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ORIGINAL ARTICLE

Novel *Agrilus planipennis* (Coleoptera: Buprestidae) early-detection tool designed on cytochrome B gene

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Abstract

Agrilus planipennis (Coleoptera: Buprestidae) is a quarantine pest that is threatening native ash populations in both North America and Europe. Early detection of this pest, which can cause severe infestations, is essential, and molecular tests applied at different developmental stages and environmental traces can contribute substantially to improving control measures to prevent or contain *A. planipennis* infestations. In this study, a specific *A. planipennis* real-time qPCR assay was developed using a Locked Nucleic Acid (LNA) probe based on the CytB (cytochrome B) gene. The detection limit of this method was 25.6 fg/µl for adult DNA extracts and 0.21 pg/µl for frass produced by *A. planipennis* larvae. The new qPCR probe test, which targets a different locus, not only allows identification of the pest and provides an indirect diagnosis through environmental DNA analysis but can also be used for cross-validation of results between different tests.

Keywords Emerald ash borer · Quarantine pest · Early detection · Molecular diagnostic tool · Phytosanitary surveillance

Introduction

The Emerald Ash Borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is a xylophagous buprestid, primary associated with trees of the genus *Fraxinus*. All European ash species are susceptible hosts of this beetle. This species is native to Asia (China, Japan, Korea, Mongolia and the Russian Far East) and has been accidentally introduced into North America and Western Russia, from where it has spread to Ukraine (Haack et al. 2002; Liu et al. 2003; Baranchikov et al. 2008; Drogvalenko et al. 2019; Evans et al. 2020) and most recently to Belarus (Zviagintsev et al. 2025). *Agrilus planipennis* is a quarantine pest listed in Part A of Annex II of Regulation (EU) 2019/2072. In addition, the European Commission carried out a risk assessment of these pests and identified 20 priority pests whose introduction can cause particularly high economic, ecological and social damage, including *A. planipennis* (Regulation (EU) 2019/1702). A recent EFSA report on the potential impact of its introduction into the EU, estimated that 87.6% of ash trees could be lost, based on the considered scenario assumptions (EFSA 2025).

Heavy infestations of *A. planipennis* cause symptoms, such as smaller or discoloured leaves, progressive crown dieback, formation of epicormic shoots on branches and stems, bark cracking and flaking due to predation by woodpeckers and even the plant's death. Furthermore, attacked trees show D-shaped insect exit holes of about 3–4 mm,

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Table 1 Samples from insects and frass used in this study, including positive (+) and negative (−) amplification results

Species	Sample code	Life stage/sample type	Supplier[b]	qPCR results
*Agrilus planipennis**	MR 000814	Adult	USDA	+
	MR 001563			+
	MR 001713			+
	MR 001638			+
	MR 001714			+
	MR 001714/2			+
	MR 001714/3			+
	MR 001714/4			+
	MR_Frass_Ap	Artificial Frass	PPS-T	+
	MR_Frass_Ap/1			+
	MR_Frass_Ap/2			+
	MR_Frass_Ap/3			+
	MR_Frass_Ap/4			+
	MR_Frass_Ap/5			+
	In 338	gStrand	PPS-T	+
*Agrilus angustulus**	MR 001710	Adult	JKI	−
	MR 001721/IIC			−
*Agrilus anxius**	MR 001715	Adult	JKI	−
	MR 001716			−
	MR 001717			−
	MR 001718			−
	MR 001719			−
	MR 001720/(BBB11)†		RU	−
	MR 001721/(BBB12)†			−
	MR 001722/(BBB20)†		GLFC	−
	MR 001723	Artificial Frass	JKI	−
	MR 001724			−
	MR 001725			−
	MR 001726			−
*Agrilus auroguttatus**	MR 001701	Adult	UCa	−
	MR 001689			−
	MR 001689/2			−
	MR 001689/3			−
	MR 001689/4			−
	MR 001689/5			−
	MR 001689/6			−
	MR 001689/7			−
	MR 001689/F	Frass	UCa	−
	MR_Frass_Au	Artificial Frass	PPS-T	−
	MR_Frass_Au/1			−
	MR 001689/Fc	Fecal residue	UCa	−
Agrilus biguttatus	MR 001722/IIC	Adult	JKI	−
	MR 001524		PPS-T	−
Agrilus convexicollis	MR 001712	Adult	JKI	−
	MR 001720/IIC			−
*Agrilus graminis**	In 308†	Adult	INRAE	−
Agrilus hastulifer	In 309†	Adult	INRAE	−
*Agrilus laticornis**	MR 001709	Adult	JKI	−
	MR 001718			−
Agrilus obscuricollis	In 310†	Adult	INRAE	−
Agrilus olivicolor	MR 001716	Adult	JKI	−
	MR 001717/1		UoF	−
Agrilus sulcicollis	MR 001717	Adult	JKI	−
Agrilus viridis fagi	MR 001711	Adult	UoF	−
	MR 001719		JKI	−

Table 1 (continued)

Species	Sample code	Life stage/sample type	Supplier[b]	qPCR results
*Anoplophora chinensis**	MR 001617	Adult	PPS-T	–
	MR 001618			–
	MR 001648			–
	MR 001620			–
	MR 000225	Larva	PPS-L	–
	MR 000259		PPS-T	–
	MR 001619			–
	MR 001678			–
	MR 001622			–
	MR 001649			–
	MR 000790	Frass	PPS-T	–
	MR 001621			–
	MR 001624			–
	MR 001639			–
	MR 001640			–
	MR 001668			–
*Anoplophora glabripennis**	MR 000226	Adult	PPS-L	–
	MR 000757	Larva		–
	MR 000301	Frass		–
	MR 001625			–
*Araecerus fasciculatus**	MR 000824	Larva	PPS-T_PLI	–
*Aromia bungii**	MR 000260	Adult	PPS-C	–
	MR 000261			–
	MR 000262			–
	MR 001674			–
	MR 000254	Larva		–
	MR 000231	Frass	PPS-C	–
	MR 000263		UoN	–
	MR 001688			–
Aromia moschata	MR 000766	Adult		–
Buprestis cupressi	MR 001707	Adult	UoF	–
Cerambyx cerdo	MR 000274	Adult	CREA_DC	–
	MR 000297			–
Cerambyx scopolii	MR 000275	Adult	CREA_DC	–
	MR 000756			–
Cerambyx welensii	MR 000755	Adult	CREA_DC	–
Cerambyx miles	MR 000829	Adult	CREA_DC	–
Chlorophorus glabromaculatus	MR 000799	Larva	PPS-T_PLI	–
*Chrysobothris affinis**	MR 001944	Larva	PPS-T_PLI	–
*Chrysobothris igniventris**	MR 001954	Larva	PPS-T_PLI	–
*Chrysobothris quadriimpressa**	MR 000853	Larva	PPS-T_PLI	–
	MR 001694			–
*Chrysobothris rugosiceps**	MR 001699	Larva	PPS-T_PLI	–
	MR 001700			–
*Chrysobothris solieri**	MR 001702	Larva	PPS-T_PLI	–
*Chrysobotris femorata**	MR 001615	Larva	PPS-T_PLI	–
Cossus cossus	MR 000831	Adult	UoP	–
	MR 000265	Larva	PPS-T	–
	MR 000289	Frass	PPS-T	–
*Graphisurus fasciatus**	MR 000809	Larva	PPS-T_PLI	–
	MR 001695			–
Hylurgus ligniperda	MR 000302	Adult	UoF	–
Hylurgopinus rufipes	MR 000855	Larva	PPS-T_PLI	–
Larinus planus	MR 001923	Larva	PPS-T_PLI	–

Table 1 (continued)

Species	Sample code	Life stage/sample type	Supplier[b]	qPCR results
*Leptostylus asperatus**	MR 001948	Larva	PPS-T_PLI	–
*Lepturges confluens**	MR 001692	Larva	PPS-T_PLI	–
Monochamus galloprovincialis	MR 000250	Adult	CREA_DC	–
	MR 000258	Adult	PPS-L	–
	MR 000818	Adult	UoF	–
Monochamus sartor	MR 000257	Adult	PPS-L	–
	MR 000256	Larva		–
Morimus asper	MR 000759	Adult	CREA_DC	–
*Neoclytus acuminatus**	MR 001704	Adult	UoF	–
*Neoclytus mucronatus**	MR 000852	Larva	PPS-T_PLI	–
*Phryneta leprosa**	MR 000828	Adult	PPS-T_PLI	–
	MR 000828/L	Larva		–
Saperda carcharias	MR 000767	Adult	UoP	–
*Saperda punctata**	MR 000784	Adult	CREA_DC	–
*Saperda scalaris**	MR 000785	Adult	CREA_DC	–
*Saperda tridentata**	MR 000294	Adult	PPS-T_PLI	–
	MR 001690	Adult		–
	MR 001691	Adult		–
	MR 000247	Larva		–
*Stictoleptura cordigera**	MR 000792	Adult	UoF	–
Zeuzera pyrina	MR 000837	Larva	UoP	–
	MR 000838	Frass		–

*Identified by sequence analysis of COI gene, using primers LCO1490/HCO2198 (Folmer et al. 1994). Provided as DNA sample. [b]*UoF* University of Florence, Italy, *JKI* Julius Kühn Institute, Germany, *UoP* University of Pisa, Italy, *USDA* U.S. Department of Agriculture APHIS PPQ, Brighton, MI, *UCa* University of California, USA, *UoN* University of Naples, Italy, *PPS-T* Plant Protection Service of Tuscany, Italy, *PPS-T_PLI* Plant Protection Service of Tuscany, Port of Leghorn interception, Italy, *CREA_DC* Consiglio per la Ricerca in agricoltura e l'analisi dell'Economia Agraria, Italy, *PPS-C* Plant Protection Service_Campania, Italy, *PPS-L* Plant Protection Service_Lombardy, Italy, *RU* Rutgers University, USA, *GLFC* Great Lakes Forestry Centre, Canada, *INRAE* Laboratoire de Biologie des Ligneux et des Grandes Cultures, France

serpentine larval galleries under the bark, detached bark and debarking.

However, tree decay due to insect infestation can only be detected after years, when the population density has increased significantly. Indeed, *A. planipennis* often remains undetected for several years in new environments (EFSA 2024). These considerations suggest that surveillance, based on visual examination and sampling of plant parts, is not sufficient for the early detection of new EAB outbreaks. Recent experiences in North America indicate that trapping is the most effective method for EAB detection (EFSA 2024). However, it must be considered that, in general, the morphological identification of *Agrilus* species appears to be challenging (EPPO Global Database), especially due to the species richness of the genus, comprising more than 3000 species (Volkovitsh et al. 2020).

Molecular identification of insects is a rising method, especially for quarantine species, for which rapid identification is crucial. In the case of xylophagous insects, the most frequently examined traces are frass samples in feeding tunnels or around entry/exit holes dug into trunks and branches (Rizzo et al. 2020; EPPO 2021). DNA shed by organisms into environment samples is considered environmental DNA (eDNA). Molecular approaches based on eDNA offer several advantages: rapid detection via direct gene amplification, reduced reliance on taxonomic expertise, and minimal disturbance to the surveyed host. Numerous diagnostic protocols exploiting frass samples or other genetic residues have been developed in recent years (Ide et al. 2016; Kyei-Poku et al. 2020; Rizzo et al. 2020; Kyle et al. 2024).

The mitochondrial DNA genes cytochrome B (CytB) and cytochrome C oxidase subunit I (COI) are both used as barcode markers for insects (Ray et al. 2024). Specific assays, targeting COI gene, to identify *A. planipennis* and detect traces of its DNA within frass samples, were developed and validated on other European wood-borer species (Peterson et al. 2023; Kupper et al. 2025). In this study, we developed a new sensitive real-time qPCR assay, based on an alternative locus (Cytochrome B), with a Locked Nucleic Acid (LNA) probe for *A. planipennis* detection. LNA probes and primers contain chemically modified bases that increase the rigidity of the ribose. This modification improves the stability, specificity, and affinity for base-pairing with target sequences (Josefsen et al. 2009). The high binding affinity allows for shorter probes, which results in higher binding specificity to the target DNA. The assay was validated using adult insect tissue and synthetic frass matrices, demonstrating its suitability for routine laboratory diagnostic applications.

Materials and methods

Samples

Target and non-target organisms (including other genera present on the same host plants) were obtained from different sources and geographical origins (Table 1). The target specimens include eight *A. planipennis* adults and six artificial frass samples. Artificial frass of EAB was produced by mixing CTAB lysates obtained from 1 g of *Fraxinus excelsior* wood chips and from *A. planipennis* adult specimen (sample MR001638—Table 1), according to Rizzo et al. (2023). Among the 131 outgroup samples, 55 belong to *Agrilus* spp. and 76 to other genera. To assess the efficiency and specificity of the assay a *GStrand* (In 338) synthetic DNA (Eurofins Genomics), equivalent to the expected amplicon and based on the CytB gene of *A. planipennis* was synthesised. All sample details are listed in Table 1.

DNA extraction

DNA extraction from all samples was carried out as described in Rizzo et al. (2023). Briefly, the lysis step was performed using a 2% CTAB buffer, followed by purification with Maxwell® RSC PureFood GMO and Authentication Kit (Promega Italia, Milan, Italy).

For artificial frass, DNA extraction was carried out following the protocol described in Rizzo et al. (2023): 800 µl of 2% CTAB extraction buffer derived from the lysate of 1 g of wood chips, was mixed with 200 µl of 2% CTAB extraction buffer derived from the lysate of *A. planipennis*. These samples were purified as previously described, using Maxwell® RSC PureFood GMO and Authentication Kit (Promega Italia, Milan, Italy). Subsequently, starting with a total DNA concentration of 10 ng/µl, eight 1:10 serial dilutions were performed by using DNA extracted from artificial frass.

Concentration and quality of the extracted DNA were quantified by using Qiaexpert (Qiagen). DNA obtained from both target and non-target organisms (Table 1) was normalised to a concentration of 5 ng/µl and used for qPCR reactions and stored at -20 °C until use. DNA amplifiability was verified by qPCR reactions using a TaqMan probe based on 18 S rDNA region (Ioos et al. 2009) as described in Rizzo et al. (2025).

Primers and probe design for real-time PCR assay

To identify the specific genomic region suitable for oligonucleotide design, several sequences were analysed and evaluated. Their selection was based on the following factors: presence of polymorphisms, possibility of comparing similar sequences from other congeneric or genetically related species, use of specific mitochondrial genomic regions (Fig. 1) and highly conserved genes that provide interspecific variability (Kralik and Ricchi 2017).

Based on the previous considerations and sequence availability, the CytB locus was selected.

CytB gene is characterised by low intraspecific variability (genetic distance < 2%) and high interspecific divergence (distance > 8%), allowing the distinction between cryptic or morphologically similar insect species (Chen et al. 2023). Primers and probe were designed, using the online software *OligoArchitec primer design* (Sigma-Aldrich, St. Louis, USA), on the CytB region of *A. planipennis* (KT363854.1) obtained from The National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov). The primers and LNA probe were synthesised by Eurofins Genomics (Ebersberg, Germany) (Fig. 2).

To evaluate the in silico specificity, a total of 640 sequences from 103 different *Agrilus* species were aligned and compared with the newly designed amplicon (Figure S1, supplementary material) by using the Geneious software. These sequences belong to species with the greatest genetic affinity, similar ecological niches or the potential for morphological misidentification (Volkovitsh et al. 2020). The amplicon specificity was verified by using the BLAST software (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST) and a partial sequences alignment is shown in Fig. 3.

Optimisation and validation of the qPCR method

Based on the melting temperatures of the oligos, the real-time PCR probe protocol has been optimised for the annealing temperature by applying a gradient (between 52 and 62 °C). The oligo concentration was evaluated as follows: 0.1 µM, 0.2 µM, 0.3 µM, and 0.4 µM for the primers and probe. For each run, two tubes containing nuclease-free distilled water (no template control - NTC), a positive and a negative amplification control were tested. Validation was performed according to the EPPO Standard PM7/98 (5). The specificity of the qPCR assay was tested for all samples listed in Table 1 by normalising their DNA concentration to 5 ng/µL.

The standard curve was generated from 5-fold serial dilutions (ranging from 10 ng/µL to 5.12 fg/µL) of a known concentration of *A. planipennis* DNA (sample MR001638) and analysed in triplicate. This curve allowed to quantify the target DNA in the artificial frass by interpolating the mean quantification cycle (Cq) values against the logarithm of the initial DNA concentration.

The limit of detection (LoD) was determined using the same standard curve by assessing the minimum amount of

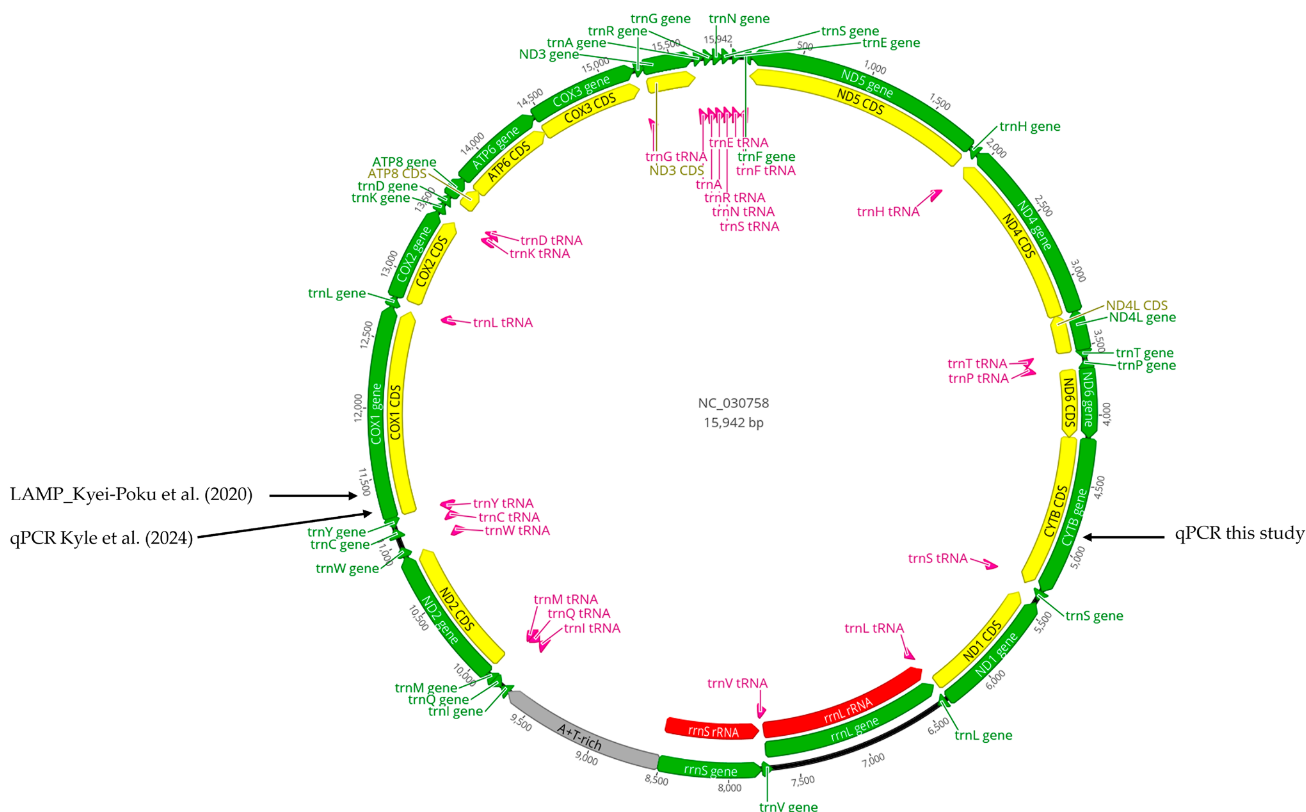


Fig. 1 Position of the developed assay sequences with evidence of the different positions of similar qPCR diagnostic assay (Kyle et al. 2024) or with different techniques (LAMP) (Kyei-Poku et al. 2020). Image produced using Geneious Prime 2025.1.3 software

Name	***Sequence***	***Amplicon size (bp)***	***Reference Sequence***
Aplanipennis_4872F	CAGTCATAGCTCTCACAA		
Aplanipennis_5034R	CCTAGTTTATTAGGGATTGATC	163	KT363854.1
Aplanipennis_4892P	FAM - att{A}cc{T}tg{T}cc{G}cacc – BHQ1		

Fig. 2 Primers (blue and red) and LNA probe (purple) designed on target CytB gene for *A. planipennis* (accession no. KT363854.1). The area highlighted in grey is equivalent to the specific gStrand (In 338)

target DNA detectable by the qPCR assay. The qPCR amplification efficiency (E) was calculated based on the slope of the standard curve according to the formula: $E = 10^{(-1/\text{slope})} - 1$.

Intra-variation (repeatability) and inter-variation (reproducibility) were performed and evaluated based on mean Cq values and their standard deviations (Dhami et al. 2016; Groth-Helms et al. 2023). Repeatability and reproducibility were performed using DNA extracts from *A. planipennis* adult, normalised to a concentration of 16 pg/μL.

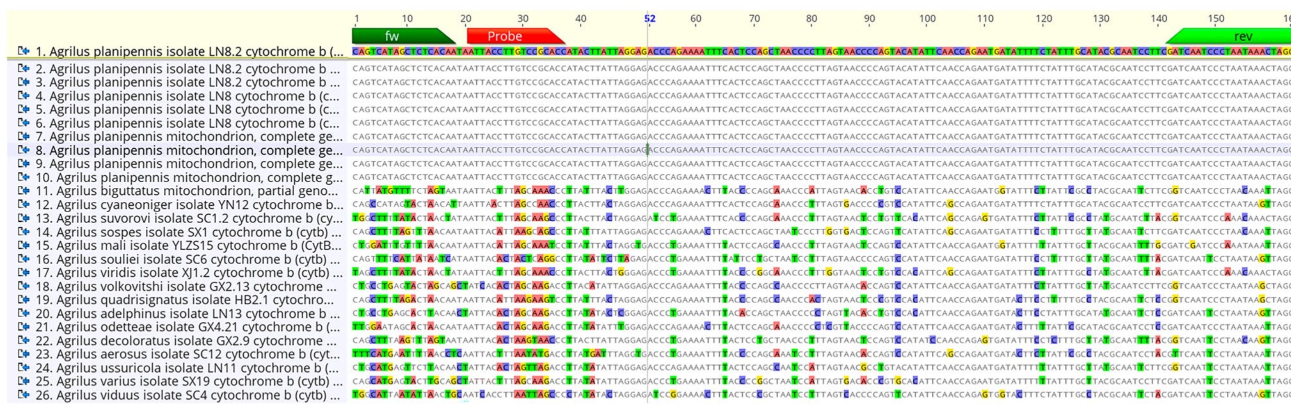


Fig. 3 Alignments between the *Agrilus planipennis* amplicon and different sequences of other *Agrilus* species. Primers are indicated in green and LNA probe in red

Table 2 Parameters considered in the blind panel: sensitivity, specificity and diagnostic accuracy

Performance criteria	Definition	Equation
Diagnostic sensitivity	Concordance between the analysis result and the assigned value of the samples, for which a POSITIVE value was assigned	TP/(TP+FN) × 100%
Diagnostic specificity	Concordance between the analysis result and the assigned value of the samples, for which a NEGATIVE value was assigned	TN/(TN+FP) × 100%
Accuracy	Concordance between analysis result and assigned value	(TP+TN)/N × 100%

TP True positives, *TN* True negatives, *FP* False positives, *FN* False negatives, *N* Total number of samples

Blind test

The blind test was carried out with 12 blind samples, at different DNA concentrations (10, 2 and 0.5 ng/µL): 6x *A. planipennis*, 2x *A. anxius*, 2x *A. sulcicollis*, 1x *A. graminis*, 1x Negative Template control (NTC). Each sample was numbered progressively and processed in triplicates. The laboratories involved in the blind panel were: (1) LAB 1 (organizer): Phytopathological laboratory of the Tuscany Regional Phytosanitary Service, headquarters of Pistoia (Italy); (2) LAB 2: Phyto pathological laboratory of the Tuscany Regional Phytosanitary Service, headquarters of Livorno (Italy); (3) LAB 3: Phytopathological laboratory of the Tuscany Regional Phytosanitary Service, headquarters of Florence (Italy); LAB 4: laboratory of the CNR- IPSP of Sesto Fiorentino—Florence (Italy).

Each laboratory conducted the qPCR analyses using the same diagnostic protocol developed in this study, but with different instruments and master mixes: (i) LAB 1_ CFX96 (Bio-Rad) thermocycler and Quantinova Probe (Qiagen) master mix; (ii) LAB 2: ARIA MMX (Agilent) thermocycler and Luna universal Probe (NEB) Master mix; (iii) LAB. 3: Line Gene 96 Plus (Bioer, Rome, Italy) thermocycler and Quantinova Probe (Qiagen) master mix; (iv) LAB 4: Rotor-Gene Q (Qiagen) thermocycler and TaqMan Universal PCR Master Mix (Applied Biosystems).

The evaluation of the performance of the participating laboratories was assessed by considering the qualitative values (Positive/Negative) recorded in the PT results sheet (Chabirand et al. 2014). The results were interpreted for each laboratory by calculating the number of true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN), according to the Table 2.

Results

Assay conditions of the qPCR LNA Probe protocol

Real-time amplification reactions with the LNA probe were optimised considering the reaction mixture, primer annealing temperatures and optimal primer concentrations. The optimal reaction mixture consisted of the following reagents, in a final volume of 20 µL: 10 µL of QuantiNova Supermix 2x (Qiagen, Hercules, USA), with primer concentrations at 0,4 µM, 0,2 µM for LNA probe and 2 µL of DNA template. As for the optimal thermal protocol, the conditions are as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and annealing at 60 °C for 40 s.

DNA extraction

Quality and amplifiability of extracted DNAs are shown in Table 3. The Cq values were obtained by real-time PCR amplifying the ribosomal 18 S gene (Ioos et al. 2009). A total of eight adult specimens of *A. planipennis* and six samples of artificial frass were evaluated.

Table 3 Performance of DNA extraction from adult *Agrilus planipennis* and artificial frass, based on mean concentrations of extracted DNA (±SD), absorbance ratio (A260/280) and mean Cq values of 18 S (Ioos et al. 2009)

Sample	Mean DNA Concentration (ng/µL)	Absorbance (A260/280)	Mean Cq (18 S)
Adult (*n*=8)	73.53±15.07	1.77±0.06	18.2±0.24
Artificial frass (*n*=6)	49.22±2.72	1.94±0.08	24.2±0.34

Table 4 *Agrilus planipennis* analytical sensitivity assays using 1:5 serial dilutions (from **10** ng/µL to **5.12** fg/µL) in triplicate (A, B, and C). The Cq values are the mean of the three threshold cycles of each dilution

Concentration values	A	B	C	Average Cq	SD (±)
10 ng/µL	18.27	18.42	18.38	18.36	0.06
2 ng/µL	20.72	20.73	20.75	20.73	0.01
0.4 ng/µL	23.07	23.15	23.11	23.11	0.03
0.08 ng/µL	25.4	25.35	25.49	25.41	0.06
0.0016 ng/µL	27.53	27.61	27.56	27.57	0.03
3.2 pg/µL	30.38	30.26	30.06	30.23	0.13
0.64 pg/µL	32.2	32.22	32.11	32.18	0.05
0.13 pg/µL	34.94	34.94	34.87	34.92	0.03
25.6 fg/µL	36.33	36.87	36.23	36.48	0.28
5.12 fg/µL	n/a	n/a	n/a	n/a	n/a

qPCR assay validation

NCBI BLAST® results showed a high specificity of the qPCR amplicon with *A. planipennis* sequences (Identity=100%; E-value=1e-75). No relevant homology was found with any closely related species. The qPCR assay developed in this study provided comprehensive and exclusive results for *A. planipennis* with a clear non-specificity towards the non-target organisms. The Limit of Detection (LoD) from *A. planipennis* DNA adult was 25.6 fg/µL (Cq mean±SD=36.48±0.28) (Table 4).

The standard curve generated from 1:5 serial dilution *A. planipennis* DNA showed the following parameters: correlation coefficient (R^2): 0.999; slope: -3.3; Y-intercept: 21.7. The qPCR assay showed a 100% of efficiency (E) (Fig. 4).

The qPCR performed on *A. planipennis* adult DNA revealed a repeatability and reproducibility of 100% (Table 5).

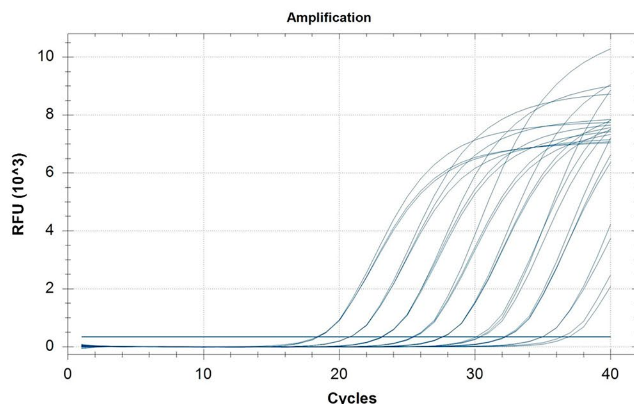
DNA extracts from artificial *A. planipennis* frass were diluted 1:10 up to the eighth dilution. *Agrilus planipennis* DNA was detected up to the fifth dilution (Cq average=33.16±0.17), that corresponds to 0.21±0.03 pg/µL (this value was found by interpolating the data from a standard curve using CFX Maestro 1.3 software). This LoD corresponds to the minimum amount of *A. planipennis* DNA detectable indirectly via frass.

Blind panel test

The blind panel test performed in 4 different laboratories showed a diagnostic sensitivity, specificity and accuracy of 100%. No false positives or false negatives were obtained (Table 6).

Discussion

In this study, we developed an alternative qPCR assay using LNA based on another region of mitochondrial DNA, cytochrome B (CytB). The CytB genomic region provides sufficient variability to allow unequivocal diagnosis of the target species; the use of an LNA probe yielded results comparable to those obtained for the detection and identification of *A. anxius* (Rizzo et al. 2025). The high specificity of the in silico analysis results was confirmed by in vivo experiments on target and non-target species. The robustness of the method was validated through proficiency tests conducted in four independent laboratories. The sensitivity of the



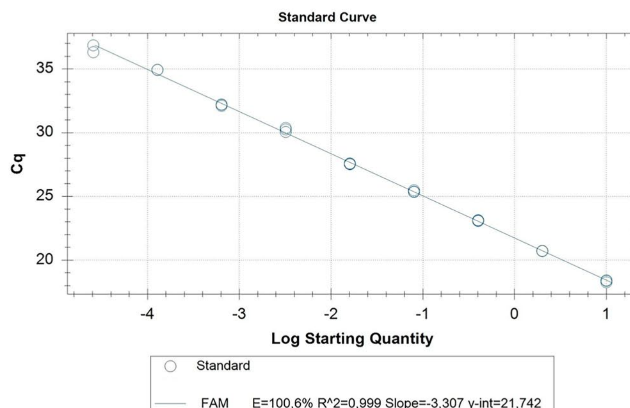


Fig. 4 Amplification curves (**A**) and standard curves (**B**) relating to the qPCR probe assay using 1:5 serial dilutions of *A. planipennis* insect adult DNA ranged between 10 ng/µL to 5.12 fg/µL

Table 5 Repeatability and reproducibility values indicating replicates and corresponding mean (Cq ± SD values) from *A. planipennis* adult DNA (normalised at 16 pg/μL concentration)

Replicates	Repeatability			Reproducibility		
	Test A	Test B	Test C	Test A	Test B	Test C
1	28.24	27.9	27.91	28.1	27.85	28.04
2	28.06	27.75	28.27	27.74	27.81	28.03
3	27.72	27.87	27.97	27.83	27.92	27.92
4	27.7	27.77	28.03	28.26	28.75	28.1
5	28	28.52	27.8	28.07	29.14	28.4
6	28.57	28.16	27.87	27.94	28.06	28.15
7	28.51	28.08	28.45	28.95	28.77	28.63
8	28.72	29.03	27.76	28.5	28.11	28.66
Cq mean	28.19	28.14	28.01	28.17	28.30	28.24
S.D.	0.36	0.41	0.22	0.37	0.48	0.27

Table 6 Results of blind panel test from 4 different laboratories by using the qPCR probe LNA assay to detect *A. planipennis*

Sample code	Species	DNA concentration	Expected result	LAB 1	LAB 2	LAB 3	LAB4
Ap1	*Agrilus planipennis*	10 ng/μL	*Positive*	+	+	+	+
Ap 2	*Agrilus planipennis*	2 ng/μL	*Positive*	+	+	+	+
Ap 3	*Agrilus graminis*	10 ng/μL	*Negative*	–	–	–	–
Ap 4	*Agrilus anxius*	10 ng/μL	*Negative*	–	–	–	–
Ap 5	*Agrilus planipennis*	0.5 ng/μL	*Positive*	+	+	+	+
Ap 6	*Agrilus anxius*	10 ng/μL	*Negative*	–	–	–	–
Ap 7	*Agrilus sulcicollis*	10 ng/μL	*Negative*	–	–	–	–
Ap 8	*Agrilus planipennis*	10 ng/μL	*Positive*	+	+	+	+
Ap 9	*Agrilus sulcicollis*	10 ng/μL	*Negative*	–	–	–	–
Ap 10	*Agrilus planipennis*	2 ng/μL	*Positive*	+	+	+	+
Ap 11	*Agrilus planipennis*	5 ng/μL	*Positive*	+	+	+	+
Ap 12	NTC	–	*Negative*	–	–	–	–

LAB 1: CFX96 (Bio-Rad) thermocycler and Quantinova Probe (Qiagen) master mix; LAB 2: ARIA MMX (Agilent) thermocycler and Luna universal Probe (NEB) master mix; LAB. 3: Line Gene 96 Plus (Bioer, Rome, Italy) thermocycler and Quantinova Probe (Qiagen) master mix; LAB 4: Rotor-Gene Q (Qiagen) thermocycler and TaqMan Universal PCR Master Mix (Applied Biosystems)

proposed qPCR protocol (LoD of 25.6 fg/μL with an average Cq of 36.48 ± 0.28) is comparable to that obtained with the LAMP test (20 fg/μL) (Peterson et al. 2023) on adult insects. This qPCR assay, can be used to validate diagnostic results obtained in the field using the LAMP technique, as it is highly specific and targets a different region of DNA than all other tests that have already been developed (Kyei-Poku et al. 2020; Kyle et al. 2024; Kupper et al. 2025). The use of a combination of assays developed on different gene regions reduces the risk associated with non-specific amplification in eDNA analysis and makes the results more reliable.

The sensitivity of the present assay on artificial frass suggests that an indirect approach by eDNA is reliable to detect *A. planipennis* in a given area. This approach was already proposed for other quarantine organisms (Kiewnick et al. 2015; Rizzo et al. 2024).

The presence of *A. planipennis* poses a serious threat to forests and ecosystems in which ash species play a key role (Hultberg et al. 2020). Prevention, quarantine measures, surveillance, and early detection are essential to reduce its impact and limit its spread especially for the European Union, which is constantly exposed to a significant risk of the introduction and spread of EAB.

In the context of phytosanitary surveillance efforts to prevent the establishment and spread of *Agrilus planipennis* within the EU (Regulation (EU) 2024/434), the method here developed may be adopted at cross-border entry points and in high-risk areas (e.g. nurseries, urban and leisure areas).

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preparations, data collection, and statistical analyses of data collected from DNA extracts were completed by D. Rizzo, C. G. Zubieta, M. Moriconi, B. Palmigiano, and A. Marrucci. Data curation and data mining, reference assembly, and manuscript formatting were done by D. Rizzo, A. Gionni, N. Luchi, F. Pecori, A. Santini, S. Feltgen, T. Panzavolta, M. Bracalini and B. Hoppe. Writing of the original draft was prepared by D. Rizzo, A. Gionni, F. Pecori, A. Santini, N. Luchi, T. Panzavolta, M. Bracalini, S. Feltgen and B. Hoppe. Revisions of manuscripts were completed by all authors.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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