Journal of Plant Diseases and Protection
https://doi.org/10.1007/s41348-024-00902-4

ORIGINAL ARTICLE

Rapid identification of *Ips sexdentatus* (Boerner, 1766) (Curculionidae) from adults and frass with real-time PCR based on probe technology

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Received: 31 August 2023 / Accepted: 28 February 2024

Abstract
A molecular tool has been developed for the molecular identification of *Ips sexdentatus* (Börner 1776) (Coleoptera Curculionidae Scolytidae), the well-known six thooted bark beetle, widely distributed in Eurasia, where it infests several species of the genus *Pinus* and occasionally a few conifer species of the genera *Abies*, *Larix* and *Picea*. The developed test can be useful both in countries where *I. sexdentatus* is handled as a quarantine species and, to greater reason, in Europe to discriminate biological traces of this commonly found beetle from those produced by regulated pests. The protocol is based on real-time PCR with TaqMan probe technology and has been developed on whole insect bodies (adults) as well as on artificial frass contaminated by DNA of the beetle. The molecular test developed here for both direct and indirect identification of *I. sexdentatus* has proven effective in terms of analytical specificity, analytical sensitivity, reliability and reproducibility. The recommended protocol is a practical diagnostic tool allowing a rapid identification of the six toothed bark beetle in the presence of any biological trace of other xylophagous pests collected at points of entry during phytosanitary surveys.

Keywords Scolytidae · Molecular diagnostics · Bark beetle detection · TaqMan technology

Introduction

Ips sexdentatus (Börner 1776) (Coleoptera, Curculionidae, Scolytidae) is a well-known bark beetle, which mainly attacks trees of the *Pinus* genus. Its main host, in northern Europe, is *Pinus sylvestris* L., while in central and southern Europe it also attacks *Pinus pinaster* Aiton*, Pinus heldreichii* Christ and *Pinus nigra* J.F. Arnold (EPPO 2023). Occasionally, the species has also been reported on *Picea*, *Abies* and *Larix* species (Chararas 1962; Ozcanet al. 2011). *I. sexdentatus* is widely distributed in Europe, with the exceptions of Ireland, Cyprus and some areas of the UK (Northern Ireland and the Isle of Man) where it is listed as a quarantine pest. The beetle is also widely distributed in Asia, Russia (Siberia, Far East), China, Japan, Mongolia, Myanmar and Thailand (EFSA 2017; EPPO 2017). *I. sexdentatus* can complete up to five generations per year in the Mediterranean area (Lévieux et al. 1985). The number of generations may be progressively reduced in territories with shorter and less hot summer, like North and Central Europe, where the species has one to two generations per year (EPPO 2023). Generally, the mating system of this polygamous species includes a nuptial chamber and two to five vertical tunnels created by the females in the phloem, each up to about 50 cm long. The beetle may carry blue-stain fungi (e.g. *Ophiostoma ips* and *Ophiostoma brunneociliatum*) which colour the wood under the gallery and may be involved in the dieback of the infested trees (Bueno et al. 2010; Croisé et al. 1998).

I. sexdentatus signs may be detected visually, after the first symptoms of the damage appeared, or by trapping

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Published online: 27 April 2024

devices baited with specific pheromones (EFSA 2017). The bark beetle can be identified morphologically and by the use of taxonomic keys (e.g. Douglas et al. 2019). Symptoms and signs on the attacked trees include discolouration of the canopy, brown frass expelled by the beetles and falling from the entrance holes. It may often be observed in association with other bark beetles on the same trees (*Ips acuminatus, Tomicus piniperda, Tomicus destruens*).

Generally, *I. sexdentatus* is not a primary pest and it is only able to attack trees already subjected to stress, both from the environmental conditions and from other parasites. It does not fall under the recommendations for regulation as a quarantine pest by the EPPO. In Europe, *I. sexdentatus* is generally associated to declining or wind-thrown trees, recently felledlog piles, or trees weakened by fires and drought (EFSA 2017). However, high density populations favoured by the large amounts of susceptible hosts may ultimately lead to successful attacks by *I. sexdentatus* seven on healthy trees which may be overcome by the simultaneous attack of a huge number of bark beetles (Rossi et al. 2009; Pineau et al. 2017). For example, the beetle has caused severe outbreaks in Italian coastal pine stands surrounding areas affected by forest fires and already debilitated by the Maritime Pine Scale *Matsucoccus feytaudi* Ducasse (Bracalini et al. 2021).

The dispersal of the insect takes place mainly through the adults who can fly continuously over long distances. In laboratory experiments, single specimens have been observed flying up to 45 km by considering the sum of a total of about 50 shorter flights (Jactel and Gaillard 1991). The beetle may be accidentally transported by human activities for even longer distances, the main pathways being raw wood materials, bark and wood packaging material.

These wood products can represent high risk for the introduction of *I. sexdentatus* into new environments where it is not currently present (for example in the USA or in Latin America). A rapid and sensitive diagnostic tool, especially of an indirect type, i.e. analysing woody frass expelled from wood products moving from one area to another, can be highly valuable for phytosanitary control operations at cross-border entry points. In this operational framework, a real-time PCR probe test was developed in this study, using species-specific primers for *I. sexdentatus*, to be used both on adults and frass.

Materials and methods

Insect samples

In 2021 and 2022 during routine inspections carried out by the Tuscan Phytosanitary Service and University of Florence, different stages (mostly adults) of *I. sexdentatus* were detected in different populations of *Pinus* spp. in Tuscany. In addition to the various evolutionary stages of the insect, the frass (Fig. 1) produced by the beetle trophic activity was collected. Species identification was carried out based on beetle mating system and morphological characters of the beetle (Douglas et al. 2019). In addition, an "artificial branch" was prepared by adding a known amount (10 ng/µL) of DNA from adult *I. sexdentatus* to the lysate in 2% CTAB extraction buffer to the branch of *Aromia bungii* (Faldermann) (Coleoptera: Cerambycidae) harvested from

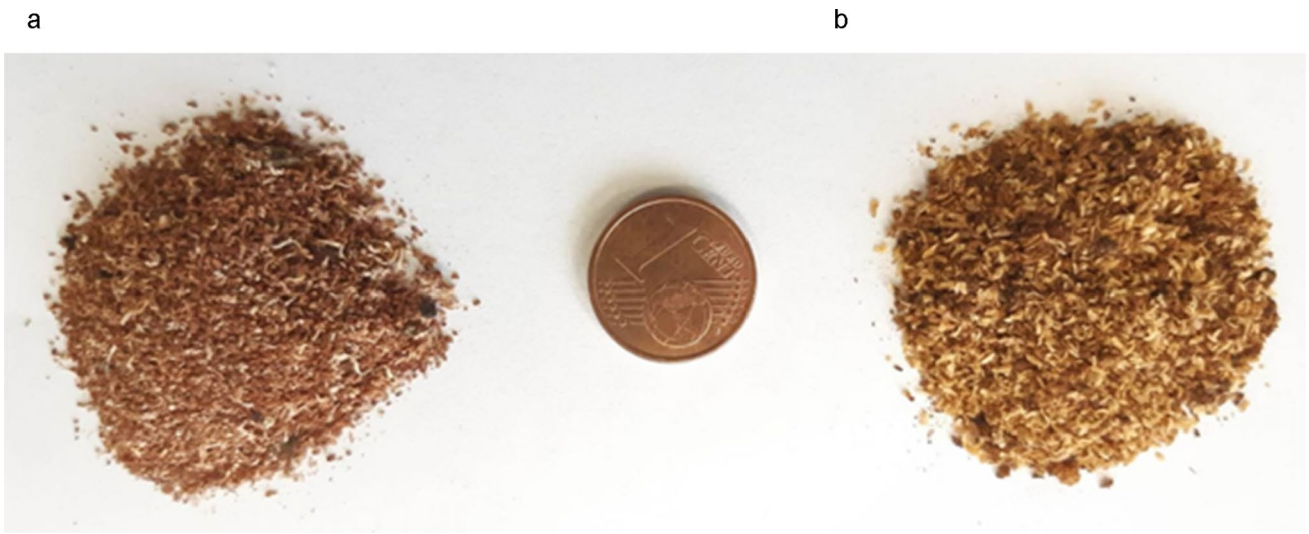

Fig. 1 *I. sexdentatus* frass (**a**) and "artificial frass" produced in laboratory using *Aromia bungii* frass and *I. sexdentatus* DNA (**b**). The size of the coin is 2 cm

Prunus armeniaca L, to a final concentration of 1 ng/µL (Fig. 1). This step allows to quantify *I. sexdentatus* DNA within frass samples, which is impossible in natural frass. Larvae, adults, and frass of various insects were used as non-target species, to evaluate the analytical specificity of the test (exclusivity). These insects were collected in the field or obtained from the collection of the University of Florence, the Phytosanitary Service of Tuscany, the University of Pisa, C.R.E.A. (Council for Research in Agriculture and the Analysis of Agricultural Economics), the University of Naples, and other national and international research centres (Table 1). When collected in the field, live specimens were frozen, preserved in ethanol 70% and stored until processed (Rizzo et al. 2020).

DNA extraction

DNA extraction on target and non-target samples (adults, larvae and frass) was performed according to the extraction protocol already described in previous papers (Rizzo et al. 2020) with different sample preparation steps depending on the starting matrices. The single insect specimen was individually ground and homogenized using Universal-U-form extraction bags (Bioreba, Reinach, Switzerland). Immediately afterwards, 3–5 mL (depending on the size and weight of the insects being investigated) of 2% CTAB (Cetyltrimethylammonium bromide) buffer (2% CTAB, 1% PVP-40, 100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA and 1% Sodium metabisulphite) was added. As for the frass samples, they were prepared differently (Rizzo et al. 2022) allowing for a homogenization phase by using steel jars with a vibromill Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) to which approximately 10 mL of 2% CTAB (Cetyltrimethylammonium bromide) buffer was added. Then, 1 mL of lysate (both from the insects/larvae and from the frass) was incubated at 65 °C for 5 min. A volume of chloroform was added, stirred by inversion, and centrifuged at 20,000 rpm for 10 min. After this step, 600 µL of the upper phase was purified using a Maxwell16 automatic extractor with the related Maxwell RSC PureFood GMO Extraction Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol (selected catalog number: AS1600). DNA was extracted in duplicate from 8 adults and 8 frass of *I. sexdentatus* deriving from each of the samples listed in Table 1. The quality and quantity of the extracted DNA were analysed using a QiaExpert spectrophotometer (Qiagen, Hilden, DE). DNA was eluted in 100 µL of nuclease-free water and used immediately in qPCR or stored at − 20 °C until use.

The qualitative evaluation was aimed at evaluating the presence of inhibitors by measuring the optical density ratio of A260/280 and A260/230 for diluted and undiluted DNA extracts. In addition, to quantitatively assess the extracted DNA and its suitability for real-time PCR tests, the DNA samples were amplified by performing a qPCR with probe targeting the highly conserved region of 18S ribosomal DNA (Ioos et al. 2009).

In addition, to investigate the operational possibilities of extracting nucleic acids from complex matrices such as insect bodies and their developmental stages, as well as the frass produced by them, three extraction methods from adult insects were compared. These methods were:

1. gDNA Clean Up Kit (Macherey Nagel) as per manufacturer's instructions;
2. FET extraction buffer protocol (Fast extraction TNA) (Edwards et al. 1991);
3. CTAB 2% protocol mentioned above (Rizzo et al. 2022).

Design of *Ips sexdentatus* primers

For the design of the oligonucletotides, we used the software OligoArchitectTM Primers and Probe Design Online (Sigma-Aldrich, St. Louis, USA) considering the sequences (Fig. 3) of genome (accession number KX035215.1) of *I. sexdentatus* (Table 2). Primer melting temperatures, amplicon length and absence of secondary structures were taken into consideration in the design of the primers and the probe. The oligos were synthesized by Eurofins Genomics (Ebersberg, Germany).

The primer specificity was analysed by the software BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) searching the most related nucleotide sequences and aligning them by the MAFFT software package (Katoh and Standley 2013) implemented with the software Geneious® 10.2.4 (Biomatters, http://www.geneious.com). The results are shown in Supplementary Material (Figure S1; Figure S2).

qPCR optimization

To determine the optimal annealing temperature, a thermal gradient was performed in the temperature range between 52 °C and 58 °C on *I. sexdentatus* DNA samples, using a CFX96 thermal cycler (Biorad, Hercules, CA, USA). To optimize the dosage for *I. sexdentatus,* two types of samples were analysed: adults and frass. All samples were tested in triplicate. The fluorescence was read at each cycle, at the end of the extension phase. The concentrations of the oligos and the probe were evaluated by testing them in combination at 0.2 µM, 0.3 µM and 0.4 µM for the primers while for the probe they were 0.1 µM, 0.2 µM and 0.3 µM. Additionally, two mastermixes with equal optimized primer and probe values were evaluated: QuantiNovaMasterMix Probe (Qiagen, Hilden, Germany) and goTaq Probe MasterMix (Promega).

Table 1 List of samples (target and non-target specimens) used in this study for the evaluation of the analytical specificity (inclusivity and exclusivity) of the test (SFR = regional phytosanitary service)

Code lot	Insect species	Order/Family	Supplier	Stage/matrix
MR 000082	*I. sexdentatus*	Coleoptera/Curculionidae	University of Florence	Adult
MR 001609	*I. sexdentatus*	Coleoptera/Curculionidae	CREA_DC_Florence	Adult
MR 001614	*I. sexdentatus*	Coleoptera/Curculionidae	University of Florence	Frass
MR 000078	*I. sexdentatus*	Coleoptera/Curculionidae	University of Florence	Adult
MR 000001	*I. sexdentatus*	Coleoptera/Curculionidae	University of Florence	Frass
MR 000002	*I. sexdentatus*	Coleoptera/Curculionidae	University of Florence	Adult
MR 000040	*I. sexdentatus*	Coleoptera/Curculionidae	University of Florence	Adult
MR 000067	*I. sexdentatus*	Coleoptera/Curculionidae	University of Florence	Frass
MR 000046	*Ips typographus*	Coleoptera/Curculionidae	SFR Tuscany	Adult
MR 000047	*I. typographus*	Coleoptera/Curculionidae	SFR Tuscany	Frass
MR 000689	*Tomicus destruens*	Coleoptera/Curculionidae	University of Florence	Adult
MR 000010	*Hylurgus ligniperda*	Coleoptera/Curculionidae	University of Florence	Adult
MR 000704	*Hylurgopinus rufipes*	Coleoptera/Curculionidae	SFR Tuscany_Leghorn	Adult
MR 000011	*Hylurgopinus rufipes*	Coleoptera/Curculionidae	SFR Tuscany_Leghorn	Adult
MR 000688	*Scolytus multistriatus*	Coleoptera/Curculionidae	CREA_DC_Florence	Adult
MR 000023	*Xyleborinus saxesenii*	Coleoptera/Curculionidae	University of Florence	Adult
MR 000061	*Xyleborinus saxesenii*	Coleoptera/Curculionidae	SFR Tuscany	Frass
MR 000075	*Xyleborus affinis*	Coleoptera/Curculionidae	SFR Tuscany_Leghorn	Adult
MR 000076	*Anisandrus dispar*	Coleoptera/Curculionidae	CREA_DC_Florence	Adult
MR 000073	*Anisandrus dispar*	Coleoptera/Curculionidae	SFR Tuscany	Frass
MR 000024	*Xyleborus monographus*	Coleoptera/Curculionidae	CREA_DC_Florence	Adult
MR 000025	*Xylosandrus compactus*	Coleoptera/Curculionidae	Isolated from monitoring	Adult
MR 000050	*Xylosandrus compactus*	Coleoptera/Curculionidae	SFR Tuscany	Frass
MR 000026	*Xylosandrus crassiusculus*	Coleoptera/Curculionidae	SFR Tuscany_Leghorn	Adult
MR 000051	*Xylosandrus crassiusculus*	Coleoptera/Curculionidae	SFR Tuscany	Frass
MR 000064	*Xylosandrus germanus*	Coleoptera/Curculionidae	University of Florence	Adult
MR 000065	*Xylosandrus germanus*	Coleoptera/Curculionidae	University of Pisa	Frass
MR 000015	*Pityokteines curvidens*	Coleoptera/Curculionidae	CREA_DC_Florence	Adult
MR 000048	*Pityophthorus juglandis*	Coleoptera/Curculionidae	University of Florence	Adult
MR 000016	*Pityophthorus juglandis*	Coleoptera/Curculionidae	SFR Tuscany	Frass
MR 000017	*Pityophthorus pityographus*	Coleoptera/Curculionidae	CREA_DC_Florence	Adult
MR 000018	*Pityophthorus pubescens*	Coleoptera/Curculionidae	University of Florence	Adult
MR 000079	*Agrilus planipennis*	Coleoptera/Buprestidae	USA	Adult
MR 000080	*Agrilus planipennis*	Coleoptera/Buprestidae	USA	Frass
MR 000081	*Agrilus auroguttatus*	Coleoptera/Buprestidae	USA	Adult
MR 000006	*Chrysobothris quadriimpressa*	Coleoptera/Buprestidae	SFR Tuscany_Leghorn	Adult
MR 000044	*Chrysobotrys femorata*	Coleoptera/Buprestidae	SFR Tuscany_Leghorn	Adult
MR 001610	*Anoplophora chinensis*	Coleoptera/Cerambycidae	SFR Tuscany	Adult
MR 000683	*Anoplophora chinensis*	Coleoptera/Cerambycidae	SFR Tuscany	Larvae
MR 000003	*Anoplophora chinensis*	Coleoptera/Cerambycidae	SFR Tuscany	Frass
MR 000041	*Anoplophora glabripennis*	Coleoptera/Cerambycidae	SFR Lombardy	Adult
MR 000063	*Anoplophora glabripennis*	Coleoptera/Cerambycidae	SFR Marche	Frass
MR 000004	*Aromia bungii*	Coleoptera/Cerambycidae	University of Naples	Adult
MR 000042	*Aromia bungii*	Coleoptera/Cerambycidae	University of Naples	Frass
MR 000005	*Aromia bungii*	Coleoptera/Cerambycidae	University of Naples	Larvae
MR 000007	*Cerambix scopolii*	Coleoptera/Cerambycidae	SFR Tuscany	Adult
MR 000008	*Cerambyx miles*	Coleoptera/Cerambycidae	CREA_DC_Florence	Adult
MR 000045	*Chlorophorus glabromaculatus*	Coleoptera/Cerambycidae	SFR Tuscany_Leghorn	Adult
MR 000012	*Lepturges confluens*	Coleoptera/Cerambycidae	SFR Tuscany_Leghorn	Adult

Table 1 (continued)

Code lot	Insect species	Order/Family	Supplier	Stage/matrix
MR 000690	*Monochamus galloprovincialis*	Coleoptera/Cerambycidae	CREA_DC_Florence	Adult
MR 000013	*Monochamus sartor*	Coleoptera/Cerambycidae	SFR–Lombardy	Adult
MR 000705	*Monochamus sutor*	Coleoptera/Cerambycidae	SFR–Lombardy	Adult
MR 000022	*Morimus asper*	Coleoptera/Cerambycidae	CREA_DC_Florence	Adult
MR 000014	*Neoclytus mucronatus*	Coleoptera/Cerambycidae	SFR Tuscany_Leghorn	Adult
MR 000047	*Phryneta leprosa*	Coleoptera/Cerambycidae	SFR Tuscany_Leghorn	Adult
MR 000019	*Saperda carcharias*	Coleoptera/Cerambycidae	University of Pisa	Adult
MR 000049	*Saperda punctata*	Coleoptera/Cerambycidae	CREA_DC_Florence	Adult
MR 000020	*Saperda scalaris*	Coleoptera/Cerambycidae	CREA_DC_Florence	Adult
MR 000021	*Saperda tridentata*	Coleoptera/Cerambycidae	SFR Tuscany_Leghorn	Adult
MR 000071	*Saperda tridentata*	Coleoptera/Cerambycidae	Isolated from monitoring	Adult
MR 000074	*Xylotrechus colonus*	Coleoptera/Cerambycidae	SFR Tuscany_Leghorn	Adult

Table 2 List of the primers and probe used in the test

Primer name	Lenght (bases)	Sequence 5′–3′	Nucleotide position	Product size (bp)	Reference sequence
Isex_3263F	18	GGACAATAGATCCAAGTG	3263–3281	129	KX035215.1
Isex_3391R	21	GTACCTAATTCAGTTCGAATA	3391–3370		
Isex_3354P	27	FAM_TTCCTACTATTCCAGATC AAGCACCAA_BHQ1	3354–3381		

Analytical specificity of the test

Analytical specificity, defined by EPPO standards (EPPO 2021) and essential to propose a test with an official feature from a molecular diagnostic point of view, must be tested both for inclusiveness (the test must be applicable to populations of targetspecies of different geographical origin) and for exclusivity (the test must discriminate between target and non-target species, especially, but not exclusively, when taxonomically related). All samples, prior to live amplifications, were normalized with a DNA/ddsH2O dilution to a working concentration of 10 ng/µL. Diagnostic reactions for evaluation of analytical specificity (inclusivity and exclusivity) were analysed in triplicate on all target and non-target samples. The master mix used in the qPCR probe reactions was the Quanti Nova MasterMix (Qiagen, Hilden, Germany). The primers and probe were diluted to 20 and 10 µM, respectively.

Analytical sensitivity of the test

The analytical sensitivity of the test was evaluated on the DNA from adults and "artificial frass" of *I. sexdentatus*. In the latter case, to simulate the natural contamination of *I. sexdentatus* ("artificial frass") in woody frass produced by the trophic activity of the various beetle stages, we have added to the lysate in extraction buffer (CTAB 2%) of frass from *P. armeniaca* produced by *A. bungii*, a volume of a solution of 10 ng/µL of DNA extracted from *I. sexdentatus* adults. This addition was aimed at obtaining a lysate "contaminated" by target insect DNA in 2% CTAB extraction buffer at a final concentration of 1 ng/µL of *I. sexdentatus* DNA. In this way, an attempt was made to quantify the presence of the insect DNA in the frass to verify both the performance from an extraction point of view and to highlight the detection limit of the presence of *I. sexdentatus* DNA in the natural frass.

The limit of detection (LOD) of the method was tested using a 1:5 serial dilution of DNA extracted from samples of *I. sexdentatus* adults and "artificial frass". Each dilution was tested in triplicate.

From the standard curve obtained, the main performance parameters of the qPCR were calculated such as the efficiency (E) and the coefficient of determination (R^2) of the qPCR using the CFX Maestro 2.0 software dedicated to the CFX96 thermal cyclers (Biorad, Hercules, CA, USA).

Test on repeatability and reproducibility of the assay

The parameters considered for intra-run (repeatability) and inter-run (reproducibility) variation were the cycle quantification means (Cq) and the corresponding standard deviation between replicates (SD). Intra-run variation (repeatability)

and inter-run variation (reproducibility) were tested on eight samples (triplicates) of adult DNA and eight samples (triplicates) of DNA from artificial frass. For reproducibility, samples were tested in two separate runs (by different operators) and mean (± SD) *C*q values were evaluated for each sample in triplicate.

Results

DNA extraction

The results of DNA extraction from whole body adults, natural and artificial frass are shown in Table 3. DNA concentration values for adults and natural frass ranged between 45–84 and 148–269 ng/µL respectively, while the A260/A280 ratios were included in the interval between 1.76–2.01 and 1.86–2.15 for the two types of matrices. The values for the quality and quantity of nucleic acids obtained from the artificial frass were similar to those obtained from the frass of *I. sexdentatus*. The data revealed the efficiency of the DNA extraction method also in a difficult matrix like frass stored at room temperature, without any particular care (Rizzo et al. 2022).

When comparing the three extraction methods, including the one used to discriminate the various matrices under study, the results were all in favour of the CTAB 2% protocol. It should be noted, however, that while the gDNA Clean Up Kit (Macherey Nagel) followed the manufacturer's instructions and the comparison was completely in favour of the CTAB 2% protocol, the second extraction method, the FET extraction buffer (Fast extraction TNA) (Edwards et al. 1991), showed much more similarity and agreement of results. In fact, in the latter case, the average *C*q values of the confront qPCRs differed by only 1–1.2 cycles. Hence, in the latter case, it is worthwhile to carry out further investigations or modifications on this protocol (FET) and for these starting matrices (object of study).

qPCR optimization

The optimized mix reaction of the proposed protocol for *I. sexdentatus* DNA was 10 µL of 2 × Quanti Nova Probe PCR Master Mix (QIAGEN, Hilden, Germany) added to 0.4 µM of primers and 0.2 µM of probe. The optimal annealing temperature was fixed at 55 °C. The complete protocol provides for an initial denaturation took place at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, and 55 °C for 40 s (Fig. 2).

Analytical specificity

The test satisfied criteria of inclusivity and exclusivity according to PM 7/98 (EPPO 2021), showing 100% of analytical specificity. All samples collected from different populations of *I. sexdentatus* resulted identified by the assay and were well distinct from the non-target species samples, without no false-positive results. The *C*q of reaction evaluated for DNA samples from *I. sexdentatus* adults and natural frass ranged between 20.88–22.01 (at a concentration of 1 ng/µL)

Table 3 Concentration and A260/280 ratio ranges (and respective averages with standard deviation) of the total nucleic acids extracted from different matrices

Matrices	Concentration		A260/A280 ratio
	Range (ng/µL)	Avg ± SD (ng/µL)	Range
I. sexdentatus (adults)	45–84	76.3 ± 12.56	1.9 ± 0.12
I. sexdentatus (natural frass)	148–269	182.2 ± 26.84	1.7 ± 0.58
I. sexdentatus (artificial frass)	175–302	220.8 ± 36.58	2.0 ± 0.47

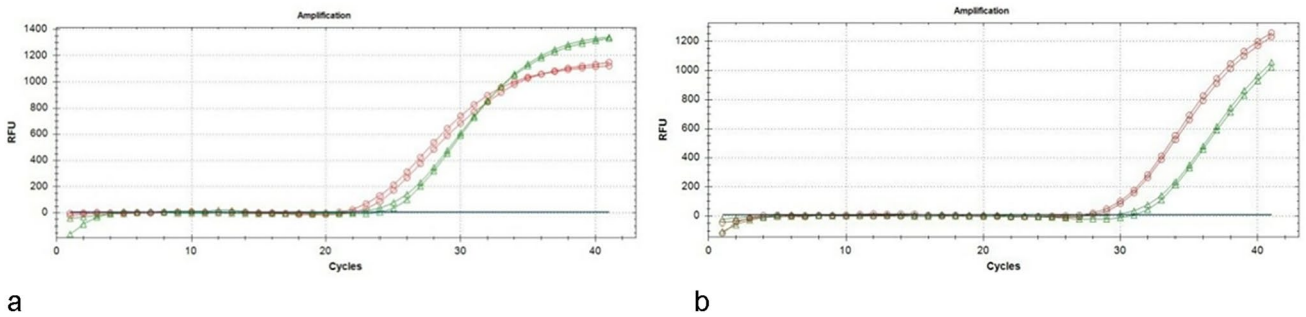

Fig. 2 Amplification curves of *I. sexdentatus*. DNA from different matrices and at two concentrations: **a** red circles: 1 ng/µL of DNA samples from adults; green triangles: 0.1 ng/µL of DNA samples from adults; **b** red circles: 1 ng/µL of DNA samples from artificial frass, green triangles: 0.1 ng/µL of DNA samples from artificial frass

Table 4 LoD values obtained for DNA samples of DNA from adults and artificial frass of *I. sexdentatus*, with serial dilutions 1:5

Adult DNA		"Artificial frass"	
Dilutions 1:5 (ng/µL)	*C*q means ± SD	Dilutions 1:5 (ng/µL)	*C*q means ± SD
10	22.24 ± 0.53	1.0	29.02 ± 0.26
2.0	23.60 ± 0.42	0.2	30.67 ± 0.53
0.4	26.41 ± 0.40	0.04	32.53 ± − 0.28
0.08	28.36 ± 0.38	0.008	34.09 ± − 0.15
0.016	30.91 ± 0.56	1.6×10^{-3}	–
3.2×10^{-3}	32.88 ± 0.43	0.32 × 10−3	–
6.4×10^{-4}	34.80 ± 0.54	0.064×10^{-3}	–
1.28×10^{-4}	36.34 ± 0.85	12.8×10^{-4}	–
2.56×10^{-5}	–	2.56×10^{-5}	–

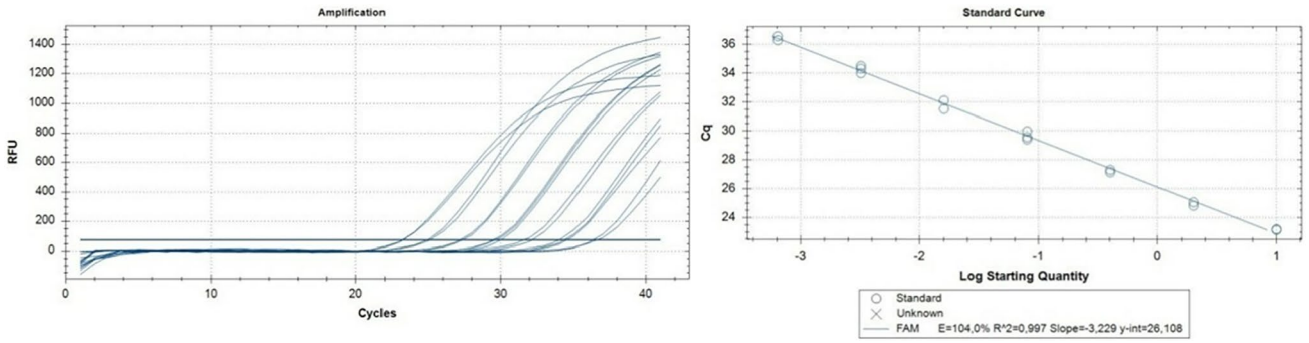

Fig. 3 Serial dilutions 1:5 of DNA extracted from *I. sexdentatus* adults. On the left, amplification curves, on the right, titration curve

and 28.69–29.83 (at a concentration of 1 ng/µL) for the two matrix samples, respectively. For the artificial frass, used at the same concentration of DNA extracts, the average values were similar to those obtained from *I. sexdentatus* natural frass, ranging from 27.29 to 27.84.

Analytical sensitivity

The LoD for the target samples of adult DNA was determined at 6.4×10^{-4} ng/µL and 1.6×10^{-3} ng/µL for artificial frass, as shown in Table 4.

From the standard curves obtained (Fig. 3), the main performance parameters of the qPCR were calculated as the efficiency (*E*) and coefficient of determination (R^2) of qPCR using CFX Maestro 2.0 (Biorad, Hercules, CA, USA).

Repeatability and reproducibility of the assay

Repeatability and reproducibility were estimated as average of *C*q values (with correspondent standard deviations, SD) for both adult and artificial frass samples; the obtained data are reported in Table 5. Low values of SD, included in the range 0.02.–0.83., demonstrate a low intra-run and inter-run variability on all tested matrices (Teter and Steffen 2017).

Table 5 Repeatability and reproducibility values (avg ± SD) of the qPCR assays were measured as average *C*q of the replicates for each series (assay adult samples and assay artificial frass samples: 0.04 ng/µL)

	Replicates no.	Repeatability (avg ± SD)	Reproducibility (avg ± SD)
Adult samples	1	28.8 ± 0.79	28.7 ± 0.12
	2	28.3 ± 0.42	28.6 ± 0.49
	3	28.2 ± 0.50	28.5 ± 0.18
	4	28.2 ± 0.32	28.1 ± 0.34
	5	27.9 ± − 0.36	28.4 ± 0.25
	6	28.6 ± 0.13	27.6 ± 0.83
	7	28.5 ± 0.30	28.4 ± 0.02
	8	28.4 ± 0.16	28.3 ± 0.25
Artificial frass samples	1	32.5 ± 0.08	32.3 ± 0.32
	2	32.6 ± 0.33	32.4 ± 0.34
	3	32.7 ± 0.47	32.4 ± 0.11
	4	32.1 ± 0.19	32.6 ± 0.16
	5	32.5 ± 0.42	32.3 ± 0.20
	6	32.5 ± 0.27	32.3 ± 0.08
	7	32.5 ± 0.24	32.3 ± 0.30
	8	32.5 ± 0.15	32.5 ± 0.07

Based on these results, the variation among the experiment replicates is acceptable (and negligible) and the test was not influenced by different operators for all the biological matrices tested.

Discussion

The international trade of timber, packaging or plants (or parts of them) is one of the main factors responsible for the transport of insect species outside their native range. Consequently, they can establish in new areas becoming invasive, often with disastrous effects (Hulme 2009).

Some insect species that have a low or insignificant phytosanitary impact in their native range may have an unexpectedly higher phytosanitary and economic impact in new areas of spread. Therefore, early diagnosis of insects at cross-border entry points or during phytosanitary monitoring activities after their accidental introduction is crucial for the management of new invasive pests. For example, early diagnosis allows timely response to the effort to mitigate the pest risk in the new territory. As a result, entomological expertise is required and samples often take a long time to prepare and examine, depending on the various developmental stages encountered, not to mention the possibility of insects present within woody tissues.

This last aspect appears onerous both in terms of time and in terms of destruction of infested or suspected material. Thus, biomolecular diagnostic tools, in the light of their ongoing advancements, can provide substantial help in the direct (Augustin et al. 2012) or indirect identification of species from their biological traces, such as frass (Rizzo et al. 2020; Ide et al. 2016a, b). In these cases, an important aspect from a laboratory point of view consists in the types of extraction protocols that are used for this type of matrix (woody frass). Indeed, such extraction methods must be high-performance but at the same time fast and reproducible. This is also in the light of the "environmental" factor in which the frass produced by the insects during the feeding phase finds itself. That is to say, in addition to the presence of PCR inhibitors to a greater extent than in other plant matrices, the degradability of the environmental DNA (eDNA) produced by *I. sexdentatus* must also be considered. The extraction protocol used in this study confirmed its performance as already demonstrated for other harmful insects and on different matrices (Rizzo et al. 2021). DNA yield was satisfactory in terms of quality and quantity. The proposed tool for the indirect diagnosis by frass of the presence of *I. sexdentatus*, moreover, appears rapid in relation to the timing of recognition of any specimens (at various developmental stages) present at the cortical level. In fact, in approximately two hours of laboratory activity, one is able to define whether or not the frass collected and analysed belongs to *I. sexdentatus*. This methodology can help both to define possible infestations in progress but also to assist in epidemiological and quantitative investigations of the pressure of the insect's populations in particular areas.

Even if the proposed qPCR test using the TaqMan probe technology is not easy to manage neither economic, despite in recent years the cost has gradually decreased, it is one of the most used techniques. Although expensive and difficult to design, this technique/approach present greater specificity in relation to precise diagnosis results. The molecular test developed here for both direct and indirect identification of *I. sexdentatus* has proven effective in terms of analytical specificity, analytical sensitivity, reliability and reproducibility.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s41348-024-00902-4.

Acknowledgements The authors would like to acknowledge the personnel of the Plant Protection Services of Tuscany for their support during the field work.

Author contributions Conceptualization of the research approach, detailed laboratory methodologies, experimental designs for this study were developed and conducted by methodologies, experimental designs for this study were developed and conducted by D. Rizzo (principal investigator) with the assistance of M.Moriconi, C. G. Zubieta, M. Carli, L. Bartolini, , C. Ranaldi, , A. Marrucci and B. Palmigiano. All sample preparations, data collection and statistical analyses of data collected from DNA extracts were completed by D. Rizzo, M.Moriconi, C. G. Zubieta, M. Carli, L. Bartolini, C. Ranaldi, A. Marrucci, B. Palmigiano, M. Bracalini, A. P. Garonna, and T.Panzavolta. Revisions of the manuscript were completed by all authors.

Funding Open access funding provided by Università di Pisa within the CRUI-CARE Agreement.

Declarations

Conflict of interest The authors declare no conflict of interest.

References

Augustin S, Boonham N, Kogel WJ, Donner P, Faccoli M, Lees DC, Marini L, Mori N, Petrucco Toffolo E, Quilici S, Roques A, Yart A, Battisti A (2012) A review of pest surveillance techniques for detecting quarantine pests in Europe. Bull EPPO 42:515–551. https://doi.org/10.1111/epp.2600

Bracalini M, Croci F, Ciardi E, Mannucci G, Papucci E, Gestri G, TiberiPanzavoltaT R (2021) *Ips sexdentatus* mass-trapping: mitigation of its negative effects on saproxylic beetles larger than the target. Forests 12(2):175. https://doi.org/10.3390/f12020175

Bueno A, Diez JJ, Fernandez MM (2010) Ophiostomatoidfungi transported by *Ips sexdentatus* (Coleoptera, Scolytidae) in *Pinus pinasterin* NW Spain. Silva Fennica 44:387–397

Chararas C (1962) Étude biologique des scolytides des conifères. Encyclopedie Entomologique, Ser. A, PaulLechevalier, Paris, pp 38

Croisé L, Dreyer E, Lieutier F (1998) Effects of drought stress and severe pruning on the reaction zone induced by single inoculations with a bark beetle associated fungus (Ophiostomaips) in the phloem of young Scotspines. Can J for Res 28:1814–1824. https://doi.org/10.1139/x98-155

Douglas HB, Cognato AI, Grebennikov V, Savard K (2019) Dichotomous and matrix-based keys to the Ips barkbeetles of the World (Coleoptera: Curculionidae: Scolytidae). Can J Arthropod Identif 38:234

EdwardsK JC, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19(6):1349. https://doi.org/10.1093/nar/19.6.1349

EFSA (2017) Pest categorisation of *Ips sexdentatus*. EFSA J 15(11):4999

EPPO (European and mediterranean plant protection organization) (2017) EPPO Global Database. https://gd.eppo.int

EPPO (2021) PM 7/98 (5) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. EPPO Bull 51:468–498

EPPO (2023) *Ips sexdentatus*. EPPO datasheets on pests recommended for regulation. https://gd.eppo.int/taxon/IPSXSE/datasheet

Hulme PE (2009) Trade, transport and trouble: managing invasive species pathways in an era of globalization. J Appl Ecol 46:10–18

Ide T, Kanzaki N, Ohmura W, Okabe K (2016a) Molecular identification of the western drywood termite (Isoptera: Kalotermitidae) by loop-mediated isothermal amplification of DNA from fecal pellets. J Econ Entomol 109:2234–2237. https://doi.org/10.1093/jee/tow167

Ide T, Kanzaki N, Ohmura W, Okabe K (2016b) Molecular identification of an invasive wood-boring insect *Lyctus brunneus* (Coleoptera:Bostrichidae: Lyctinae) using frass by loop-mediated isothermal amplification and nested PCR assays. J Econ Entomol 109:1410–1414. https://doi.org/10.1093/jee/tow030

Ioos R, Fourrier C, Iancu G, Gordon TR (2009) Sensitive detection of *Fusarium circinatum* in pine seed by combining an enrichment procedure with a real-time polymerase chain reaction using dual-labeled probe chemistry. Phytopathology 99:582–590. https://doi.org/10.1094/PHYTO-99-5-0582

Jactel H, Gaillard J (1991) A preliminary study of the dispersal potential of *Ips sexdentatus* (Boern) (Col., Scolytidae) with an automatically recording flight mill. J Appl Entomol 112:138–145

Katoh KM, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30(4):772–780. https://doi.org/10.1093/molbev/mst010

Lévieux J, Lieutier F, Delplanque A (1985) *Les Scolytes* ravageurs du *Pin sylvestre*. Revue Forestière Francaise 37:431–440

Ozcan GE, Eroglu M, Akinci HA (2011) Use of pheromone-baited traps for monitoring *Ips sexdentatus* (Boerner) (Coleoptera: Curculionidae) in oriental spruce stands. Afr J Biotechnol 10:16351–16360

Pineau X, Bourguignon M, Jactel H, Lieutier F, Sallé A (2017) Pyrrhic victory for bark beetles: successful standing tree colonization triggers strong intraspecific competition for off spring of *Ips sexdentatus*. For Ecol Manag 399:188–196

Rizzo D, Taddei F, Da Lio D, Nugnes F, Barra E, Stefani L, Bartolini L, Griffo RV, Spigno P, Cozzolino L, Rossi E, Garonna AP (2020) Identification of the red-necked longhorn beetle *Aromia bungii* (Faldermann, 1835) (Coleoptera: Cerambycidae) with Real-Time PCR on frass. Sustainability 12:6041. https://doi.org/10.3390/su12156041

Rizzo D, Da Lio D, Bartolini L, Salemi C, Del Nista D, Aronadio A, Pennacchio F, Binazzi F, Francardi V, Garonna AP, Rossi E (2021) TaqMan probe assays on different biological samples for the identification of three ambrosia beetle species, *Xylosandrus compactus* (Eichoff), *X. crassiusculus* (Motschulsky) and *X. germanus* (Blandford) (Coleoptera Curculionidae Scolytidae). 3Biotech 11:259. https://doi.org/10.1007/s13205-021-02786-9

Rizzo D, Da Lio D, Bartolini L, Francia C, Aronadio A, Luchi N, Campigli S, Marchi G, Rossi E (2022) Extraction methods to obtain high DNA quality from different plant tissues. Plant Pathol: Methods Protoc, Methods in Mol Biol. https://doi.org/10.1007/978-1-0716-2517-0_5

Rossi JP, Samalens JC, Guyon D, Van Halder I, Jactel H, Menassieu P, Piou D (2009) Multiscale spatial variation of the bark beetle *Ips sexdentatus* damage in a pine plantation forest (Landes de Gascogne, SouthwesternFrance). For Ecol Manage 257:1551–1557

Teter S, Steffen L (2017) Real-time qPCR: guidelines for a comparison of reagent performance. Retrieved from Promega Corporation website: https://ita.promega.com/resources/pubhub/applications-notes/an299-real-time-qpcr-guidelines-for-a-comparison-of-reagent-performance/