	
trnQ–rps16	TTAT–TTTT	KY215793	225	F: 20	F: ATTAGCAAGACCCCGGCTTT	JOE
				R: 21	R: TGGGAAGTTGGAAGGAATGGG	

2.5. Multiplex PCR amplification

PCR amplifications were performed using the QIAGEN Multiplex PCR Kit [13] in accordance with the manufacturer's guidelines. Since the selected target loci for *Xanthium strumarium* and *Xanthium spinosum* were confirmed to be compatible with a multiplex PCR format, all reactions were prepared in a final volume of 50 µL. Each PCR reaction mixture consisted of 25 µL Multiplex PCR Master Mix, 5 µL primer mix (final concentrations of 1 µM

for D35, psbA–trnH, trnL–trnF, and trnQ–rps16, and 1.5 µM for ITS-2), 15 µL nuclease-free water, and 5 µL genomic DNA (1 ng/µL).

PCR amplifications were carried out using a Veriti™ Thermal Cycler (96-Well Fast, Thermo Fisher Scientific, USA) under optimized conditions based on primer melting temperatures (Tm). The thermal cycling protocol consisted of an initial activation step at 95 °C for 15 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 90 s, and extension at 72 °C for 60 s. A final extension step was performed at 60 °C for 30 min.

2.6. Capillary electrophoresis

PCR products were analyzed using a 3500 Genetic Analyzer (Thermo Fisher Scientific, USA). Prior to analysis, samples were prepared by combining 10 µL formamide, 0.5 µL ROX size standard, and 1 µL PCR product, followed by loading onto the instrument. Capillary electrophoresis was performed using POP-4 polymer in 36 cm capillaries under F-matrix conditions. Injection was carried out at 3 kV for 5 s, and electrophoretic separation was conducted at 15 kV for 40 min at 60 °C. The resulting electropherograms were evaluated using GeneMapper IDX® software.

2.7. PCR and Capillary Electrophoresis Analysis in Different Species

To evaluate the discriminatory power and interspecies comparability of the DNA barcoding–based molecular identification approach, samples from taxonomically distinct species Salvia officinalis (Lamiaceae) and *Ephedra major* (Ephedraceae) were included in the analyses.

Although *Salvia officinalis* is not taxonomically closely related to *Xanthium* species, its placement within the class Magnoliopsida made it suitable as a comparative species for assessing the molecular specificity and selectivity of the developed DNA barcoding approach. Its inclusion enabled evaluation of whether amplification occurred exclusively in target *Xanthium* species or whether false-positive results were generated in taxonomically distant taxa. Additionally, the widespread distribution of Salvia officinalis in Türkiye, its accessibility, and the ability to obtain high-quality genomic DNA contributed to its selection.

Ephedra major, belonging to a distinct taxonomic group, was included to further assess interspecies discrimination. All plant materials were prepared under appropriate conditions prior to analysis, and genomic DNA was extracted from all samples using the same DNA isolation protocol. Following DNA quantification and quality assessment, samples with sufficient concentration and purity were used for PCR analyses. Identical experimental conditions were applied throughout the isolation and amplification processes to ensure comparability of results.

PCR amplifications and capillary electrophoresis analyses were performed in accordance with the protocol developed and validated in this study.

2.8. Method validation

Validation of each locus was conducted in accordance with the guidelines of the Scientific Working Group on DNA Analysis Methods [SWGDAM] [14,15]. Within this framework, analytical threshold, dynamic range, sensitivity, stochastic threshold, and repeatability parameters were systematically evaluated.

3. Results

In this study, PCR optimization and validation analyses were conducted for the amplification of loci selected for DNA barcoding in individuals numbered 1–45 belonging to *Xanthium strumarium*, and individuals numbered 46–50 belonging to *Xanthium spinosum*, whose species identifications had previously been established using a morphological identification key. The loci D35, psbA–trnH, trnL–trnF, ITS-2, and trnQ–rps16 were configured into multiplex PCR systems using appropriate primer combinations, and successful amplification was achieved for each multiplex system.

Throughout the optimization and validation processes, all PCR and capillary electrophoresis analyses were performed under identical experimental conditions, and the resulting electrophoretic profiles were evaluated on a locus-by-locus basis. In addition, to assess the interspecies discriminatory capability of the method, taxonomically distinct species (Salvia officinalis and Ephedra major) were included in the analyses.

3.1. DNA isolation and quantification in Xanthium species

Genomic DNA was successfully isolated from plant materials obtained from all 50 individuals. Post-extraction DNA quantification results demonstrated that all samples were of sufficient concentration and purity for subsequent PCR analyses.

DNA concentrations varied among samples, ranging from 0.127 ng/µL to 7.073 ng/µL.

3.2. Multiplex PCR optimization and identification

The selected loci were adapted to a multiplex PCR system by considering primer melting temperatures (Tm) and expected amplicon sizes. During the optimization process, primer concentrations and PCR cycling conditions were adjusted, and conditions yielding balanced inter-locus peak heights were identified (Tables 2 and 3).

Table 2. Optimized PCR reaction components

Component	Volume per reaction (µL)
Master Mix	25
Distilled water	15
DNA	5
Primer mix*	5
Total volume	***50***

**Primer mix contained locus-specific primer pairs at final concentrations of 1 µM for D35, psbA–trnH, trnL–trnF, and trnQ–rps16, and 1.5 µM for ITS-2.*

Table 3. Optimized PCR cycling conditions

Step	Process	Temperature (°C)	Duration
1	Initial denaturation	95	15 min
2	Amplification (45 cycles)	94	30 s
		55	90 s
		72	60 s
3	Final extension	60	30 min
4	Hold	4	Indefinite

Under optimized conditions, electropherograms revealed that allele peaks obtained from *Xanthium strumarium* and *Xanthium spinosum* samples were clear, reproducible, and consistently above the analytical threshold (Figure 3).

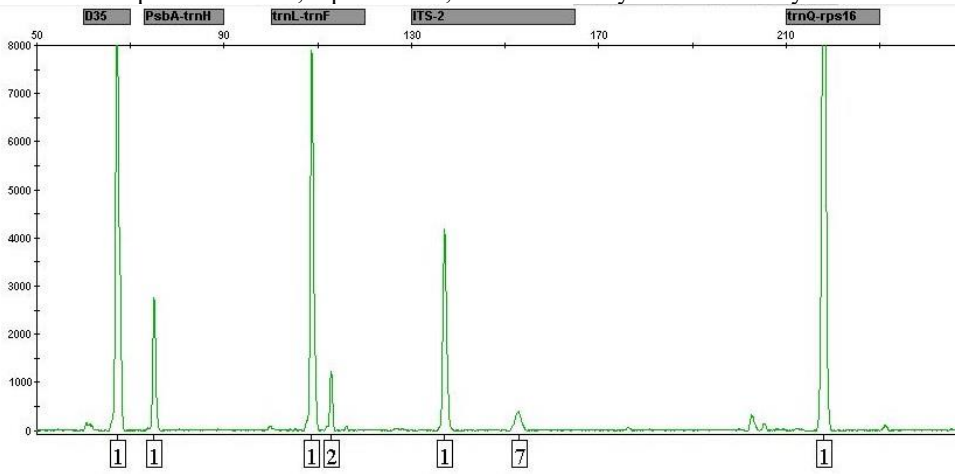

Figure 3. Representative DNA profiles obtained from plant samples.

3.3. Allele Size Distribution

Table 4 presents the allele size ranges obtained for the five loci analyzed in this study. The selected barcode regions produced consistent and reproducible amplification profiles; however, substantial overlap in allele-size distributions was observed between *X. strumarium* and *X. spinosum*. Therefore, fragment-size variation at these loci did not provide sufficient resolution for reliable species-level discrimination between the two taxa in the present dataset.

Table 4. Comparison of allele-size distributions between morphologically identified *X. strumarium* and *X. spinosum* samples

Locus	*X. strumarium* (n=45)	*X. spinosum* (n=5)
D35	66–67	66–67
psbA–trnH	74–75	74–75
trnL–trnF	108–112	108–112
ITS-2	136–155	136–154
trnQ–rps16	217–218	217–218

Capillary electrophoresis analysis of PCR products enabled DNA profiling for each species. Examination of electropherograms obtained from *Xanthium strumarium* and *Xanthium spinosum* revealed a single, consistent profile pattern within individuals of each species (Table 5).

Table 5. Allele sizes obtained from individual samples

Individual	D35	psbA–trnH	trnL–trnF	ITS-2	trnQ–rps16
1	67–67	75–75	108–112	137–152	218–218
2	67–67	75–75	108–112	136–152	218–218
3	67–67	75–75	108–112	137–153	218–218
4	67–67	75–75	108–112	137–153	218–218
5	67–67	75–75	108–112	137–153	218–218
6	67–67	75–75	108–112	137–153	218–218
7	67–67	75–75	108–112	137–154	218–218
8	67–67	75–75	108–112	137–155	218–218
9	67–67	75–75	108–112	137–153	218–218
10	67–67	75–75	108–112	136–153	217–217
11	67–67	74–74	108–112	136–153	217–217
12	67–67	75–75	108–112	137–153	218–218
13	67–67	75–75	108–112	137–153	218–218
14	67–67	75–75	108–112	136–153	218–218
15	67–67	75–75	108–112	137–153	217–217
16	67–67	75–75	108–112	137–154	218–218
17	67–67	75–75	108–112	136–154	218–218
18	67–67	75–75	108–112	136–154	218–218
19	67–67	75–75	108–112	136–154	218–218
20	67–67	75–75	108–112	136–153	218–218
21	67–67	75–75	108–112	136–154	217–217
22	67–67	75–75	108–112	137–154	218–218
23	67–67	75–75	108–112	137–154	218–218
24	67–67	75–75	108–112	137–153	218–218
25	67–67	75–75	108–112	136–153	218–218
26	67–67	74–74	108–112	137–154	218–218
27	67–67	75–75	108–112	137–154	218–218
28	67–67	75–75	108–112	137–154	218–218
29	67–67	74–74	108–112	136–153	217–217
30	67–67	74–74	108–112	136–154	217–217
31	67–67	75–75	108–112	136–153	218–218
32	67–67	75–75	108–112	137–153	218–218
33	67–67	75–75	108–112	136–153	217–217

Table 5. Continued

34	67–67	75–75	108–112	136–154	217–217
35	67–67	75–75	108–112	136–153	218–218
36	67–67	75–75	108–112	136–153	217–217
37	67–67	74–74	108–112	136–153	217–217
38	66–66	74–74	108–112	137–153	218–218
39	66–66	74–74	108–112	137–154	217–217
40	66–66	74–74	108–112	136–154	218–218
41	66–66	74–74	108–112	136–154	218–218
42	66–66	75–75	108–112	137–153	217–217
43	66–66	75–75	108–112	137–153	217–217
44	66–66	74–74	108–112	137–152	217–217
45	66–66	74–74	108–112	136–153	217–217
46	67–67	75–75	108–112	137–153	218–218
47	67–67	74–74	108–112	136–153	217–217
48	67–67	74–74	108–112	136–153	217–217
49	66–66	74–74	108–112	136–154	218–218
50	67–67	75–75	108–112	136–153	217–217

3.4. Interspecific and intraspecific variation between *X. strumarium* and *X. spinosum*

To explicitly evaluate the discriminatory capacity of the multiplex system between *Xanthium strumarium* and *Xanthium spinosum*, allele-size distributions were compared separately at each locus and across the complete multilocus profiles. Considerable overlap was observed between the two morphologically identified species. At D35, both species exhibited 66- and 67-bp alleles; at psbA–trnH, both showed 74- and 75-bp alleles; trnL–trnF exhibited the same 108–112-bp profile in all analyzed individuals; and both species shared the observed ITS-2 and trnQ–rps16 allele ranges.

Importantly, none of the five loci produced an allele that was exclusive to *X. spinosum* within the present dataset. Likewise, all multilocus profiles observed among the five *X. spinosum* individuals were also detected among one or more *X. strumarium* individuals. Therefore, although intraspecific variation was detected at several loci, the magnitude and distribution of this variation overlapped with the variation observed between the two species.

Accordingly, no species-specific allele, multilocus profile, or diagnostic decision threshold could be defined that would allow an unknown sample to be assigned reliably to either *X. strumarium* or *X. spinosum* using the present fragment-size data alone. The present multiplex assay should therefore be interpreted as a molecular characterization and screening system for *Xanthium* samples rather than as a validated species-discrimination assay for these two taxa.

3.5. PCR and capillary electrophoresis results in different species

DNA barcoding–based molecular analyses were performed using genomic DNA extracted from plant samples of *Salvia officinalis* (sage) and *Ephedra major*. Because identical isolation, amplification, and analytical conditions were applied to all samples, the resulting data were directly comparable.

No allele peaks were observed in the electropherograms obtained from *Salvia officinalis* and *Ephedra major*, indicating a clear distinction between these species and *Xanthium strumarium /Xanthium spinosum* (Figures 4 and 5).

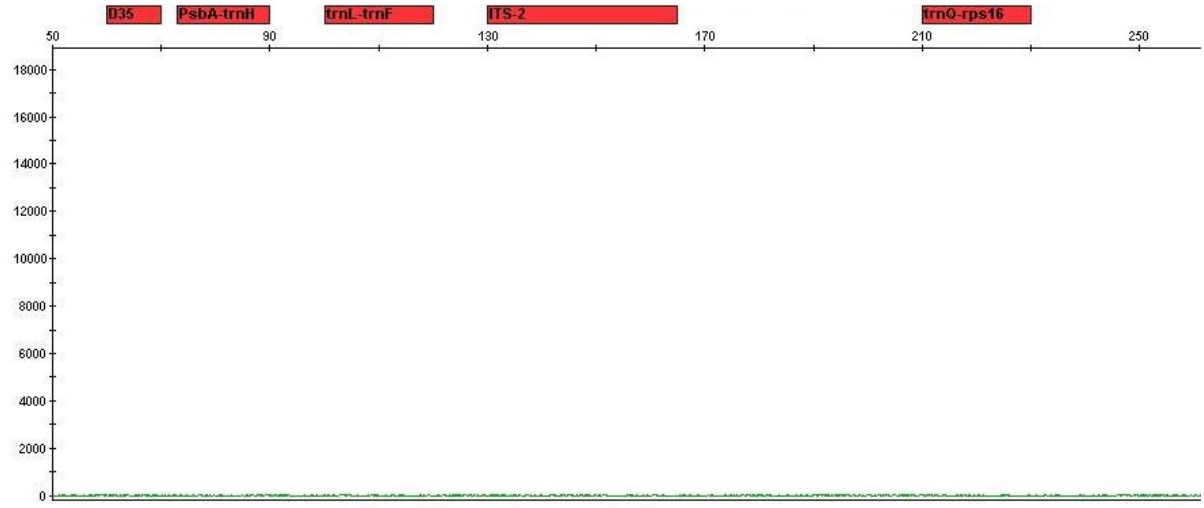

Figure 4. Absence of amplification in *Ephedra major*

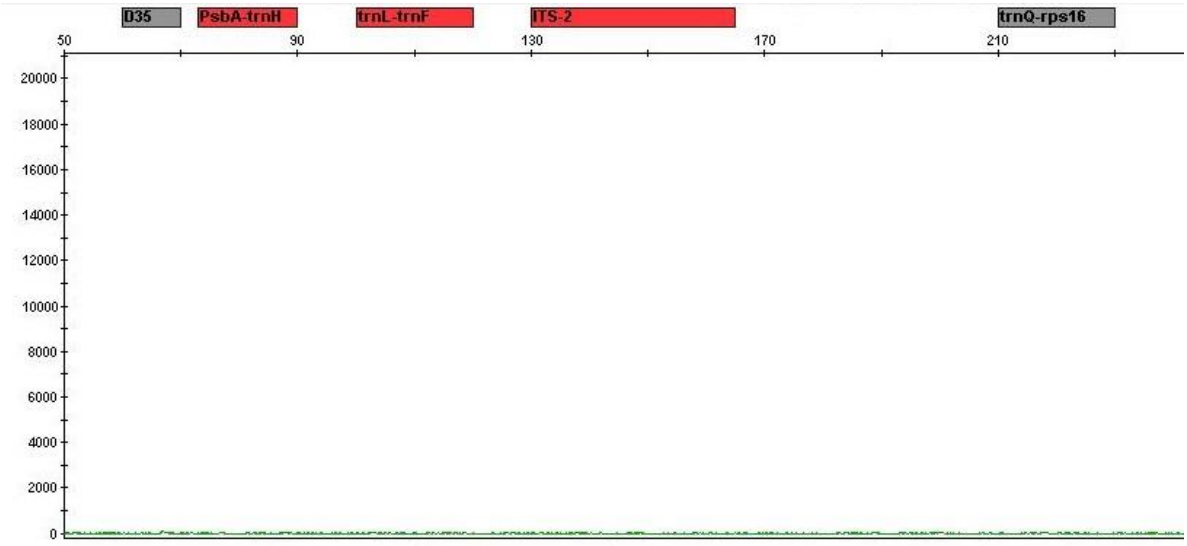

Figure 5. Absence of amplification in *Salvia officinalis*

3.6. Method validation

Validation experiments were performed to evaluate the limit of detection, tested concentration range, sensitivity, stochastic effects, and within-laboratory reproducibility of the multiplex system.

3.6.1. Analytical threshold (Limit of detection (LOD))

Three negative-control samples were evaluated to determine the analytical threshold of the genetic analyzer. The highest RFU (Relative Fluorescence Unit) values observed in the three negative controls were 109, 135, and 73 RFU. The mean of these values was 105.67 RFU and the standard deviation was 31.13 RFU. Based on the calculation used in the thesis, the analytical threshold was determined as 93.39 RFU.

3.6.2. Dynamic range

Five DNA concentrations, 0.1, 0.5, 1, 2, and 3 ng/µL, were evaluated. Three replicate PCR reactions were performed for each concentration, resulting in a total of 15 reactions. The mean peak heights obtained at 0.1, 0.5, 1, 2, and 3 ng/µL were 6757.56, 8332.08, 4691.13, 8140.06, and 9864.20 RFU, respectively (Table 6; Figure 6).

The RFU values did not show a monotonic increase with increasing DNA concentration. In particular, the mean RFU value obtained at 0.5 ng/µL was higher than that obtained at 1 ng/µL.

Table 6. Mean peak heights obtained at five DNA concentrations

DNA concentration	Mean peak height (RFU)
3 ng/µL	9864.2
2 ng/µL	8140.06
1 ng/µL	4691.13
0.5 ng/µL	8332.08
0.1 ng/µL	6757.56

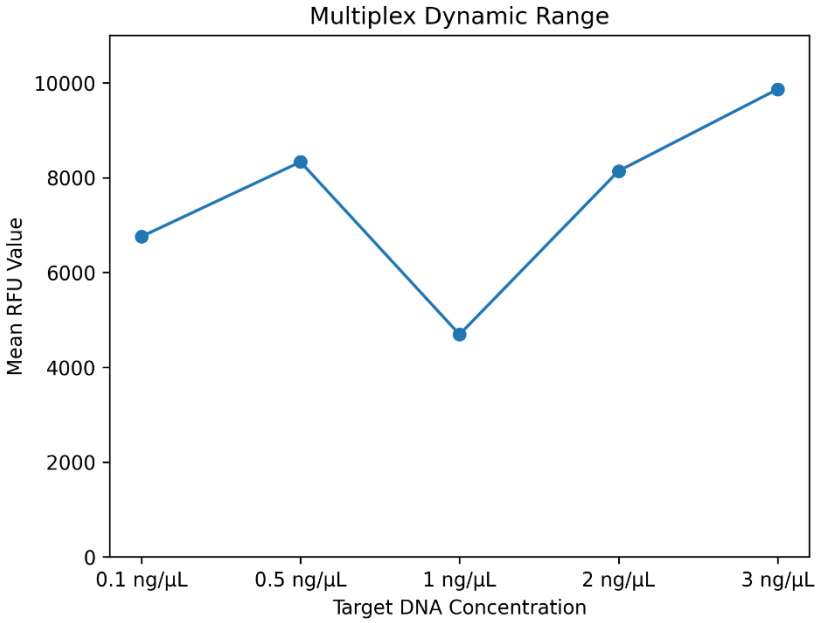

Figure 6. Dynamic range of the multiplex system based on mean RFU values

3.6.3. Sensitivity (LOQ)

Following the dilution analyses used in the dynamic range study, the two lowest DNA concentrations at which alleles were obtained at all loci were determined as 0.5 ng/µL and 1 ng/µL. Three samples were analyzed at each concentration. The calculated sensitivity values were 7987.41 RFU for 1 ng/µL and 10666.57 RFU for 0.5 ng/µL. The sensitivity result was determined as 0.5 ng/µL.

3.6.4. Stochastic Threshold

The stochastic threshold was evaluated using DNA samples at the two lowest concentrations that produced complete profiles, 0.5 and 1 ng/µL. Heterozygosity was observed at the trnL–trnF and ITS-2 loci, and paired allele peak-height ratios and their standard deviations were calculated.

For 1 ng/µL, the mean paired allele peak-height ratio was 4.14 with a standard deviation of 2.20. For 0.5 ng/µL, the mean ratio was 10.96 with a standard deviation of 11.34.

3.6.5. Repeatability

Three samples that had completed analysis were reprocessed by a different analyst, at a different time point, using the same laboratory and instrumentation. The results obtained from both analyses were fully concordant, demonstrating repeatability of the method.

4. Conclusions and discussion

The molecular evaluation of plant-derived materials has become increasingly important in biodiversity research, plant systematics, environmental investigations, and forensic botany. Advances in molecular biology have enabled plant-derived materials to be used as complementary and, in certain contexts, discriminative evidence in forensic investigations, thereby establishing forensic botany as a modern discipline supported by molecular methods such as DNA barcoding [3,6].

Although the assay was initially developed with the aim of molecularly characterizing and discriminating *X. strumarium* and *X. spinosum*, the present fragment-size data revealed substantial overlap between the two species. None of the five loci yielded a species-exclusive allele, and all multilocus profiles observed in the five *X. spinosum* samples were also represented among the *X. strumarium* samples. Thus, the observed interspecific variation did not exceed the level of intraspecific variation sufficiently to establish a reliable species-classification rule.These findings indicate that the current multiplex system is useful for reproducible molecular characterization of *Xanthium* material, but should not be considered a validated diagnostic assay for distinguishing *X. strumarium* from *X. spinosum*. Species-level discrimination may require additional loci with greater interspecific divergence, sequencing-based analysis of the barcode regions, or the development of species-specific markers.However, pronounced phenotypic plasticity and interspecific morphological similarity may reduce the reliability of identification based solely on conventional morphological characteristics [8].

Within the genus *Xanthium* (Asteraceae), morphological overlap between *Xanthium strumarium* and *Xanthium spinosum* has been consistently reported as a taxonomic challenge [16,17]. The molecular data obtained in this study demonstrate that the selected loci generated internally consistent and reproducible electrophoretic profiles across the analyzed samples. These findings indicate that DNA barcoding can complement morphology-based identification and reduce ambiguity associated with environmentally influenced or overlapping phenotypic characteristics. They also support the combined use of multiple barcode regions for the molecular characterization of closely related plant taxa, in line with previous reports [5,6].

Accordingly, a multiplex PCR system incorporating five barcode loci (D35, *psbA–trnH*, *trnL–trnF*, ITS-2, and *trnQ–rps16*) was optimized to achieve balanced amplification across loci. The successful simultaneous amplification of all five loci at low DNA concentrations demonstrates the analytical feasibility of the multiplex system under controlled laboratory conditions. However, the present study was conducted using well-characterized plant material and did not include mock forensic samples, experimentally degraded material, environmental exposure, mixed samples, contaminated specimens, or trace-level forensic evidence. the developed multiplex system provides a preliminary methodological framework whose potential forensic applicability should be evaluated in future studies using experimentally degraded, environmentally exposed, mixed, contaminated, and low-template plant materials.

The analytical performance of the multiplex system was evaluated through analytical threshold, dynamic range, sensitivity, stochastic threshold, repeatability, and reproducibility assessments. Five DNA concentrations (0.1, 0.5, 1, 2, and 3 ng/µL) were examined in triplicate, resulting in a total of 15 PCR reactions. Complete multilocus profiles were obtained at concentrations as low as 0.5 ng/µL, supporting the sensitivity of the assay under the tested laboratory conditions. However, the mean RFU values did not show a strictly concentration-dependent pattern. In particular, the mean peak height obtained at 0.5 ng/µL was higher than that observed at 1 ng/µL. Therefore, the 0.1–3 ng/µL interval should be interpreted as the tested concentration range over which interpretable profiles were obtained rather than as a linear quantitative dynamic range.

The analytical threshold was determined as 93.39 RFU based on the highest background peaks observed in three negative controls. Although this threshold provided a practical criterion for allele detection in the present study, the limited number of negative controls restricts the statistical robustness and broader generalizability of this value. Future validation studies should therefore include a larger number of negative controls and replicate measurements to establish a more robust laboratory-specific analytical threshold.

Repeat analyses of three samples performed by a different analyst at a different time point using the same laboratory and instrumentation produced concordant profiles, supporting within-laboratory consistency of the assay. However, external reproducibility could not be evaluated because the planned analysis in an independent laboratory using different instrumentation and personnel was not completed. Consequently, the present validation should be considered preliminary, and further inter-laboratory studies are required before the analytical performance of the system can be generalized beyond the conditions evaluated here.

Method specificity was preliminarily assessed by the absence of amplification in taxonomically distant species (*Salvia officinalis* and *Ephedra major*), indicating that no cross-amplification occurred in the non-target taxa tested in this study. This finding supports the selectivity of the developed assay under the tested conditions; however, evaluation of additional closely related and co-occurring taxa would be necessary before broader taxonomic specificity can be established.

While forensic botany studies have traditionally focused on pollen analysis, morphological identification, or case-based observations [18,19], DNA barcoding based molecular approaches remain relatively limited and are often restricted to economically significant or trafficked plant species [20,21]. By applying a multilocus molecular approach to *Xanthium strumarium* and *Xanthium spinosum*, this study contributes to the molecular characterization of morphologically variable plant taxa and expands the potential use of DNA-based identification in botanical investigations. The study also aligns with calls for integrating molecular tools into plant-based evidence evaluation and taxonomic assessment [7,22].

Recent studies similarly demonstrate that species-level discrimination in plants cannot always be achieved using universal or conventional barcode loci, particularly in closely related or recently diverged taxa. Dokane [23], evaluating ITS, matK, rbcL, and trnH–psbA for forensic plant species identification, reported that marker performance varied

according to plant material quality and that multilocus combinations provided greater discriminatory value than individual loci. In particular, ITS, trnH–psbA, and matK provided the most effective combination for fresh material, whereas ITS and matK performed better with lower-quality specimens. These findings emphasize that no single barcode locus is universally optimal for forensic plant identification and support the use of complementary loci in taxonomically challenging groups.

Similarly, Liu et al. [24] showed that universal plant DNA barcodes may provide insufficient species-level resolution in taxonomically complex groups and demonstrated the potential benefit of taxon-specific barcode regions. Comparable limitations were also reported by Yu et al. [25] in closely related *Schima* species, where standard DNA barcodes and even plastome-level super-barcodes showed limited discriminatory success. These studies support the interpretation of the present *Xanthium* results, in which substantial overlap between interspecific and intraspecific fragment-size variation prevented reliable assignment of samples to *X. strumarium* or *X. spinosum*. Accordingly, the absence of species-specific fragment profiles in the present study likely reflects the limited discriminatory resolution of the selected loci at the fragment-size level rather than analytical failure of the multiplex system. Higher-resolution sequence-based analysis and/or taxon-specific markers may therefore be necessary for robust species discrimination within *Xanthium*.

In conclusion, the developed multiplex PCR system provides reproducible molecular profiles for *Xanthium* samples and demonstrates satisfactory analytical performance under the tested laboratory conditions. The assay may serve as a useful multilocus DNA barcoding–based framework for the molecular characterization and preliminary taxonomic assessment of *Xanthium* material, particularly when morphological features are limited or unavailable. However, comparison of interspecific and intraspecific allele-size variation showed extensive overlap between *X. strumarium* and *X. spinosum*, and no species-specific allele or multilocus profile was identified. Because the present validation was performed using controlled plant samples, the applicability of the assay to degraded, environmentally exposed, mixed, contaminated, or trace-level forensic botanical evidence remains to be established experimentally. Consequently, the present fragment-size–based assay cannot currently support reliable assignment of an unknown sample to either species. Future studies should include larger and more balanced sample sets, broader geographic representation, additional closely related *Xanthium* taxa, and higher-resolution sequence-based analysis or species-specific markers to establish robust diagnostic criteria for species-level identification.

Artificial Intelligence Statement
( ) This article did not use artificial intelligence.
( ) This article does use artificial intelligence. During the preparation of this work, the authors used ChatGPT (OpenAI, GPT-5.5) solely to improve the visual presentation of Figures 1 and 6. No AI-generated text, data analysis, interpretation, or scientific content was incorporated into the manuscript.
Prompt(s) employed:"Redesign graphs as a clear, publication-quality academic schematic while preserving the original scientific content and relationships."After using this tool, the authors reviewed and edited the output as needed and take full responsibility for the content of the publication.

Acknowledgement: We would like to thank Üsküdar University Institute of Addiction and Forensic Sciences for the use of the laboratory and Istanbul University Department of Pharmaceutical Botany for morphological identification.

Funding: No Funding Received

Conflicts of interest: No Conflict of Interest

Author contributions: Tuğba ÜNSAL SAPAN conceptualized, designed, supervised, and edited the study. İrem CEYHAN collected the materials, performed DNA extraction, quantification and capillary electrophoresis. Nurdan SANDIKÇI assisted with experimental work. Tuğba ÜNSAL SAPAN drafted the manuscript, and Tuğba ÜNSAL SAPAN, Nurdan SANDIKÇI, and İrem CEYHAN revised and finalized it. All authors reviewed and approved the final manuscript.

Ethical statement: This study does not require ethical approval

Data Availability Statement (Please see the Data Availability Policy section for details.)
( ) Option A (Public Repository): "The research data supporting this study are publicly available in [Name of Repository: e.g., Zenodo, Figshare, Dryad] with the permanent DOI link [URL/DOI]."
(x) Option B (Upon Request): All data supporting the findings of this study are included within the article and its tables and figures.
( ) Option C (Third-Party Data): "The data used in this study were obtained from [Name of Institution/Source] and are subject to third-party restrictions regarding redistribution."

( ) Option D (No Data): "No new data were created or analyzed in this study. Data sharing is not applicable to this article."

References

[1] Elkins, K. M. (2013). *Forensic DNA biology: A laboratory manual*. Elsevier.

[2] Malmström, B. G. (Ed.). (1997). *Nobel lectures: Chemistry 1991–1995*. World Scientific.

[3] Budowle, B., Schutzer, S. E., Einseln, A., Kelley, L. C., Walsh, A. C., Smith, J. A. L., … Campos, J. (2003). Building microbial forensics as a response to bioterrorism. *Science, 301*(5641), 1852–1853. doi:10.1126/science.1090083

[4] Chen, S., Yao, H., Han, J., Liu, C., Song, J., Shi, L., … Leon, C. (2010). Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLOS ONE, 5*(1), Article e8613. doi:10.1371/journal.pone.0008613

[5] Kress, W. J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A., & Janzen, D. H. (2005). Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences of the United States of America, 102*(23), 8369–8374. doi:10.1073/pnas.0503123102

[6] Chase, M. W., Cowan, R. S., Hollingsworth, P. M., van den Berg, C., Madriñán, S., Petersen, G., … Wilkinson, M. (2007). A proposal for a standardised protocol to barcode all land plants. *Taxon, 56*(2), 295–299. doi:10.1002/tax.562004

[7] Hollingsworth, P. M., Graham, S. W., & Little, D. P. (2011). Choosing and using a plant DNA barcode. *PLOS ONE, 6*(5), Article e19254. doi:10.1371/journal.pone.0019254

[8] Aquila, I., Ausania, F., Di Nunzio, C., Serra, A., Boca, S., Capelli, A. M., … Ricci, P. A. (2014). The role of forensic botany in crime scene investigation: Case report and review of literature. *Journal of Forensic Sciences, 59*(3), 820–824. doi:10.1111/1556-4029.12401

[9] Ünsal Sapan, T., Acarkan, E., & Sandıkçı, N. (2025). Determination of efficient DNA recovery method in *Pyracantha coccinea* for use in forensic botany. *Anatolian Journal of Botany, 9*(1), 6–10. doi:10.30616/ajb.15406

[10] Intron Biotechnology, Inc. (2012). *i-genomic plant DNA extraction mini kit: Instruction manual for genomic DNA extraction from plant tissues using silica membrane* (2nd ed.).

[11] Invitrogen. (2021). *Qubit dsDNA HS assay kits: User guide*. Thermo Fisher Scientific. Qubit dsDNA HS Assay Kit User Guide (Pub. No. MAN0002326 C.0).

[12] National Center for Biotechnology Information. (2025). *National Center for Biotechnology Information*. U.S. National Library of Medicine. https://www.ncbi.nlm.nih.gov

[13] QIAGEN. (2016). *QIAGEN multiplex PCR handbook*. https://www.qiagen.com/us/resources/kithandbook/en-qiagen-multiplex-pcr-handbook

[14] Özyer, S., Uslu, Z. S., Ünsal Sapan, T., Bülbül, Ö., & Filoğlu, G. (2023). The development of the novel 22 X-indel multiplex system for forensic genetics. *Legal Medicine, 62*, Article 102224. doi:10.1016/j.legalmed.2023.102224

[15] Scientific Working Group on DNA Analysis Methods. (2015). *Validation guidelines for DNA analysis methods*. https://www.swgdam.org/_files/ugd/4344b0_813b241e8944497e99b9c45b163b76bd.pdf

[16] Auld, B. A., McRae, C. F., & Say, M. M. (1988). Possible control of *Xanthium spinosum* by a fungus. *Agriculture, Ecosystems & Environment, 21*(3–4), 219–223. doi:10.1016/0167-8809(88)90089-8

[17] Kim, Y. S., Kim, J. S., Park, S.-H., Choi, S.-U., Lee, C. O., Kim, S.-K., … Ryu, S. Y. (2003). Two cytotoxic sesquiterpene lactones from the leaves of *Xanthium strumarium* and their in vitro inhibitory activity on farnesyltransferase. *Planta Medica, 69*(4), 375–377. doi:10.1055/s-2003-38879

[18] Laurence, A. R. (2018). Forensic palynology in the United States: The search for geolocation. *Microscopy and Microanalysis, 24*(S1), 1186–1187. doi:10.1017/S1431927618006414

[19] Mildenhall, D. C. (2006). *Hypericum* pollen determines the presence of burglars at the scene of a crime: An example of forensic palynology. *Forensic Science International, 163*(3), 231–235. doi:10.1016/j.forsciint.2005.11.028

[20] Gilmore, S., Peakall, R., Robertson, J., & Short, J. (2003). Short tandem repeat DNA markers are hypervariable and informative in *Cannabis sativa*: Implications for forensic investigations. *Forensic Science International, 131*(1), 65–74. doi:10.1016/S0379-0738(02)00397-3

[21] Schield, C., Campelli, C., Sycalik, J., Randle, C., Hughes-Stamm, S., & Gangitano, D. (2016). Identification and persistence of *Pinus* pollen DNA on cotton fabrics: A forensic application. *Science & Justice, 56*(1), 29–34. doi:10.1016/j.scijus.2015.11.005

[22] Mackenzie, G., Boa, A. N., Diego-Taboada, A., Atkin, S. L., & Sathyapalan, T. (2015). Sporopollenin: The least known yet toughest natural biopolymer. *Frontiers in Materials, 2*, Article 66. doi:10.3389/fmats.2015.00066

[23] Dokane, K. (2024). Analysis of markers for forensic plant species identification. Forensic Science International, 358, 112007. https://doi.org/10.1016/j.forsciint.2024.112007

[24] Liu, Z.-F., Ma, H., Zhang, X.-Y., et al. (2022). Do taxon-specific DNA barcodes improve species discrimination relative to universal barcodes in Lauraceae? Botanical Journal of the Linnean Society, 199(4), 741–753.

[25] Yu et al. (2022). Species discrimination in Schima (Theaceae): Next-generation super-barcodes meet evolutionary complexity. Molecular Ecology Resources. DOI: 10.1111/1755-0998.13683