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A Probe-Based qPCR Method for Rapid Detection of *Ips typographus* (Coleoptera: Curculionidae, Scolytinae) in Border Inspections and Forest Surveillance

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Abstract

Ips typographus is one of the most destructive bark beetles affecting conifer forests in Europe, where climatic disturbances and the movement of infested wood can rapidly shift populations from endemic levels to severe outbreaks. Early detection through border inspections and forest monitoring is essential to prevent new introductions and limit the spread of established populations. Here, we developed and validated a probe-based TaqMan qPCR assay, targeting the mitochondrial *COI* barcode region, for the rapid and species-specific detection of *I. typographus* from both insect material and environmental DNA recovered from frass and exit-hole wood chips. Validation followed EPPO PM7/98(5) guidelines, assessing analytical specificity, sensitivity, repeatability, reproducibility, and inter-laboratory transferability. High analytical specificity was demonstrated against a broad panel of non-target species, and reliable amplification was obtained across different tested matrices. The method showed strong analytical sensitivity, with limits of detection of 0.32 pg/µL for adult-derived DNA and 1.6 pg/µL for artificial frass. Repeatability, reproducibility, and inter-laboratory blind testing further confirmed the diagnostic reliability of the method. This validated qPCR assay provides a rapid and sensitive molecular tool for the early detection of *I. typographus*, supporting border inspection and phytosanitary diagnostic laboratories in forest biosecurity activities.

Keywords: border inspection post; *cytochrome c oxidase subunit I*; environmental DNA; European spruce bark beetle; forest biosecurity; molecular diagnostics; real-time PCR; TaqMan assay

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1. Introduction

Bark beetles (Coleoptera: Curculionidae, Scolytinae) are small phloem-feeding insects that typically develop on weakened or stressed trees when populations remain at low densities [1]. At endemic levels, they contribute to nutrient cycling and play an ecological role in maintaining forest health [1,2]. However, several species can shift into major pests in coniferous forests when density-dependent mass attacks occur, allowing them to overcome host defenses and kill otherwise healthy trees [3,4]. This outbreak potential, combined with their cryptic lifestyle and frequent association with timber, wood products, and packaging, facilitates unintentional long-distance dispersal via trade and keeps bark beetles a persistent threat in forest biosecurity [5–7]. For this reason, monitoring and border inspections are critical components of phytosanitary defense [8].

The European spruce bark beetle (ESBB), *I. typographus* (Boerner), is one of the most destructive scolytines in Eurasian conifer forests [9]. It belongs to the tribe Ipini, whose genera share morphological traits and adaptations to conifer hosts [10], complicating species-level discrimination during routine monitoring [11]. The pest primarily colonizes Norway spruce (*Picea abies*) but may also infest other Pinaceae trees, including *Abies*, *Larix*, and *Pinus* spp., under favorable conditions [12,13]. Normally, ESBB populations persist at endemic levels where stressed or dead trees provide sufficient breeding substrate [12,14]. However, large-scale abiotic disturbances such as windstorms can trigger destructive outbreaks [9], as observed after storm "Vaia" in 2018, where the pest contributed to the loss of more than 8.5 million m^3 of timber across the Italian Alps regions [15].

ESBB is widely distributed across continental Europe and is one of the wood-boring pests most frequently intercepted during phytosanitary checks at the Border Inspection Posts (BIPs) [6,7]. Although it is not regulated as a high-concern organism in most EU Member States, it remains a protected-zone quarantine pest in Ireland and the United Kingdom [16,17]. Accordingly, imports of host plants and wood commodities are subject to restrictions and targeted border surveillance [18]. More broadly, *I. typographus* remains a pest of phytosanitary concern due to its ecological and economic impact [13,17,18]. In Italy, for example, intensive field monitoring and control measures are currently implemented to mitigate its infestations in affected areas [19].

One important requirement of early detection programs to prevent biological invasion is the need for rapid and accurate identification of intercepted species [8]. In the case of generic surveillance, initial screening relies on morphological identification by expert taxonomists, which can be reliable for well-preserved adults but becomes problematic when specimens are damaged, fragmentary, or limited to immature stages [20]. Larval identification, for example, requires highly specialized expertise and may be limited by the lack of comprehensive keys. As a result, morphology-based diagnosis can be time-consuming and may lack sensitivity when only trace evidence is available [21,22].

Recent advances in molecular diagnostics provide powerful complementary approaches for rapid and sensitive pest detection, including real-time quantitative PCR (qPCR) methods that can exploit environmental DNA (eDNA) recovered from biological traces [23–26]. Frass, in particular, is an abundant and easily collected substrate for non-invasive detection of scolytines [27,28]. Although molecular diagnostic tools have been validated for several invasive and high-risk quarantine beetles [28–30], a dedicated qPCR assay for *I. typographus* has not yet been available for routine phytosanitary use and is needed to support both border diagnostics and field surveillance.

Here, we address this gap by developing and validating a species-specific TaqMan qPCR assay for the rapid identification of *I. typographus* from both insect material and eDNA-associated matrices. The assay was designed to operate across adults, frass, and exit-hole chips, enabling both direct and indirect detection, and was validated following EPPO PM7/98(5)

standards [31] with respect to specificity, sensitivity, repeatability, and reproducibility, including assessment under operational conditions. Our objective was to deliver a robust, field-relevant molecular tool that can be readily integrated not only into diagnostic workflows supporting BIPs but also in forest surveillance, where timely discrimination of ESBB from morphologically similar bark beetles is often critical for rapid management decisions.

2. Materials and Methods

2.1. Samples

All biological materials used in this study are listed in Table S1. Specimens of *I. typographus* were collected by the Plant Health Service of the Tuscany Region in collaboration with the University of Florence, as part of ongoing phytosanitary surveillance from 2021 to 2023. Monitoring focused on natural stands of *Abies* spp. and *Pinus* spp., where multiple developmental stages were present. Such field collections are essential to ensure that the assay is validated on ecologically relevant material, reflecting the diversity of natural infestation contexts. Additional adult specimens were sourced from the entomological collection of the Department of Agricultural Sciences, University of Naples "Federico II", to increase the genetic and geographical diversity of the material examined. Additional frass samples were supplied by CREA. In total, 206 samples were analyzed, including 116 *I. typographus* specimens and 90 non-target samples representing different xylophagous beetle taxa and substrate types (Table S1). All ESBB adults were identified morphologically following [32]. Frass samples were air-dried at room temperature (23–26 °C) for one week and subsequently stored under the same environmental conditions in 50 mL tubes wrapped with cotton until DNA extraction.

To explore indirect detection through insect eDNA, wood fragments originating from beetle exit holes ("exit-hole chips") were also collected from *Picea abies* and *Larix decidua*. This additional matrix was included because such material often remains on bark surfaces after emergence and may contain insect eDNA traces, offering a non-invasive substrate for detection in the absence of visible frass or specimens.

2.2. Preparation of Artificial Frass Controls

Natural frass is intrinsically heterogeneous, with variable moisture content, microbial load, particle composition, and target DNA concentration, all of which may reduce reproducibility in sensitivity tests. To minimize this variability and obtain a standardized matrix for calibration purposes, we prepared an artificial frass control by mixing 200 µL of *I. typographus* lysate (extracted in 2% CTAB buffer, see above) with 800 µL of lysate from sterile "healthy" wood chips drilled from *Abies* logs. The resulting 1:5 mixture produced a homogeneous frass-like material containing a known DNA concentration. It served as a positive control for the calibration of analytical sensitivity, allowing direct comparison between experimental runs and ensuring reproducible DNA quantification.

2.3. Non-Target Panel for Specificity Testing

Analytical specificity was evaluated using a broad panel of non-target taxa listed in Table S1, including both closely related scolytines and unrelated xylophagous beetles. This inclusivity was crucial because non-target species often co-occur with *I. typographus* in timber/wood products subject to inspection.

The panel included adults, larvae, frass, and exit-hole chips, thereby replicating the complexity of real-world diagnostic scenarios.

2.4. DNA Extraction and Sample Processing

Total DNA was extracted following the protocol described by Rizzo et al. (2024) [27], with minor modifications. Each specimen or matrix type was processed individually in

2.0 mL microcentrifuge tubes using porcelain beads (1.4–5 mm depending on sample size) in a Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) at 20 Hz for 20 s. Mechanical homogenization was chosen because it ensures thorough tissue disruption across a wide range of substrates, including chitinous adults and fibrous wood-derived matrices. Homogenates were suspended in 1 mL of FET buffer (Tris base 0.2 M, NaCl 0.2 M, EDTA 30 mM, SDS 1%, PVP-40 1%, and sodium metabisulfite 1%). From this, 500 µL was mixed with an equal volume of CTAB buffer (2% CTAB; 2% PVP-40; 100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; and 1% sodium metabisulfite). After vortexing, lysates were incubated at 65 °C for 5 min, extracted with chloroform, and centrifuged at 20,000× *g* for 10 min. The aqueous phase (600 µL) was precipitated with isopropanol, centrifuged again at 20,000× *g* for 10 min, and the pellet was vacuum-dried for 10 min before resuspension in 100 µL nuclease-free water.

Each sample was extracted in duplicate to confirm reproducibility. DNA quality and purity were assessed spectrophotometrically (QiaExpert, Qiagen, Hilden, Germany) by measuring A260/280 and A260/230 ratios. The amplifiability of extracts was verified by conventional PCR using conserved 18S rDNA primers [23,33,34], confirming DNA suitability for downstream qPCR analyses.

2.5. Primer and Probe Design and In Silico Validation

The mitochondrial *cytochrome c oxidase subunit I* (*COI*) gene was chosen as a diagnostic marker because it provides strong resolution at the species level in insects and is widely used for DNA barcoding and supported by extensive reference sequence availability in public databases [35]. A primer-TaqMan probe system specific for *I. typographus* was generated using OligoArchitect™ (Sigma-Aldrich, Darmstadt, Germany) from the reference *COI* sequence (GenBank accession: MK315170.1) (Table 1; Figure 1). The primers and probe were designed to minimize secondary structures and self-dimerization while producing a short, 151 bp amplicon suitable for detection in degraded eDNA templates and were synthesized by Eurofins Genomics (Ebersberg, Germany).

Table 1. Primer and TaqMan probe set for *Ips typographus* used in this study.

Name	Nucleotide Sequence	Length (bp)	Tm (°C)	GC Content (%)	Self-Dimer (ΔG)
Ityp_973F	CTAGGTTTAAGAGGAATGC	19	58.8	42.1	−1.7
Ityp_1123R	TTCGGTGAGAAGAGAATC	18	59.8	44.4	−0.6
Ityp_997P	FAM–CGTTACTCAGATTATCCAGATGCGT–BHQ1	25	67.6	44.0	−0.5

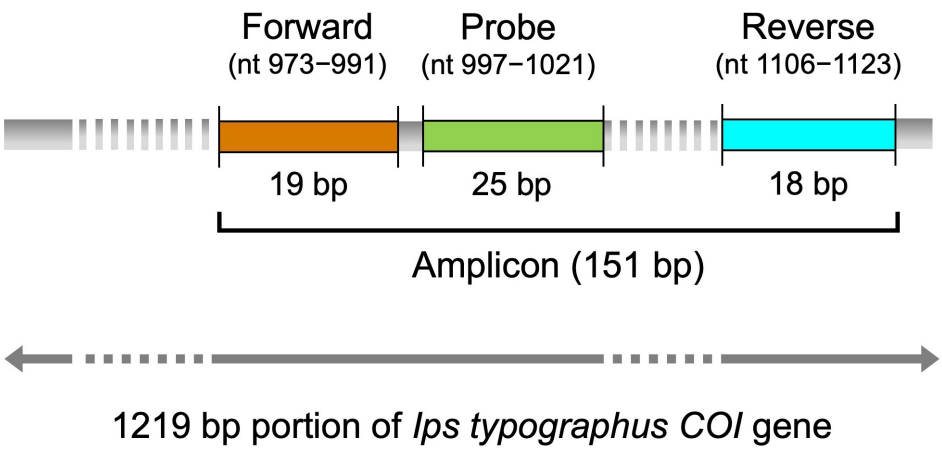

Figure 1. Primer–probe design targeting a 151 bp fragment of the *Ips typographus COI* gene.

To strengthen the *in silico* evaluation of analytical specificity against the most closely related and diagnostically challenging taxa, *COI* sequences from *I. typographus* and representative species belonging to the Ipini tribe (genera *Ips*, *Orthotomicus*, *Pityogenes*, and *Pityokteines*) were retrieved from GenBank and aligned with the target region used for primer–probe design. Multiple sequence alignments were generated with MAFFT implemented in Geneious® 10.2.4, and visually inspected to assess conservation within *I. typographus* at the primer- and probe-binding sites and, conversely, the presence of mismatches and/or indels in non-target species. In addition, the specificity of the selected amplicon was preliminarily assessed using the NCBI BLASTn (Basic Local Alignment Search Tool; v.2.17.0, http://www.ncbi.nlm.nih.gov/BLAST, accessed on 8 January 2026) tool against publicly available nucleotide sequences. This analysis was used as a complementary *in silico* step to verify whether the primer–probe target region showed significant similarity to non-target species beyond those included in the alignment-based analysis.

2.6. qPCR Assay Optimization

For qPCR optimization, DNA extracted from five adult ESBB specimens was used as template, and each condition was evaluated in three technical replicates. To identify the optimal annealing temperature, a thermal gradient ranging from 50 to 58 °C was tested. Primer concentrations were also optimized by evaluating each oligonucleotide at final concentrations ranging from 0.1 to 0.4 µM, while the hydrolysis probe was maintained at a fixed concentration of 0.2 µM. Each reaction contained 1 µL of template DNA normalized to 5 ng/µL. Amplifications were carried out on a CFX96 real-time PCR system (Bio-Rad, Segrate, Italy) in a final reaction volume of 20 µL using QuantiNova Probe PCR Master Mix (Qiagen, Hilden, Germany). For each run, three no-template controls (NTCs), consisting of 1 µL of nuclease-free water, were included to monitor for contamination or non-specific signal generation. Ambiguous or inconsistent amplification profiles were retested to confirm the result. Amplification data were analyzed using CFX Maestro software version 2.0 (Bio-Rad, Segrate, Italy), with automatic threshold and baseline settings applied to the FAM channel. A reaction was considered positive when the amplification plot showed a typical sigmoidal profile with a clear inflection point and exponential fluorescence increase, associated with a Cq value < 40. Following optimization, assay performance was additionally verified using Luna® Universal Probe qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) under identical conditions.

2.7. Analytical Validation

Analytical validation followed EPPO Standard PM7/98(5) [31]. Inclusivity was tested using *I. typographus* samples from different pest populations, while exclusivity testing included non-target species listed in Table S1, selected for ecological overlap and taxonomic proximity. All DNA samples were normalized to 5 ng/µL and analyzed in triplicate. This standardization ensured that observed differences in Cq reflected true assay performance rather than template variation.

Serial fivefold dilutions of adult and artificial-frass DNA were used to generate standard curves and determine the amplification efficiency (E) and correlation coefficient (R^2) values. Analytical sensitivity was determined by defining the limit of detection (LoD) as the lowest DNA concentration producing consistent amplification (Cq $\leq$ 40). Each dilution point was initially tested in three technical replicates. To confirm the LoD, three independent experimental replicates were additionally performed for each dilution close to the detection threshold.

2.8. Repeatability and Reproducibility

Assay precision was evaluated using adult DNA near the LoD (3.2 pg/µL) with three technical replicates per independent sample (n = 8). Intra-run (repeatability) and inter-run

(reproducibility) precision were summarized using the coefficient of variation (CV) Formula (1):

$$\mathrm{CV}(\%) = \frac{\mathrm{SD}}{\mathrm{Mean\ Cq}} \times 100 \quad (1)$$

A CV $\leq$ 2% was considered indicative of high analytical precision, following common diagnostic thresholds [36].

2.9. Inter-Laboratory Blind Validation

To confirm diagnostic reliability and transferability under operational conditions, a blind validation was performed by two accredited phytosanitary laboratories: the Central Phytosanitary Service (Pistoia, Italy) and the Phytosanitary Service of the Port of Livorno.

Each laboratory received twelve coded DNA samples (six *I. typographus* and six non-target species listed in Table S1) at a standardized concentration of 5 ng/µL. All reactions were performed in triplicate under the same cycling conditions but using different thermocycler models (CFX96, Bio-Rad, Segrate, Italy; AriaMx, Agilent, Santa Clara, CA, USA).

A sample was defined as positive if Cq $\leq$ 40 was detected in $\geq$2/3 replicates and negative if no amplification occurred within 40 cycles. Diagnostic values were evaluated by calculating sensitivity, specificity, and accuracy using standard contingency-based formulas (2), where TP represents true positives, TN indicates true negatives, FP is false positives, and FN is false negatives.

$$\mathrm{Sensitivity} = \frac{\mathrm{TP}}{(\mathrm{TP}+\mathrm{FN})};\ \mathrm{Specificity} = \frac{\mathrm{TN}}{(\mathrm{TN}+\mathrm{FP})};\ \mathrm{Accurancy} = \frac{(\mathrm{TP}+\mathrm{TN})}{(\mathrm{TP}+\mathrm{FN}+\mathrm{TN}+\mathrm{FP})} \quad (2)$$

No fixed Cq threshold was imposed, and results were interpreted based on the mean Cq of triplicates and associated SD, reflecting realistic decision-making in diagnostic laboratories.

3. Results

3.1. DNA Isolation

The extraction protocol yielded DNA of suitable quality and quantity from all biological matrices analyzed (Table 2). Mean concentrations ranged from approximately 40 ng/µL in adult samples to around 60 ng/µL in frass and exit-hole wood chips. Spectrophotometric purity ratios (A260/A280 = 1.68–2.12) indicated minimal contamination by proteins or other inhibitors. Natural and artificial frass produced comparable DNA yields and purity values, demonstrating that the artificial substrate reliably reproduced the extraction behavior of authentic frass and could therefore serve as a standardized matrix for analytical sensitivity testing. Amplification of the conserved 18S rDNA fragment confirmed the presence of amplifiable DNA in all extracts. Notably, natural frass stored at room temperature for a long period (about one month) yielded strong amplification signals, showing that unprocessed material can serve as a reliable source of eDNA for diagnostic purposes.

Table 2. DNA yield and purity obtained from *Ips typographus* biological matrices. DNA concentration ranges, mean values ± SD, and A260/A280 purity ratios are reported (n = 3 independent extractions per matrix).

Matrix	DNA Concentration (ng/µL)	Mean ± SD (ng/µL)	A260/A280 Ratio (Range)
Adults	13.40–65.70	39.55 ± 26.15	1.72–2.12
Natural frass	32.14–80.15	56.15 ± 24.01	1.68–2.09
Artificial frass	26.30–92.56	59.43 ± 33.13	1.76–2.11
Exit-hole wood chips	43.56–76.32	59.94 ± 16.38	1.82–2.08

3.2. qPCR Optimization

Optimization trials identified 0.4 µM of each primer as the most effective concentration, yielding the lowest Cq values and the highest fluorescence signals among the conditions tested (Table S2). The optimal annealing temperature was 55 °C. Under these conditions, 20 µL of reaction contained 10 µL of QuantiNova Probe PCR Master Mix, 0.4 µM of each primer, 0.2 µM of the probe, and 1 µL of the template DNA.

The final amplification protocol consisted of an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 55 °C for 40 s. This protocol produced reproducible amplification across all tested DNA extracts, while no signal was detected in the no-template controls. Comparable amplification profiles were also obtained using Luna® Universal Probe qPCR Master Mixes, confirming the transferability of the assay across different reagent chemistries.

3.3. Analytical Validation

The assay achieved complete inclusivity and exclusivity per EPPO PM 7/98(5) standards [35], confirming its adaptability to both direct and eDNA sources. All ESBB samples were successfully detected across matrices and origins, while no amplification was observed for any non-target species included in the exclusivity panel (Table S1).

After normalization to 5 ng/µL, amplification profiles were comparable across matrices (Table 3). As expected, adult-derived DNA produced earlier amplification, whereas wood-associated matrices yielded higher Cq values. Nevertheless, Cq distributions overlapped across matrices and showed limited dispersion within each group, demonstrating stable amplification even in substrates known to contain potential PCR inhibitors. In particular, exit-hole wood chips exhibited low intra-matrix variability, while artificial frass showed greater dispersion, reflecting its heterogeneous composition. Importantly, no systematic loss of performance was observed in any matrix, and all substrates consistently yielded detectable amplification at the normalized DNA concentration.

Table 3. Cq values obtained for *Ips typographus* DNA extracted from different biological matrices following normalization to 5 ng/µL. Cq ranges and mean values ± SD are reported.

Matrix	Cq Range	Cq Means ± SD
Adults	18.58–22.45	20.76 ± 1.33
Natural frass	29.64–33.45	31.72 ± 1.39
Artificial frass	23.43–35.91	29.38 ± 4.55
Exit-hole wood chips	31.55–32.80	31.91 ± 0.63

In silico COI alignments of closely related Ipini revealed multiple mismatches and indels at primer- and probe-binding sites, providing a mechanistic explanation for the absence of cross-reactivity and supporting the observed analytical specificity (Figures S1–S4). Consistently, BLASTn analysis of the selected amplicon did not reveal significant matches to non-target taxa among the top-ranked results, further supporting the specificity of the primer–probe system.

3.4. Assay Sensitivity

Analytical sensitivity was assessed using five-point 1:5 serial dilutions of adult and artificial-frass DNA (Table 4). The limit of detection (LoD) was 3.2×10^{-4} ng/µL for adult DNA and 1.6×10^{-3} ng/µL for artificial frass. Standard curves showed strong linearity across dilution ranges (Figure 2), with coefficients of determination (R^2) equal to 0.999 for both matrices.

Table 4. Analytical sensitivity and limit of detection (LoD) of the *Ips typographus* qPCR assay determined using five-point 1:5 serial dilutions of adult and artificial-frass DNA.

Adult DNA		Artificial-Frass DNA	
Dilutions 1:5 (ng/µL)	**Cq Means ± SD**	**Dilutions 1:5 (ng/µL)**	**Cq Means ± SD**
5	20.06 ± 0.25	25	17.18 ± 0.11
1	22.41 ± 0.18	5	19.71 ± 0.2
0.2	24.81 ± 0.7	1	22.17 ± 0.16
0.04	27.33 ± 0.3	0.2	24.71 ± 0.07
0.008	29.63 ± 0.3	0.04	27.31 ± 0.29
0.0016	32.16 ± 0.01	0.008	29.68 ± 0.17
0.00032	34.46 ± 0.3	0.0016	31.56 ± 0.21
0.000064	-	0.00032	-

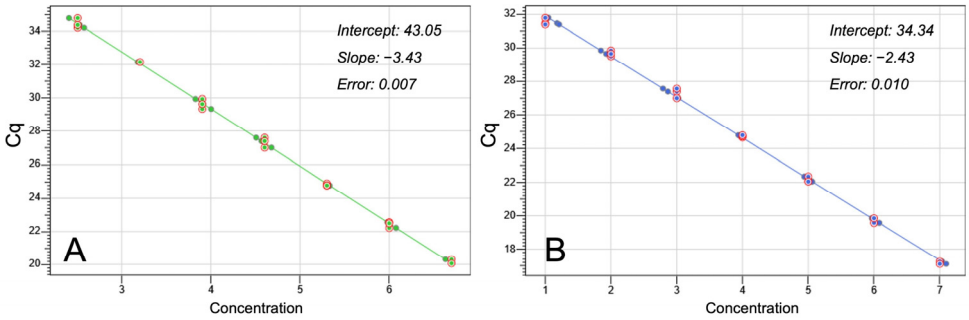

Figure 2. Standard curves generated from 1:5 serial dilutions of DNA extracted from *Ips typographus* adults (**A**) and artificial frass (**B**). Cq values are plotted against the logarithm of DNA concentration.

Amplification efficiency was 95.7% for adult DNA and exceeded the theoretical optimum for artificial frass (158.8%), likely reflecting matrix-related effects at low template concentrations. These results demonstrate that the assay detects *I. typographus* DNA at very low concentrations, supporting its suitability for early detection in surveillance and inspection contexts.

3.5. Repeatability and Reproducibility

The assay demonstrated high analytical precision in both repeatability and reproducibility analysis (Table 5). Intra-run assays yielded a mean Cq of 34.47 ± 0.57 with a coefficient of variation (CV) of 1.65%. Inter-run analyses performed on different days, by different operators and thermocyclers, showed similarly low variability (mean Cq = 33.54 ± 0.48; CV = 1.42%). Only one replicate failed to amplify, likely due to stochastic loss of template near the detection threshold.

Table 5. Repeatability (intra-run) and reproducibility (inter-run) of the *Ips typographus* qPCR assay evaluated using adult DNA at 3.2 pg/µL. Values represent mean Cq ± SD and coefficient of variation (CV%).

Replicates	Repeatability (Intra-Run)		Reproducibility (Inter-Run)	
	Cq Means ± SD	**CV (%)**	**Cq Means ± SD**	**CV (%)**
1	34.19 ± 0.35	1.03	33.55 ± 0.6	1.78
2	34.71 ± 0.96	2.77	33.63 ± 0.9	2.66
3	34.46 ± 0.06	0.17	33.96 ± 0.39	1.13
4	34.53 ± 0.9	2.62	33.22 ± 0.57	1.72
5	34.26 ± 0.38	1.12	33.70 ± 0.44	1.31

Table 5. *Cont.*

Replicates	Repeatability (Intra-Run)		Reproducibility (Inter-Run)	
	Cq Means ± SD	CV (%)	Cq Means ± SD	CV (%)
6	34.85 ± 0.69	1.98	33.31 ± 0.33	0.98
7	34.23 ± 0.72	2.11	33.35 ± 0.13	0.38
8	34.53 ± 0.48	1.40	33.60 ± 0.47	1.40

3.6. Inter-Laboratory Blind Test

Inter-laboratory blind validation confirmed the diagnostic robustness and transferability of the assay. Both accredited phytosanitary laboratories correctly identified all *I. typographus* samples, including adult-derived DNA and artificial frass, while no amplification was observed for any non-target species. Mean Cq values obtained in the blind test are reported in Table S3.

These findings demonstrate that the assay is fully operational under routine diagnostic conditions and can be reliably implemented in BIPs and forest health surveillance laboratories.

4. Discussion

A growing number of qPCR methods have been developed in recent years for the rapid identification of invasive and quarantine pests. These approaches have demonstrated high reliability in identifying target species from both insect material and eDNA contained in biological residues [23,24,29,37,38]. Such methods are particularly well suited to bark beetles [27,38], whose cryptic lifestyle and close association with wood products often limit the recovery and analysis of intact specimens [5,7]. Against this background, the present study aimed to develop and validate a probe-based qPCR assay specifically targeting *I. typographus*, with the dual objective of supporting operational phytosanitary diagnostics and strengthening forest surveillance programs where early detection is critical for outbreak management. The diagnostic approach was developed in compliance with the MIQE guidelines for qualitative real-time PCR assays [39], which define key performance criteria including amplification efficiency, LoD, and analytical and diagnostic specificity.

Although rapid identification of ESBB has previously been addressed using molecular approaches, existing methods were developed for different diagnostic purposes. For example, Zink et al. (2019) [40] proposed a duplex droplet digital PCR (ddPCR) assay optimized for high-throughput screening of bulk trap catches, a context distinct from border inspections and trace-based diagnostics. Another study focused on conventional PCR strategies to overcome mitochondrial barcoding failures induced by genomic contamination of insect symbionts or parasites [41], facilitating downstream sequencing rather than rapid decision-making. In contrast, the assay developed here relies on a TaqMan probe-based qPCR system validated according to EPPO PM7/98(5) standards [31] and explicitly designed for routine phytosanitary applications. The closed-tube format of the TaqMan technology minimizes contamination risk, enhances specificity through dual primer–probe recognition, and is fully compatible with real-time PCR platforms commonly used in diagnostic laboratories [27,29,42].

The DNA extraction protocol applied in this study yielded comparable quality and quantity rates from all tested samples, demonstrating effective isolation of amplifiable insect DNA across all matrices. This performance is consistent with previous molecular studies on bark and ambrosia beetles, which demonstrated that frass and wood-derived residues represent reliable sources of eDNA for qPCR-based diagnostics [27,28].

Regarding assay performance, complete analytical specificity and inclusivity were achieved, as demonstrated by wet-lab testing against a broad non-target panel. These

findings were further supported by *in silico* analysis of the primer- and probe-binding regions, which revealed multiple mismatches and indels in representative species of the closely related Ipini genera *Ips*, *Orthotomicus*, *Pityogenes*, and *Pityokteines*. Given the high morphological and genetic similarity within this tribe [32,43,44], the concordance between empirical exclusivity testing and sequence-based mismatch analysis provides a robust mechanistic basis for confidence in species-level discrimination. Such high specificity is particularly critical in border diagnostics, where false-positive detections may lead to unnecessary regulatory actions [45].

The LoDs obtained for adult and artificial-frass DNA samples (0.32 and 1.6 pg/µL, respectively) were comparable to those reported for qPCR assays targeting other *Ips* species, such as *I. sexdentatus* [27]. This concordance likely reflects shared methodological approaches, including amplification of mitochondrial targets with similar copy numbers and the use of comparable probe-based qPCR chemistries, resulting in broadly similar analytical sensitivity across congeneric bark beetles under equivalent experimental conditions. Importantly, the method performed consistently across a range of biologically relevant matrices, including natural frass and exit-hole wood chips. The suitability of these substrates aligns with previous studies on ambrosia beetles (e.g., *Xylosandrus* spp.), which established frass-derived eDNA as a reliable and non-invasive source for molecular diagnostics [28].

Inter-laboratory blind validation confirmed the reproducibility and diagnostic reliability of the assay under operational conditions, an increasingly recognized benchmark for readiness in phytosanitary diagnostics.

Artificial frass was introduced in this study as a standardized reference matrix to minimize the inherent variability of natural frass and improve the reproducibility of sensitivity testing. Nevertheless, because the matrix was generated from wood-derived material, it may still have retained compounds commonly associated with plant substrates, such as phenolic compounds, polysaccharides, or other PCR-interfering substances [46,47]. This may explain both the broader Cq dispersion observed for artificial frass and the supra-theoretical amplification efficiency (>150%), which is outside the expected range for qPCR assays. Such overestimation may arise when matrix-associated compounds alter amplification dynamics across the dilution series, particularly at low template concentrations, leading to non-linear behavior and an artificially steep standard curve [46–48]. In this context, the result should be interpreted as a matrix-related effect rather than as evidence of enhanced assay performance. However, this effect did not compromise the low detection limits or the overall diagnostic performance of the method, as the assay was developed for qualitative detection of ESBB, where the primary objective is the reliable discrimination between positive and negative samples rather than the absolute quantification of target DNA. By contrast, natural frass and other biologically relevant matrices showed lower Cq variability, suggesting that, under the extraction conditions adopted, it behaved as a more stable diagnostic substrate despite its biological heterogeneity.

Finally, the successful amplification of *I. typographus* DNA from frass samples stored for one month confirms the persistence of fecal DNA in wood-associated environments, consistent with observations reported for other wood-boring beetles such as *Aromia bungii* (Coleoptera: Cerambycidae) [29]. The air-dried frass processed in this study, stored at room temperature prior to DNA extraction, allowed reliable amplification, supporting its practical use as a diagnostic substrate. However, the stability of insect DNA in this matrix under a broader range of environmental variables, including light exposure, humidity, and temperature fluctuations, was not systematically investigated here and should be assessed in future studies to better define the operational limits of the method.

5. Conclusions

The probe-based qPCR protocol developed and validated in this study provides a sensitive and highly specific molecular tool for the detection of *I. typographus* from both insect material and indirect biological matrices. Its compliance with MIQE and EPPO standards, combined with successful inter-laboratory validation, supports its immediate applicability within BIPs and Plant Health Service laboratories, where rapid and reliable identification is essential for informed phytosanitary decision-making.

Future studies should focus on integrating this diagnostic tool into multiplex screening platforms, assessing its performance on bulk environmental samples, evaluating its implementation in routine workflows, and extending validation to geographically broader ESBB populations to further confirm conservation of the primer–probe target region across the species' distribution.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f17040440/s1, Supplementary Table S1: Target and non-target Coleoptera samples used for the development and validation of the real-time qPCR assay for *Ips typographus*; Supplementary Table S2: qPCR optimization data for the *Ips typographus* TaqMan assay; Supplementary Table S3: Results of the inter-laboratory blind panel performed to evaluate diagnostic sensitivity, specificity, and reproducibility of the *Ips typographus* qPCR assay; Supplementary File S1: *In silico COI* sequence alignments supporting analytical exclusivity of the *Ips typographus* qPCR assay. Figure S1. Multiple sequence alignment of the *mitochondrial cytochrome oxidase I* (*COI*) gene showing *Ips typographus* haplotype aligned with closely related *Ips* species. The positions of the forward primer (fw), TaqMan probe, and reverse primer (rev) developed in this study are indicated. Conserved regions in *I. typographus* contrast with diagnostic nucleotide substitutions within the probe- and/or primer-binding sites of non-target *Ips* spp., supporting assay exclusivity; Figure S2. *COI* sequence alignment between *Ips typographus* and representative species of the genus *Orthotomicus*. The forward primer, probe, and reverse primer binding sites are highlighted. Multiple substitutions and indels within the probe and amplicon region of *Orthotomicus* sequences explain the absence of cross-amplification observed during exclusivity testing; Figure S3. *COI* sequence alignment of *Ips typographus* and selected *Pityogenes* species, including the target region of the qPCR assay. Non-target sequences exhibit substantial divergence at the primer- and probe-binding sites; Figure S4. Alignment of *COI* sequences from *Ips typographus* and *Pityokteines* species. The location of the qPCR primers and TaqMan probe is shown. The presence of multiple mismatches and indels in non-target taxa supports the analytical specificity of the assay toward *I. typographus*.

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Abbreviations

The following abbreviations are used in this manuscript:

BHQ1	Black Hole Quencher 1
BIP/BIPs	Border Inspection Post(s)
CTAB	Cetyltrimethylammonium bromide
CV	Coefficient of variation
Cq	Quantification cycle (qPCR cycle threshold value)
CREA	Council for Agricultural Research and Economics (Italy)
eDNA	Environmental DNA
EPPO	European and Mediterranean Plant Protection Organization
ESBB	European spruce bark beetle (Ips typographus)
FAM	6-carboxyfluorescein (fluorophore label)
LoD	Limit of detection
MAFFT	Multiple Alignment using Fast Fourier Transform (alignment software)
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
Pg	Picogram

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