

Identification of the ambrosia beetle *Anisandrus dispar* (Fabricius) (Coleoptera Curculionidae Scolytinae) using TaqMan™ probe assay on biological samples

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The European shot-hole borer *Anisandrus dispar* (Fabricius) (Coleoptera Curculionidae Scolytinae) is a well-known ambrosia beetle living on shrubs and several ornamental and fruiting trees where it can cause heavy damages. Like other harmful xyleborine species, *A. dispar* can represent a potential threat outside its native region. Molecular diagnostic tools can lead to accurate identification of xylophagous species hidden in wooden matrix in phytosanitary surveys at entry points. A molecular assay based on qPCR TaqMan™ Probes was developed for the identification of *A. dispar* from different matrices. To setup and perform the test, DNA extraction was carried out from adults, larvae, and artificial samples of wood chips from oak healthy plants whose lysates were contaminated with a known amount of DNA of *A. dispar* adults. The assay has proven inclusive for *A. dispar*, and exclusive towards the non-target organisms, showing 100% analytical specificity. The limit of detection was 0.32 pg μL^{-1} for the samples of insect adult DNA of *A. dispar*, and at 0.8 pg μL^{-1} for the samples containing lysates of *Quercus* spp. and 0.1 ng μL^{-1} of *A. dispar* adult DNA. Repeatability and reproducibility showed low values independently from the matrix used for DNA extraction, confirming the possible use in diagnostics of biological samples even if not directly related to the presence of *A. dispar* developmental stages. The presented approach may be adjusted and applied for phytosanitary purposes to other quarantine pests and rapidly detect possible infestations in vegetal matrices globally traded.

Keywords: European Shot-hole Borer, Xyleborini, Quarantine Species, Molecular Diagnostics, Biological Traces

Introduction

Increasing threats to forest and fruiting trees around the world is driven by the introduction and establishment of exotic Ambrosia and bark beetles (Curculionidae: Scolytinae and Platypodinae) outside native territories. The Ambrosia beetle *Anisandrus dispar* (Coleoptera Curculionidae Scolytinae) is a Palearctic species widely distributed in Europe and Asia (Smith et al. 2020) and established across North Amer-

ica, where it should be considered a high-risk invasive quarantine species (Rabaglia et al. 2006, Gomez et al. 2018). The host plant range include many species of deciduous trees and conifers (French & Roeper 1975, Wood & Bright 1992, Sauvard 2004, Galko et al. 2014). Although *A. dispar* usually attacks decaying trees, this beetle can colonize and severely infest wounded, injured or apparent healthy plants (Tanasković et al. 2016). Infestations may be par-

ticularly serious on hazelnut crops (Bucini et al. 2005, Speranza et al. 2009, Saruhan & Akyol 2012) and damages have been recorded in pear orchards (Tanasković et al. 2016). Like other harmful xyleborine species, *A. dispar* can represent a potential threat outside its native range (Gohli et al. 2016).

The identification of xylophagous species in phytosanitary practice is generally rather complicated. Traditionally adult identification is time consuming with continuous comparison among similar morphological characters to distinguish a genus from a subfamily or a species from cogeneric ones (Cognato et al. 2020, Smith et al. 2020). Today the use of molecular diagnostic tools lead to accurate and ease identification and time savings. In all those cases where only signs of damage (empty galleries or with traces of frass) are available or juveniles hidden inside woody tissues with the issue to extract the intact bodies from galleries, traditional approaches show their limits. The molecular diagnosis becomes particularly useful if this tool can be applied to obtain results from indirect evidence. In fact, the identification of xylophagous species from frass has already been developed for several species (Ide et al. 2016a, Ide et al. 2016b, Rizzo et al.

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Tab. 1 - List of samples of the target species *Anisandrus dispar* used for DNA extraction.

Sample	Specimens in the sample	Sample content
i	<i>Anisandrus dispar</i>	DNA from single adults and larvae
ii	<i>A. dispar</i> and <i>Q. cerris</i>	Mix at a ratio 1:5 of lysates from <i>A. dispar</i> adult and artificial sawdust, both of CTAB 2%
iii	<i>A. dispar</i> and <i>Q. cerris</i>	Mix of 0.1 ng/ μ L adult DNA and <i>Q. cerris</i> lysate in CTAB 2%

2020a, 2020b, Taddei et al. 2021). Moreover, the use of biological traces (exuviae, incomplete bodies, frass or faeces) for the identification at species level can be decisive, especially when fast, reliable and unambiguous diagnosis are needed to identify the presence of quarantine or invasive species in time (Rizzo et al. 2020a, Rizzo et al. 2021). From a phytosanitary point of view the setup of molecular protocols ready to use may support early pest detection carried out by any Plant Protection and Inspection Service (Wu et al. 2017).

More than 30 species belong to the genus *Anisandrus* including forest threatening pests (Smith et al. 2020). Morphologically *A. dispar* could be confused with the smaller *A. maiche* Kurentzov (Gomez et al. 2018, Smith et al. 2020). Recently, this Asian species has been recovered in European Russia and Ukraine (Mandelstam et al. 2018), Italy (Colombari et al. 2022, Ruzzier et al. 2022) and Switzerland (EPPO 2022). Consequently, a precise molecular identification of *A. dispar* is considered to be of crucial importance to support its easy discrimination among congeneric exotic pests and avoid accidental introductions of non-native *Anisandrus* species across Europe.

Here we propose a molecular diagnostic test based on qPCR with TaqMan™ Probe technology developed for the identification of *A. dispar* using DNA extracted from samples of larvae, adults, and artificial sawdust obtained from healthy *Quercus cerris* branches, and subsequently contaminated with known amounts of *A. dispar* adult DNA. This approach could be shared and applied to several other organisms/insects for the development of standard identification methods.

Materials and methods

Biological samples

Adults and larvae of *Anisandrus dispar* were collected during routine monitoring

inspections carried out by the Phytosanitary Service in Tuscany, or by other research institutions in the respective Regions (Tab. 1). Larvae and adults were stored in 70% ethanol solution at room temperature until use. Artificial sawdust samples to simulate the frass of *A. dispar* were obtained by holing healthy branches (5-10 cm diameter) from oak trees (*Quercus cerris*) with a manual drill. All samples were stored at room temperature in the lab until use, for a period ranging from 2 to 8 months to simulate the conditions in which natural samples could be collected.

The non-target insects (adults, larvae and/or frass in the case of xylophagous species) used for testing the analytical specificity of the assay, belonged to the biomolecular collection of the Phytopathological Lab of the Phytosanitary Service of the Tuscany Region, Italy. The non-target insects chosen were several other xylophagous beetles and moths, as specified in Tab. S1 (Supplementary material).

DNA extraction from target and non-target samples

Genomic DNA from all target samples was extracted using the modified protocol suggested by Li et al. (2008), until the addition of chloroform and the subsequent centrifugation at 11,500 g for 5 min. Some 600 μ L of the upper phase was purified using the Maxwell® RSC PureFood GMO and Authentication Kit in combination with the automated purificator MaxWell 16 (Promega, Madison, WI, USA). Details of the purification have been previously described (Rizzo et al. 2020a, 2020b). The DNA of non-target samples was extracted using the same procedure used for target samples, but in different times.

DNA extraction was carried out with the same methodology on different matrices as shown in Tab. 1: (i) single larvae and adults of *A. dispar*; (ii) lysate of CTAB 2% buffer of *A. dispar* adult added at lysate of CTAB 2% buffer from artificial sawdust at a

ratio 1:5; (iii) *A. dispar* adult DNA (0.1 ng μ L⁻¹) mixed with artificial sawdust lysate in 2% CTAB buffer.

Serial dilutions 1:5 were made, and DNA extraction was carried out in duplicate for each dilution step, to verify the analytical sensibility of the extraction method. Extract dilutions were 5⁻¹ - 5⁻⁷ for samples (ii) and 0.1 ng μ L⁻¹ - 0.24 fg μ L⁻¹ for samples (iii).

These different samples used for DNA extractions were chosen to test the sensibility of the method in discriminating *A. dispar* target specimens (DNA and lysate) from host plant lysate, simulating the indirect diagnosis of the woodborer from the natural frass (unavailable in our experiment).

The quality of the extracted DNA was assayed in qPCR after a dilution 1:20 of DNA in ddH₂O; a dual-labeled probe (18S uni F/-R) and a YakimaYellow-BHQ1 dual labeled probe (18S uni-P) targeting a highly conserved region of the 18S rDNA was used in the reaction (Taddei et al. 2021, Ios et al. 2009). The amplification tests of DNA samples were used as control and allowed to verify the presence of inhibitors in relation to both the C_q (quantification cycle) detected and the slope of the relative amplification curves.

Design of primers and probe and relative optimization

The software Oligo Architect Online® (Sigma-Aldrich, St. Louis, MO, USA) was used to design the primer pairs and probes, targeting the conserved sequences (voucher BMNH 1047383 mitochondrion, complete genome) of *A. dispar* genome (accession number KX035217.1) calculating the product size, the melting temperature and primer length. The absence of secondary structure was also considered when possible. For the development of the qPCR probe method, the sequences used are shown in Tab. 2.

An *in-silico* test of the primer pairs was then performed with the BLAST® software (Basic Local Alignment Search Tool - <http://www.ncbi.nlm.nih.gov/BLAST>) to assess the specificity of the designed primer pairs and probe.

The *in-silico* specificity was further verified searching for the most related nucleotide sequences by means of the BLAST software, using as query the expected amplicons of the probe qPCR protocol. The sequences were aligned using the MAFFT program (Katoh & Standley 2013) implemented within the software Geneious® ver.

Tab. 2 - List of the primers and probes designed for qPCR TaqMan protocol for *Anisandrus dispar*.

Method	Primer/Probe name	Length (bp)	Sequence 5' - 3'	Nucleotide position	Product size (bp)
TaqMan Probe	Xdisp_2302F	21	CCACCATTAGCTGCCAATATC	2302 - 2323	151
	Xdisp_2452R	21	GTTCGGGCTTTATTCTGTAG	2452 - 2431	
	Xdisp_2325P	27	HEX_CCACGAAGGAGCATCTGTTGACCTAGC_BHQ1	2325 - 2352	



Fig. 1 - Alignments resulting from the *in silico* theoretical TaqMan amplicon and sequences of the related organisms present in GenBank.

10.2.6 (Biomatters, Auckland, NZ – <http://www.geneious.com>). The results are shown in Fig. 1 and Fig. 2.

qPCR protocols

To determine the optimal annealing temperatures for the developed protocol, the temperature gradient from 52 to 62 °C were tested on 8 DNA samples extracted from two adults of *A. dispar* (4 samples of type (i) for each specimen – Tab. 1). Oligos and probes were used at different concentrations: 0.2 μM, 0.3 μM and 0.4 μM each. Samples were tested as technical duplicates. The amplification reactions in qPCR Probe were performed with a CFX96 (Bio-rad, Hercules, CA, USA) thermocyclator in a final volume of 20 μL. Data obtained were

analyzed with the software CFX Maestro ver. 2.0 (BioRad) using automatic thresholds and baselines for FAM (Fluorescein Amidite Dye). Samples were considered positive when the correspondent qPCR curves showed a clear inflection point and an increasing kinetics, and C_q values <32.

Validation of the method for the qPCR Probe

In view of the possibility to apply the test in routine diagnostics, performance criteria such as analytical sensitivity, analytical specificity, repeatability and reproducibility were determined. Validation was performed according to EPPO standard PM7/98 (5) updated in 2021 for insect samples (larvae and adults) and for the artificial

sawdust. The parameters true positives, false negatives, false positives, and true negatives were considered according to the quoted EPPO standard (EPPO 2021).

Analytical sensitivity (limit of detection, LoD) of the test was estimated on 1:5 serial dilutions of samples (i), (ii) and (iii) previously described in Tab. 1.

DNA extracts were diluted to 5 ng μL⁻¹ for DNA from *A. dispar* adults. Two μL of template were added to each sample and 3 replicates were set up for each sample. The evaluation range was included between 10 ng μL⁻¹ up to 25.6 fg μL⁻¹. All measurements were made using the QIAxpert® system (QIAGEN, Hilden, Germany).

The repeatability was tested on 8 DNA samples extracted from 2 adult (5 ng μL⁻¹)

the increasing rate of accidental introduction of ambrosia beetles throughout the world, a phenomenon reported regularly for temperate climate zones (Rassati et al. 2015, Meurisse et al. 2018). The economic and ecological consequences of the establishment by invasive Scolytinae represent serious threats to agriculture and forestry; therefore, timely alien species detection and identification in import is crucial (Poland & Rassati 2019, Cognato et al. 2020).

The availability of easy-to-handle discrimination methods is crucial for the identification of invasive scolytine species. However, the traditional taxonomic keys essential to identify adults are reserved to experienced staff figures not always on hand (Vega & Hofstetter 2014, Cognato et al. 2020). Moreover, the problematic identification of intercepted juvenile stages of these pests is another open issue (Pennacchio et al. 2012, Wu et al. 2017).

Nowadays, the constant updating of still incomplete DNA sequence databases may facilitate accurate beetle identification with time (Briski et al. 2016) as well as the continuous developing/tuning of enhanced molecular protocols may better support the inspection efforts at points of entry where a rapid identification is required (Rabaglia et al. 2019, Avtzis & Lakatos 2021).

Different DNA extraction methods have been compared and proposed by authors to suggest less expensive, reliable and not time-consuming methods providing highest purity of extracts needed for accurate bark beetle identification (Albo et al. 2020, Amini et al. 2020).

The test developed for the molecular identification of *Anisandrus dispar* in this study showed good performance in terms of inclusivity and exclusivity, analytical sensibility, repeatability, and reproducibility. Although the test is not economic, its reliability and rapidity make it a possible candidate as official standard identification method for *A. dispar* from different matrices. In a previous paper (Rizzo et al. 2021), a molecular diagnostic test based on qPCR with TaqMan probe technology has been developed for three species belonging to the genus *Xylosandrus* (Coleoptera Curculionidae Scolytinae). The results obtained for these Ambrosia beetles were comparable, in terms of analytical sensibility, to those obtained for *A. dispar*.

For the identification of *A. dispar*, an artificial frass created in the lab has been used to simulate the natural one. This choice has been supported by previous experiments where both types of frass were processed (Rizzo et al. 2020c). The comparison of the obtained experimental data related to DNA extraction showed excellent results. This further evidence supports the idea that the artificial sawdust can be used to finalize the molecular identification test, since the artificial samples are not subjected to the degradation by environmental factors (temperature, humidity) as well as by enzyme activity that can denature part of in-

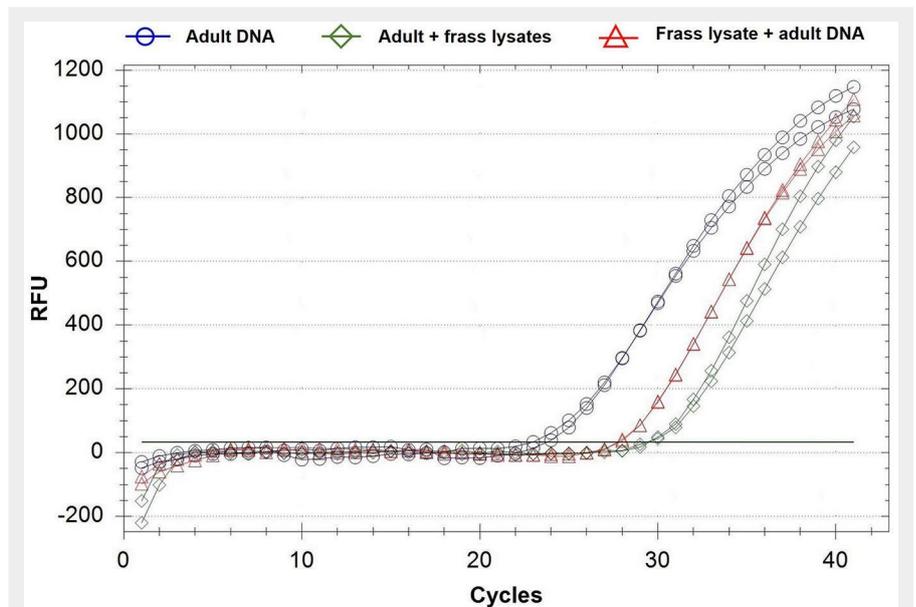


Fig. 3 - Amplification curves of *Anisandrus dispar* DNA from different matrices. Lines with blue circles: samples i) with 1 ng/ μ L of DNA from adult specimens; lines with green diamonds: sample ii) lysate from *A. dispar* adults added to lysate of *Quercus cerris* both in CTAB 2%; lines with red triangles: samples iii) lysate in CTAB 2% of *Q. cerris* contaminated to 0.1 ng/ μ L of DNA from *A. dispar* adults.

sect DNA (Strangi et al. 2013). The obtained results confirm the soundness of this approach and underlines the need to proceed with the fine-tuning of other easy to arrange DNA extraction protocols for phytosanitary purposes. These new tools might be certainly applied in all those cases where a possible presence of quarantine pests in different plant matrices is suspected (e.g., at ports of entry).

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization of the research approach, detailed laboratory methodologies, experimental designs for this study were developed and conducted by DR (principal investigator) with the assistance of ADA, LB, CR, AM, IS, and DDL. All sample preparations, data collection and statistical analyses of data collected from DNA extracts were completed by DR, ADA, DDL, and LB. DR, APG, FP and ER wrote the manuscript and formatted the draft. Revisions of the manuscript were completed by all authors.

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Supplementary Material

Tab. S1 - List of target and non-target insects and biological traces (frass) used in the test.

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