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The rapid identification of *Anoplophora chinensis* (Coleoptera: Cerambycidae) from adult, larva and frass samples using TaqMan Probe assay

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Abstract

A molecular diagnostic method using TaqMan probe qPCR is presented for the identification of *Anoplophora chinensis* (Förster) from whole body insects (adults and larvae) and frass samples stored under different conditions. The results showed a perfect amplification of DNA from all samples; the repeatability and reproducibility of the protocol were very good, with standard deviations of inter-run and intra-run variability less than or equal to 0.5. The assay allowed to discern all *A. chinensis* samples from those of the other non-target wood-borer species, with 100% correspondence to the homologous sequences. No amplification or cross reactions were observed with *A. glabripennis* (Motschulsky), which is the most related species among those tested. The protocol was validated by an internal blind panel test which showed a good correspondence between the results obtained by different operators in the same lab. The analytical sensitivity for the lab frass with the Probe qPCR, namely the lowest amount of *A. chinensis* DNA that can be detected (LoD), was 0.64 pg/µL with a Cq of 34.87. The use of indirect evidence for the identification of a pest is an important feature of the method, which could be crucial to detect the presence of wood-boring insects. This diagnostic tool can help prevent the introduction of *A. chinensis* into new environments or delimit existing outbreak areas thanks to indirect frass diagnosis.

Keywords: Citrus longhorned beetle, quarantine pests, frass, molecular tool, insect pest diagnostics

Introduction

Anoplophora chinensis (Förster, 1771) (Coleoptera Cerambycidae), the citrus longhorned beetle, is a polyphagous woodborer of great economic importance worldwide (Haack et al. 2010). It is native to eastern Asia; however, it has also spread to many countries (EFSA 2019). Today the species has become established in many Asiatic countries (China, Indonesia, Korea, Malaysia, Myanmar, the Philippines, Taiwan, and Vietnam) and in Italy (in Lazio, Lombardy and two Tuscan provinces). In the rest of Europe and the United States (EFSA 2019; Hérard and Maspero 2019; EPPO 2021) it has either never been present, eradicated or is in the process of being eradicated. The threat posed by its accidental importation is high, and the species is considered a quarantine pest in many countries. The host plants include many forest and ornamental species as well as fruiting trees of economic importance (Haack et al. 2010). According to Sjöman et al. (2014), there are 108 woody host species belonging to 73 genera known in the literature. The *A. chinensis* life cycle typically lasts one to two years, and up to three in the northern areas. The adults emerge from the summer to mid-autumn. After mating, oviposition occurs in the lower part of the trunk. Single eggs are deposited by the female under the bark. A female can deposit up to 100 eggs on different host plants. The eggs hatch after 2-3 weeks, and the larvae bore galleries towards the roots of healthy plants, often compromising the life of the plant and the quality of the wood (Haack et al. 2010). The citrus longhorned beetle is recognised to have a low diffusion capacity (EFSA 2019), and it has been observed mainly in urban areas or at the edges of woods, but never within forests. The trade in bonsai or other plants is indicated as the main diffusion method of *A. chinensis*, followed by the wood packaging (EFSA 2019). Mitigating the risk of these accidental introductions involves a robust regulatory framework for plant protection and efficient quarantine measures, as well as reliable pest identification.

The morphological identification of *A. chinensis* larval stages requires a specific expertise, although a user-friendly dichotomous key based on morphological characters of larvae is available to distinguish between *A. chinensis* and related species (Pennacchio et al. 2012).

47 This study presents an original diagnostic protocol using qPCR with a hydrolysis probe (TaqMan)
48 for identifying *A. chinensis* from whole-body insects (larvae or adults) and frass. Frass was used by
49 Strangi et al. (2013) for the molecular identification of the citrus longhorned beetle with endpoint
50 PCR. Frass is a matrix containing mainly chewed wood fragments produced by newly emerging
51 adults leaving the pupation chamber, or wood fragments and faeces that wood-boring larvae
52 produce inside the tunnels and push outside. In either case, frass has proven to be a suitable matrix
53 for the identification of other xylophagous species (Blake et al. 2014; Ide et al. 2016a,b) in addition
54 to *A. chinensis* (Kethidi et al. 2003) and *A. glabripennis* (Rizzo et al. 2020b). The use of frass for
55 identification enables sample collection without destroying the host plant (Nagarajan et al. 2020),
56 although the DNA can be degraded by negative environmental conditions and/or the presence of
57 DNA amplification inhibitors (Strangi et al. 2013).

58 **Materials and methods**

59 **Biological samples.** Samples of *A. chinensis* as well as of non-target species used in the assays are
60 shown in Table 1. Samples were collected in Tuscany (the provinces of Pistoia and Prato), between
61 2015 and 2019, during the monitoring and management activities carried out by the Tuscan
62 Phytosanitary Service as part of the mandatory control measures. The specimens, including larvae
63 and adults, were collected, frozen, and then preserved in 70% ethanol at room temperature. The
64 frass samples were derived from three different sources: a) the so-called "environmental" samples
65 were sampled from infested *Acer* sp., *Aesculus* sp. and *Populus* sp. trees in the field, with the frass
66 collected directly from the tunnels or on the ground, at the foot of the plant; b) the "lab" samples
67 were obtained by rearing *A. chinensis* in the lab on poplar stumps infested with the citrus long-
68 horned beetle and enclosed in a wired mesh to prevent adult flight; c) a third type of frass was
69 collected in the field and stocked at room temperature for five years ("five-year-old" samples). Fig.
70 1 shows the different structure of the environmental frass (Fig. 1A) and the lab frass (Fig.1B). To
71 evaluate the possible role of environmental factors on DNA degradation and assay performance, the
72 three sample types were kept separate throughout the experiment.

73 The frass samples (irrespectively of their origin) were collected and preserved in glass tubes at
74 room temperature until the DNA had been extracted.
75 The non-target DNA samples were extracted from adults, larvae and, when available, from the frass
76 of other species belonging to the insect molecular collection at the Phytopathological Lab of the
77 Tuscan Phytosanitary Service. The non-target samples were used for inclusiveness and diagnostic
78 specificity tests for comparison with *A. chinensis*. These samples were collected in several regions
79 in Italy by local Phytosanitary Service Inspectors during their routine monitoring or, in some cases,
80 were provided by research institutions. In all samples, the insects were identified on the basis of
81 specific morphological characters.
82 **DNA extraction from insect samples and frass**
83 DNA was extracted from both insect samples and frass, according to the protocol described in detail
84 in a previous work Rizzo et al. 2020a), modified with a CTAB 2% extraction buffer and the
85 automated purificator MaxWell 16 (Promega, Madison, WI, USA).
86 To test the quality of the extracted DNA from the insect samples, an aliquot of each extract was
87 diluted in double distilled water (1:20) and tested in a qPCR reaction, using a dual-labeled probe
88 and targeting a highly conserved region of 18S rDNA (Ioos et al. 2009).
89 To evaluate the amplifiability of nucleic acid extracts, qPCR reactions on DNA frass samples were
90 performed using a TaqMan dual-labeled probe, targeting a highly conserved region of the
91 cytochrome oxidase gene (Ioos et al. 2009). The amplifiability tests carried out on both frass and
92 whole-body insect samples, were used to check the efficiency of extractions and to verify the
93 presence of inhibitors through the detected Cq and the slope of the amplification curves.
94 The concentration of DNA of each sample was determined using QIAxpert System (Qiagen,
95 Hilden, Germany).

Design of primers and hydrolysis probe. The primer pairs and probe were designed preferentially within conserved region sequences of *A. chinensis* using the *OligoArchitect™ Primers and Probe Online* software (Sigma-Aldrich, St. Louis, USA) with the following parameters: 80 to 200 bp product size, melting temperature (Tm) from 55 to 65°C, primer length 18 to 22 bp, and absence of secondary structure when possible. The primers/probe used in this study are reported in Table 2. The genomic region used as the template was the sequence of *A. chinensis* mitochondrion, complete genome deposited in GenBank (accession No. KT726932.1). An *in silico* test of the primer pairs was then performed with BLAST® (Basic Local Alignment Search Tool: http://www.ncbi.nlm.nih.gov/BLAST) to assess the specificity of the designed primer pairs (Fig.2). As a further check on the specificity *in silico*, the nucleotide sequences related to the qPCR Probe (337F/374P/447R) were aligned using MAFFT (Katoh and Standley 2013) implemented in Geneious® 10.2.6 (Biomatters: http://www.geneious.com) (Kearse et al. 2012).

Optimization of the assay. In the qPCR probe protocol, temperatures ranging from 52° to 60°C were used to determine the optimal annealing temperatures. Two technical replicates were performed for all reactions using a CFX96 (Biorad, Hercules, CA, USA) thermal cycler. Fluorescence was read at each cycle, at the end of the extension step.

The concentrations of the oligos and probe were tested at 0.2 µM, 0.3 µM and 0.4 µM for oligos and 0.1 µM, 0.2 µM and 0.3 µM for the probe. To evaluate the robustness of the methods, two different master mixes from two companies were used for a comparison: the Quanti Nova MasterMix Probe (Qiagen, Hilden, Germany) was compared with the iTAQ MasterMix (Biorad, Hercules, CA, USA).

The diagnostic specificity of the qPCR probe protocol was tested for both target and non-target species to compare the inclusivity and exclusivity in the different matrices analyzed. Samples (target and non-target) were normalized with a dilution DNA/ddsH_2O at a working concentration of 10 ng/µL before real time amplification.

Validation of TaqMan assay. The analytical sensitivity, specificity and repeatability/reproducibility were determined according to EPPO Standard PM 7/98(4) (EPPO 2019).

Diagnostic inclusivity and exclusivity were assayed in triplicate on all target and non-target samples. To test the performance parameters (specificity, sensitivity, and accuracy) of the assay, an internal panel was used with blind samples processed by different operators at different times. A total of 38 samples were assayed in the study, and in particular: for the non-target species, two samples of *Cossus cossus* L., two of *Aromia bungii* (Faldermann), four of *A. glabripennis* (Motschulsky) frass; for the target species *A. chinensis*, twenty-two samples of frass (ten of the environmental frass, ten of the lab frass and two of the five-year-old frass), four adults and four larvae were tested (Table 1). Each sample was numbered and handed over to another operator to be processed.

The master mix used in qPCR was QuantiNova MasterMix for the probe (Qiagen, Hilden, Germany). Primers and probes were diluted at 20 µM and 10 µM, respectively. A total amount of 100 µL of each extracted DNA (at a normalized concentration of 10 ng/µL) was used internally for the blind test. The true positive parameters, false negatives, false positives, and true negatives were considered (EPPO 2019).

Standard parameters such as the cycle quantification mean (Cq), and the standard deviation (SD) were taken into account to calculate the intra-run variation (repeatability) and inter-run variation (reproducibility).

To estimate the repeatability, eight samples were tested in triplicate in two separate runs and the SD was calculated for each sample. In estimating the reproducibility, the data of two analyses carried out by different operators at different times were compared.

LoD was estimated using a 10-fold 1:5 serial dilution of DNA extracted from an "artificial frass", obtained by adding a DNA extract (100 ng/µL) from the frass of another wood borer (*Aromia bungii*) to 10 ng/µL of DNA extract from *A. chinensis* larvae. Each dilution was tested in triplicate over 10 1: 5 dilutions (from 10 ng/µL of *A. chinensis* larvae to 0.01 fg/µL). From the standard curve obtained, the main performance parameters of the qPCR were calculated as the efficiency (E) and coefficient of determination (r^2) of qPCR reaction using CFX Maestro 1.0 (Biorad, Hercules, CA, USA). Data were analyzed using the statistical software SPSS version 26.0 (SPSS Inc., Chicago, IL, USA).

Results

DNA extraction from frass and insect samples. The DNA extracted from frass was of a high quality and sufficient quantity (the maximum was reached in environmental frass samples with an average concentration of 313.24±39.85 ng/µL and an average 260/280 nm absorbance ratio of 2.02±0.02). The efficiency of the extraction protocol was confirmed by qPCR (COX) probe with an average Cq of 18.36±2.01.

The DNA extraction protocol from insect bodies was also efficient: the mean concentrations were 96±26 ng/µL and 147±3.38 ng. The A260/280 ratios were 1.76±0.15 and 1.96±0.18 for larvae and adults, respectively. The DNA extracts from *A. chinensis* larvae and adults were perfectly amplified with a mean Cq value of 15.76±1.37 with 18S rDNA. The performances of the extractions from the environmental and lab frass samples were similar, with the DNA quantities from the environmental frass samples being slightly higher than the lab samples (Table 3).

Optimization of the diagnostic methods for *Anoplophora chinensis*. The optimized thermocycling conditions of qPCR were set at 95°C for 2 min, followed by 40 cycles at 95°C for 10 s and 58°C for 40 s. All reactions were performed in a final volume of 20 µL and contained 1X Quanti Nova Probe PCR Master Mix (Qiagen, Valencia, CA, USA), 0.4 µM and 0.2 µM of the primer and probe respectively, and 2 µL of template DNA.

Validation of TaqMan assay. The results obtained in the internal blind panel showed a specificity, sensitivity, and diagnostic accuracy equal to 100% (EPPO 2019). No significant differences were observed among the different types of *A. chinensis* DNA frass (environmental, lab and five-year-old frass).

Repeatability and reproducibility were estimated only for *A. chinensis* DNA frass samples, which showed very low values (Table 4). The repeatability values, as well as the reproducibility values, measured as the standard deviation (SD), varied between 0.00 and 0.48.

The sensitivity of the assay, tested with the serial dilutions 1:5 of the DNA from *A. chinensis* control frass, produced a good linear regression curve whose coefficient of determination (r^2) was 0.99 (Fig. 3). The efficiency value was 82.3%. The analytical sensitivity for the lab frass with the probe qPCR, i.e. the lowest amount of *A. chinensis* DNA that can be detected (LoD), was 0.64 pg/µL (Table 5).

Discussion

The need for standardized and reliable methods for pest identification is key in internationally recognized phytosanitary practices, as demonstrated by the general guidelines formulated by IPPC and FAO in the International Standard of Phytosanitary Measures (ISPM 27) (FAO 2016) and established by EPPO, for Europe and the Mediterranean Region, in its diagnostic protocols (PM7). These guidelines outline the principles of pest diagnostics, with the basic requirements being standardization, reliability, rapidity, sensitivity, and repeatability (Hodgetts et al. 2016).

In the case of insect pests, the diagnostic methods should enable insect pests to be recognised at each development stage or on the basis of evidence such as biological residues (such as frass or faeces). The molecular tools match previous requirements perfectly, providing many interesting diagnostic solutions (Augustin et al. 2012). Current molecular methodologies for identifying quarantine pests involve techniques that can be easily used in basic molecular laboratories (Rizzo et al. 2020a).

194 In this study, a protocol was established for the identification of the citrus longhorned beetle. The
195 proposed assay was shown to be efficient not only on larvae and adults of the pest, but also on its
196 frass, for an indirect diagnosis. However, the frass is a "difficult" matrix, in which the amount of
197 DNA is scarce with inhibitors capable of preventing DNA amplification. Moreover, the frass may
198 be exposed to environmental factors (i.e., temperature, humidity, solar radiation) especially when it
199 is ejected from the exit holes, which means that the DNA may be degraded. These constraints were
200 overcome in the protocol proposed using an efficient DNA extraction method, which enabled DNA
201 of a high quality and quantity to be obtained from frass . The strength of the extraction from faecal
202 pellets and wood residues is the purification step using a CTAB buffer followed by the addition of
203 chloroform in order to facilitate the appropriate extraction of DNA also from a complex matrix
204 such as frass. The DNA concentrations obtained from *A. chinensis* frass were comparable to those
205 obtained for a similar species, *A. glabripennis* (Rizzo et al. 2020b), and another Cerambycidae,
206 *Aromia bungii* (Rizzo et al. 2021) using the same extraction method. The qPCR assay was able to
207 differentiate between all the *A. chinensis* samples and those of the other non-target wood-boring
208 species, with 100% diagnostic specificity. No amplification or cross reactions were observed with
209 *A. glabripennis*, which is the most related species among those tested. The protocol was also
210 validated by an internal blind panel test which showed a good correspondence between the results
211 obtained by different operators in the same lab. The repeatability and reproducibility of the assay
212 were satisfactory, as the standard deviations of inter-run and intra-run variability were less than or
213 equal to 0.5 (Teter and Steffen 2017). Considering that the linear regression fitted to the standard
214 curve data should have a $r^2 \geq 0.98$ for qPCR (Bustin et al. 2009), the linearity of the qPCR standard
215 curve was very good, with a coefficient of determination r^2 of 0.99. On the other hand, the reaction
216 efficiency was 82.5% which falls within the acceptable, albeit rather low, range (D'haene et al.
217 2010). Several factors could explain the low assay amplification efficiency (Lamarche et al. 2015),
218 however in this specific case, it is due to the particular matrix, which obviously contains PCR
219 inhibitors. The smallest amount of *A. chinensis* DNA detectable in the frass (LoD) was 0.64 pg/µL

220 corresponding to an average Cq of 34.87. This is particularly important in terms of proposing the
221 method as a standard diagnostic tool.
222 The proposed qPCR using a hydrolysis probe (TaqMan) protocol provides an accurate and reliable
223 diagnosis of *A. chinensis* infestation on frass. This matrix provided a very surprisingly rich source
224 of DNA, with a satisfactory quality and quantity, regardless of the age and/or storage conditions of
225 the sample. In this regard, it was a deliberate decision not to use specific storage conditions (i.e.,
226 storage below -20°C) for the frass, in order to demonstrate how the presence of *A. chinensis* can be
227 detected simply by analysing frass in normal operating situations.
228 These findings confirmed previous results obtained on *A. glabripennis* (Rizzo et al. 2020b). Unlike
229 the results of other studies (Strangi et al. 2013) on degradation and the presence of inhibitors in a
230 frass matrix, our extraction method worked efficiently on all the assayed frass samples.
231 Among invasive alien insects, wood-boring species are considered particularly harmful, because
232 wood packaging and goods are transported globally, and these insects are difficult to detect
233 (Augustin et al. 2012; Hulme 2009). A rapid, relatively easy, and efficient diagnosis is thus highly
234 desirable. Such a tool could be crucial for use at entry points or sites (nurseries, parks, etc.) exposed
235 to risks, or directly in the field when an infestation is in the process of spreading (Hérard et al.
236 2006).
237 We thus believe that our protocol could open new perspectives in the diagnostics of wood-boring
238 insect pests, enabling harmful species to be indirectly detected from the weak traces left in the
239 environment which could be used as fingerprints. The availability of large sets of such molecular
240 tools could greatly enhance control operations, especially when the risk of the introduction or
241 diffusion of quarantine species is high.

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Declarations

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Data availability. The data of this study are available from the corresponding author upon request.

Conflicts of Interest. The authors declare no conflict of interest.

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