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Alien pest molecular diagnostics: can DNA traces be exploited to assess the damage caused by the western conifer seed bug on stone pine fructification?

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Abstract

The exotic western conifer seed bug (WCSB) *Leptoglossus occidentalis* Heidemann is considered one of the most important pests of stone pine nut production in the Mediterranean region. However, aside from radiographic analysis, none of the diagnostic tools, already implemented in North America to assess the real impact of the WCSB on conifer seeds, have found application on the European stone pine yet. For the first time, specific DNA-based diagnostic protocols to detect WCSB DNA traces were developed in this study, in the effort of characterizing and monitoring WCSB damage on stone pine fructification in Europe. We designed two primer pairs targeting the WCSB DNA sequences for the mitochondrial gene coding for the subunit I of cytochrome c oxidase (*COI*), according to the data available on the main online databases. Specificity and sensitivity of these primers were assessed via End Point and Real Time PCR. As negative control, DNA from stone pine tissues was used as well as from five other insect species. The *COI* primer pair used in End Point PCR gave amplicons of the expected length only when WCSB DNA was used as template. Moreover, it successfully detected WCSB DNA into the insect liquid saliva, with a sensitivity as low as 100 fg/µl. However it failed to detect WCSB DNA when tested on WCSB-attacked seed samples. Conversely, the Real Time PCR protocol, still targeting the WCSB *COI* gene, was proved to be more sensitive (10 fg/µl) and also led to promising results when tested on attacked plant tissues. In fact, a distinctive melting temperature peak was found for all WCSB positive samples, including WCSB fed upon and feces contaminated seeds. The same primer pair was used for High Resolution Melting (HRM) analysis, which allowed the detection of intra-species single nucleotide polymorphisms (SNPs) in WCSB samples of different geographical origins, thus highlighting the potential of HRM analysis for insect genotyping.

Key words: *Leptoglossus occidentalis*, *Pinus pinea*, cone and seed pests, HRM, insect genotyping, *COI*, DNA barcoding.

Introduction

The western conifer seed bug (WCSB), *Leptoglossus occidentalis* Heidemann (Rhynchota Coreidae) was accidentally introduced in Italy in 1999 (Bernardinelli and Zandigiacomo, 2001), then it spread to most of Europe within a decade (Fent and Kment, 2011). WCSB adults and nymphs attack cones of many conifer species, piercing the scales and then feeding on both cone tissues and developing seeds (Bates *et al*., 2000; 2001; Lait *et al*., 2001a). Known as a seed pest in coniferous tree seed orchards in North America (Koerber, 1963; Blatt and Borden, 1996; Bates *et al*., 2000; Strong *et al*., 2001; Strong, 2006), it also plays a role in conelet abortion and abscission, as reported by Bates *et al*. (2002b). Similarly, it is considered a major factor in the losses of stone pine (*Pinus pinea* L.) nut production in the Mediterranean region (Bracalini *et al*., 2013). Furthermore, some authors report how the WCSB may also pose a threat to natural regeneration of conifer stands in Europe, by exploiting favorable climatic factors as well as the lack of natural enemies (Tamburini *et al*., 2012; Lesieur *et al*., 2014b).

However, a diagnostic tool to specifically assess WCSB impact and damages on stone pine fructification is still to be implemented, in the efforts of monitoring this pest on European conifers as well as evaluating future control programs. The available diagnostic methods for WCSB have been developed on North American conifers and applied mainly on these species. More importantly, their reliability is still questionable. Radiographic analysis is the only diagnostic method which have been tested so far also on European conifers, where its limits about specificity were confirmed (Lesieur *et al*., 2014b). As reported by Showalter and Sexton (1990), the radiographic approach allows the identification of partially depleted seeds of conifers fed on by the WCSB, but those completely emptied by this insect cannot be absolutely distinguished from naturally aborted seeds (Bates *et al*., 2000). Similarly, the polyclonal antibody raised against salivary glands extracted from the WCSB is able to detect the presence of residual salivary proteins in WCSB attacked seeds, but positive signals were also obtained with seeds fed on by another hemipteran (*Leptoglossus corculus* Say) (Lait *et al*., 2001a; 2001b; Bates *et al*., 2002a; Lait *et al*., 2003). Chemical staining of cones directly points out WCSB stylet wounds on North American conifers (Campbell and Shea, 1990). As WCSB usually inserts its stylets between stone pine cone scales, this detection method could be thus less successful when applied to stone pine cones.

Molecular DNA-based diagnostics are becoming an essential step for the unambiguous identification of pests, as an alternative or sometimes complementing traditional taxonomic approaches based on morphological characters. A short sequence (658 base pairs) of the mitochondrial gene coding for the subunit I of cytochrome c oxidase (*COI*) is now generally accepted as a global standard marker for the taxonomic identification of animal species, including insects (Folmer *et al*.,

1994; Hebert *et al*., 2003; 2004a; 2004b; Ward *et al*., 2005; Park *et al*., 2011). On this *COI* fragment, which can be easily sequenced with current technologies, informative and unique species-specific variations have been found, with the final aim to develop a large and standardized database to be used for comparison in animal species identification, according to the so called "DNA barcoding" (Hebert *et al*., 2003). More generally, molecular DNA-based diagnostics could be successfully applied in a wide range of fields, with commercial, sanitary, social, environmental, epidemiological, and agricultural purposes. In fact, they may help distinguishing morphologically cryptic species, as well as unveiling ecological and trophic relationships where insects are involved. Moreover, species identification can be achieved for different insect life stages (*e.g.* eggs, larvae, nymphs, and pupae) otherwise not so easily determined, and even using only fragmentary residues instead of the whole insect specimen. Undoubtedly, these features associated to diagnostic DNA-based technologies are essential for the rapid and accurate identification of exotic and invasive insect pests, affecting human, animal or plant health, and can efficiently support their prompt and effective control (for a recent review, see Boykin *et al*., 2012). In this frame, some DNA-based methods were recently developed for the specific identification and characterization of several insect pests for plants (Walsh *et al*., 2005; Yu *et al*., 2005; Huang *et al*., 2010; Naaum *et al*., 2012; Zhang *et al*., 2012; Naaum *et al*., 2014). Nevertheless, none of them was ever tested for the identification of their targets directly on attacked plant tissues.

Since no DNA-based detection assays were available for the WCSB, in this study we developed two specific and sensitive protocols for the identification of this pest by End Point and Real Time PCR. In the effort to develop a molecular tool able to unequivocally characterize and monitor the extent of WCSB damages on stone pine fructification, we investigated their potential for an *in planta* application on stone pine seeds to detect WCSB DNA from its residual biological traces. To this purpose, we took into account as template also the WCSB DNA, which was here for the first time verified to be present as susceptible of amplification in the insect's liquid saliva. Finally, high resolution melting analysis (HRMA) was coupled to Real Time to evaluate further applications of our protocols.

Materials and methods

Specimens

WCSB adult specimens were collected in Italy (sites A and B in Tuscany Region: A= 43°47'15"N 11°44'33"E and B= 44°03'32"N 10°54'59"E; site C in Veneto Region: C= 45°50'17"N 11°49'46"E) and Hungary (site D, in the Hajdú-Bihar Province: 47°33'30"N 21°38'22"E). Specimens were handpicked both on host trees (during summer) as well as at various overwintering sites (in autumn). Both male and females individuals were used in this study as no differences are known for their feeding activity. Laboratory rearings were also established to provide constant availability of WCSB specimens. Several adult specimens of five other insect species were collected in Tuscany and here used as negative controls: *Palomena prasina* (L.) (Rhynchota Pentatomidae Pentatominae), *Ernobius impressithorax* Pic, *Ernobius parens* (Mulsant et Rey) (Coleoptera Ptinidae Ernobinae), *Pissodes validirostris* (Sahlberg) (Coleoptera Curculionidae Molytinae), and *Dioryctria mendacella* (Staudinger) (Lepidoptera Pyralidae Phycitinae). All of these species, except *P. prasina*, are commonly associated with stone pine fructification (Zocchi, 1961; Roques *et al*., 1983; Turgeon *et al*., 1994; Innocenti and Tiberi, 2002; Bracalini *et al*., 2013). *P. prasina* was included since it is more closely related to the WCSB, both being hemipterans. In fact, no Mediterranean hemipterans are phylogenetically close to the WCSB, or share its ecological traits. Moreover, *P. prasina* was observed feeding occasionally on pine cones during our field samplings, thus resulting a good candidate as a control species.

Force feeding

Stone pine seed samples (10 mg each) were supplied to WCSB adults during laboratory force-feeding trials. One seed sample was supplied as sole food source for each WCSB individual, inside a transparent plastic cup, for 8 hours. Every session of force-feeding included 20 seed samples, to be checked every ten minutes so that signs of feeding activity could be promptly recorded. At the end of each session, pierced samples were collected and stored at –20 °C. In addition, samples contaminated by WCSB excrements were also collected, and stored separately. Both pierced and contaminated samples were used to test our DNA-based diagnostic protocols. Non-pierced samples were discarded since, as control, additional fresh seeds were used, which had never been in contact with our WCSB specimens.

Molecular biology general techniques

Genomic DNA was extracted from insect and seed tissues using the Puregene® Genomic DNA Purification Kit, (Gentra Systems Inc., Minneapolis, MN, USA), according to manufacturers' instructions. Hind femora and/or thorax were used to extract DNA from adult insects (WCSB, *P. validirostris*, *P. prasina*), while whole specimens were used for adults of the two *Ernobius* species as well as *D. mendacella*.

DNA was also extracted from WCSB liquid saliva, collected from live specimens using a parasympathomimetic drug (Madhusadhan *et al*., 1994). The insects were held in place with crossed mounting pins, their proboscis inside glass microcapillary tubes, then 80 µl of 4% pilocarpine chlorhydrate were applied ventrally to the insects' abdomen. Salivation was induced both by the drug application and by applying a stream of warm air (40 °C) to the immobilised insect. DNA was extracted from the saliva still inside the glass tube, using Puregene® kit as reported above.

DNA concentration of each sample was evaluated both spectrophotometrically, using a NanoDrop™ ND-1000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and visually by agarose gel electrophoresis [1% agarose

Table 1. WCSB specific primers designed in this study: their main features and performances.

Primer pair	LeptoF-LeptoR	LeptoRTF-LeptoRTR
PCR protocol	End Point PCR	Real Time PCR
Forward Seq. (5'→3')	TACCCTTTACTTTATTTTTG	AAATAAATGCTGATATAAAATAG
Reverse Seq. (5'→3')	GGTATTACAGCACTTCTACTTCTTCT	AATGGCTAAATCAACAGAGGCT
Amplicon length (bp)	658	171
Annealing temperature (°C)	55	60
Sensitivity (fg/ µl)	100	10

(w:v) in TAE 1X] (Sambrook and Russell, 2001), stained with ethidium bromide (0.5 µg/ml). Electrophoresis gel analysis was performed on Gel Doc™ XR+ System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), using for comparison a standard molecular ruler (GeneRuler 1 kb Plus DNA Ladder, Thermo Fisher Scientific, Inc.).

DNA fragments were purified from agarose gel with PureLink® Quick Gel Extraction Kit (Thermo Fisher Scientific, Inc.), cloned using InsTAclone® PCR Cloning Kit (Thermo Fisher Scientific, Inc.), and then sequenced on both strands by the dideoxy sequencing termination method (Eurofins MWG Biotech, Ebersberg, Germany).

By using Beacon Designer 7.5 software (Premier Biosoft International, Palo Alto, CA, USA), the primer pairs LeptoF/LeptoR and LeptoRTF/LeptoRTR were designed according to the WCSB nucleotidic *COI* sequences available on GenBank and EMBL databases, to be then used in End Point and Real Time PCR, respectively (table 1). Given the very high degree of homology, one WCSB *COI* sequence was chosen as the best representative (Accession Number JQ996145.2). Alignments and comparisons with sequences from other insects were performed with CLUSTALW2 (Larkin *et al*., 2007), for the identification of conserved regions in WCSB sequences, upon which the primers were designed. BLAST algorithm (Altschul *et al.*, 1990) was used for homology searches.

End Point PCR

Using the primer pair LeptoF/LeptoR, End Point PCR amplifications were carried out in a 25 µl reaction volume, which contained DNA template in variable amounts according to the specific experimental purposes, 0.25 µM of each primer, 0.5 µM of each dNTP, 1 unit of DreamTaq® Green DNA Polymerase and DreamTaq® Green Buffer (1X) (Thermo Fisher Scientific, Inc.). Amplifications were performed on a C1000 thermal cycler (Bio-Rad Laboratories, Inc.). Cycling conditions were as follows: an initial denaturation step at 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, to end with a final step of 72 °C for 10 min. PCR products were visualised on 1.5% agarose gel electrophoresis in TAE 1X, the amplicons of the expected length cloned and then sequenced for confirmation.

The specificity of the End Point PCR protocol here developed was tested on WCSB genomic DNA, obtained from wild specimens (sampling sites A, B, C, and D), and from laboratory reared specimens. As putative negative controls, DNAs from other five insect species, as well as stone pine DNA extracted from healthy seeds were used as template. Genomic DNA extracted from WCSB saliva was also used as template in End Point PCR runs.

The detection limits and the sensitivity of this End Point PCR procedure were evaluated by using as template different amounts of WCSB DNA (from 100 pg to 10 fg/µl, with 2 µl DNA solution for 25 µl reaction).

Real Time PCR and HRM analysis

The primer pair LeptoRTF/LeptoRTR was used in Real Time PCR runs, carried out on a CFX96 thermal cycler (Bio-Rad Laboratories, Inc.), in white multiwells PCR plates (96 wells), with 10 µl reaction volume, containing 0.5 µM of each primer, 1X SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc.) according to manufacturer's instructions, and variable amounts of DNA template according to the specific experimental purposes. The thermal cycler program used was 95 °C for 3 min, followed by 45 cycles of 10 s at 95 °C, and 15 s at 60 °C. The amount of fluorescence for each sample, given by the incorporation of EvaGreen® into dsDNA, was measured at the end of each cycle and analyzed via CFX-Manager Software v1.6 (Bio-Rad Laboratories, Inc.). Standard melting curves of PCR amplicons were obtained with temperatures ranging from 60 °C to 95 °C, and data were collected every 0.5 °C temperature increment. Each sample was tested in three independent experiments, with at least three replicates each. For High-Resolution Melting (HRM) analysis, data were still collected in the temperature range from 60 °C to 95 °C, but acquisition was performed every 0.2 °C increase in temperature, with a 10 s step. Data were then analyzed by HRM analysis software (Bio-Rad Laboratories, Inc.), which automatically clusters the samples according to their melting profiles and assigns a confidence score to each sample. The confidence level threshold here considered for a sample to be included in a cluster was 99.5%.

The specificity of the Real Time PCR protocol here developed was tested on both reared and feral WCSB genomic DNAs, and using as putative negative controls genomic DNAs from the other five insect species considered in this study, as well as stone pine DNA. The detection limits and the sensitivity of this Real Time PCR procedure were evaluated by using as template different amounts of WCSB DNA (from 100 pg to 1 fg/µl, with 2 µl DNA solution for 10 µl reaction). Moreover WCSB target DNA was also PCR tested as spiked with stone pine DNA (20 ng/reaction), to further confirm both the specificity and the sensitivity of this Real Time PCR protocol. Two negative water controls (sterile distilled water) were included within each PCR run.

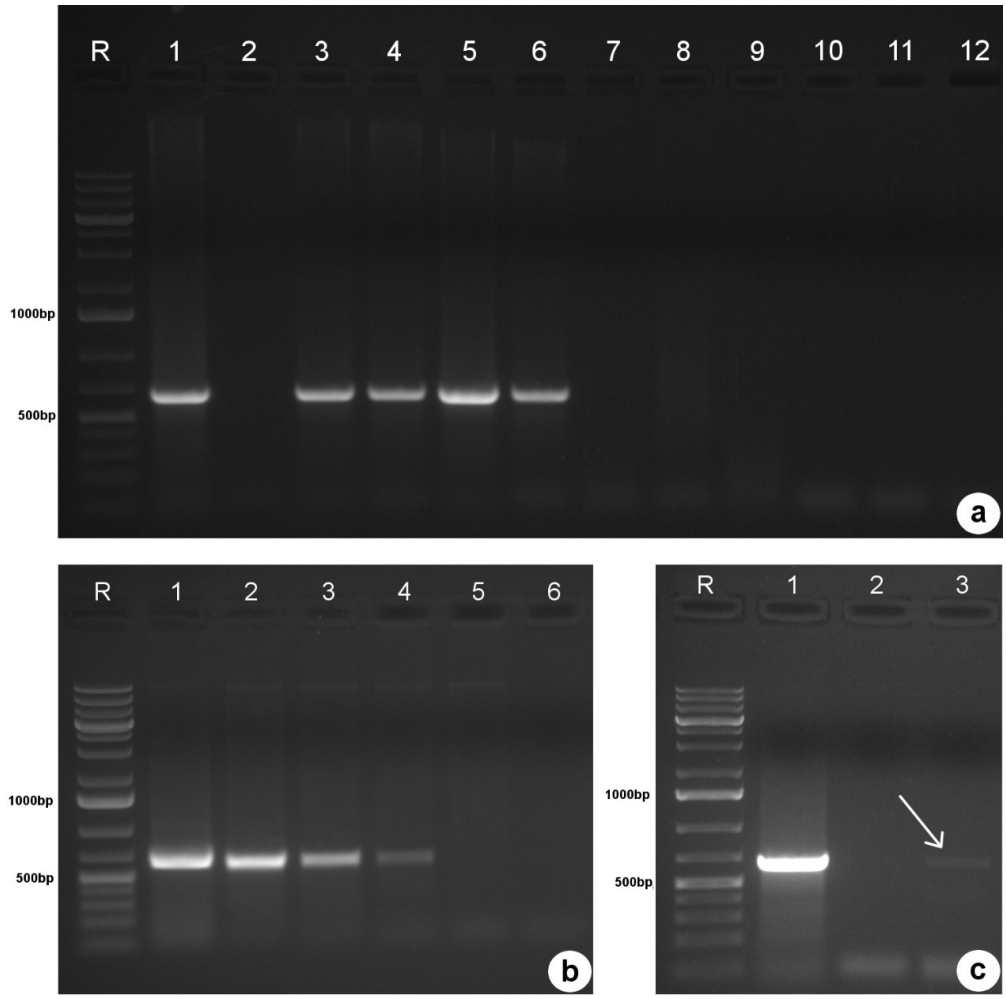

Figure 1. Agarose gel electrophoresis of the PCR products obtained with the primer pair LeptoF/LeptoR. **(a)** Specificity test on 20 ng DNA extracted from: (1) Laboratory reared WCSB; (3 - 6) WCSB, from sites D, C, B and A respectively; (7) *D. mendacella*; (8) *P. validirostris*; (9) *E. parens*; (10) *E. impressithorax*; (11) *P. prasina*; (12) Sound stone pine seed. (2) Sterile PCR grade water; (R) Ruler. **(b)** Sensitivity test on tenfold dilution series of WCSB DNA from laboratory reared specimens as template (2 µl DNA solution for 25 µl reaction volume). Lanes: (1) 100 pg/µl; (2) 10 pg/µl; (3) 1 pg/µl; (4) 100 fg/µl; (5) 10 fg/µl; (6) Sterile PCR grade water; (R) Ruler. **(c)** Test on DNA extracted from WCSB saliva. Lanes: (1) WCSB DNA (20 ng/reaction); (2) Sterile PCR grade water; (3) DNA extracted from WCSB liquid saliva (the arrow points to the amplification signal); (R) Ruler.

In planta assays

The End Point and Real Time PCR protocols here developed were both evaluated for the detection of WCSB DNA traces on attacked stone pine seeds. To this aim DNA extracted from seeds fed on by the WCSB during force-feeding sessions and from seeds externally contaminated with WCSB excrements were used as template. As negative control, DNA extracted from additional sound seeds was assayed as well. Moreover, at least two negative controls (sterile distilled water) were included within each PCR run.

Results and discussion

End Point PCR protocol for WCSB detection

The specificity for WCSB of the primer pair LeptoF/LeptoR was firstly evaluated *in silico*, and no significant homologies for these primers were found among insect pests (data not shown). Then, their performances were assessed in End Point PCR runs, where a single amplicon of the expected length (658 bp) was exclusively obtained when WCSB DNA was used as template, and whose identity was confirmed by sequencing (Sequences for Site B and D specimens were deposited in GenBank, with Accession Numbers KP262419 and KP262420, respectively). In no case, DNA from both stone pine healthy-looking seeds and from the five control insects, interfered with these primers giving aspecific amplifications (figure 1a). Thus, these data demonstrated *COI* gene and the primer pair LeptoF/LeptoR to be a highly specific marker for WCSB molecular detection, as far as Mediterranean cone and seed pests are concerned.

When several tenfold dilutions of WCSB DNA (ranging from 100 pg to 10 fg per µl) were used as template, the detection threshold for the primer pair LeptoF/LeptoR was as low as 100 fg/µl (figure 1b). The

high sensitivity of this End Point PCR protocol was further confirmed by the positive amplification signals obtained by using as template WCSB DNA extracted from its liquid saliva (figure 1c). Although WCSB saliva samples always yielded electrophoretically undetectable levels of DNA, the high sensitivity of this specific PCR protocol allowed the successfull and constant amplification of the expected *COI* fragment, suggesting this protocol to be highly promising for the detection of WCSB DNA traces into attacked stone pine fructification.

Real Time PCR protocol for WCSB detection

In general terms, Real Time PCR is 10 to 100-fold more sensitive than End Point PCR, and has a dynamic range of more than four decimal orders of magnitude, in detecting the same DNA target. The results obtained in the detection of WCSB DNA into its liquid saliva by End Point PCR were definitely encouraging, but more efforts had to be spent to increase the sensitivity of this molecular diagnostic approach for the WCSB. Accordingly, the primer pair LeptoRTF/LeptoRTR was also

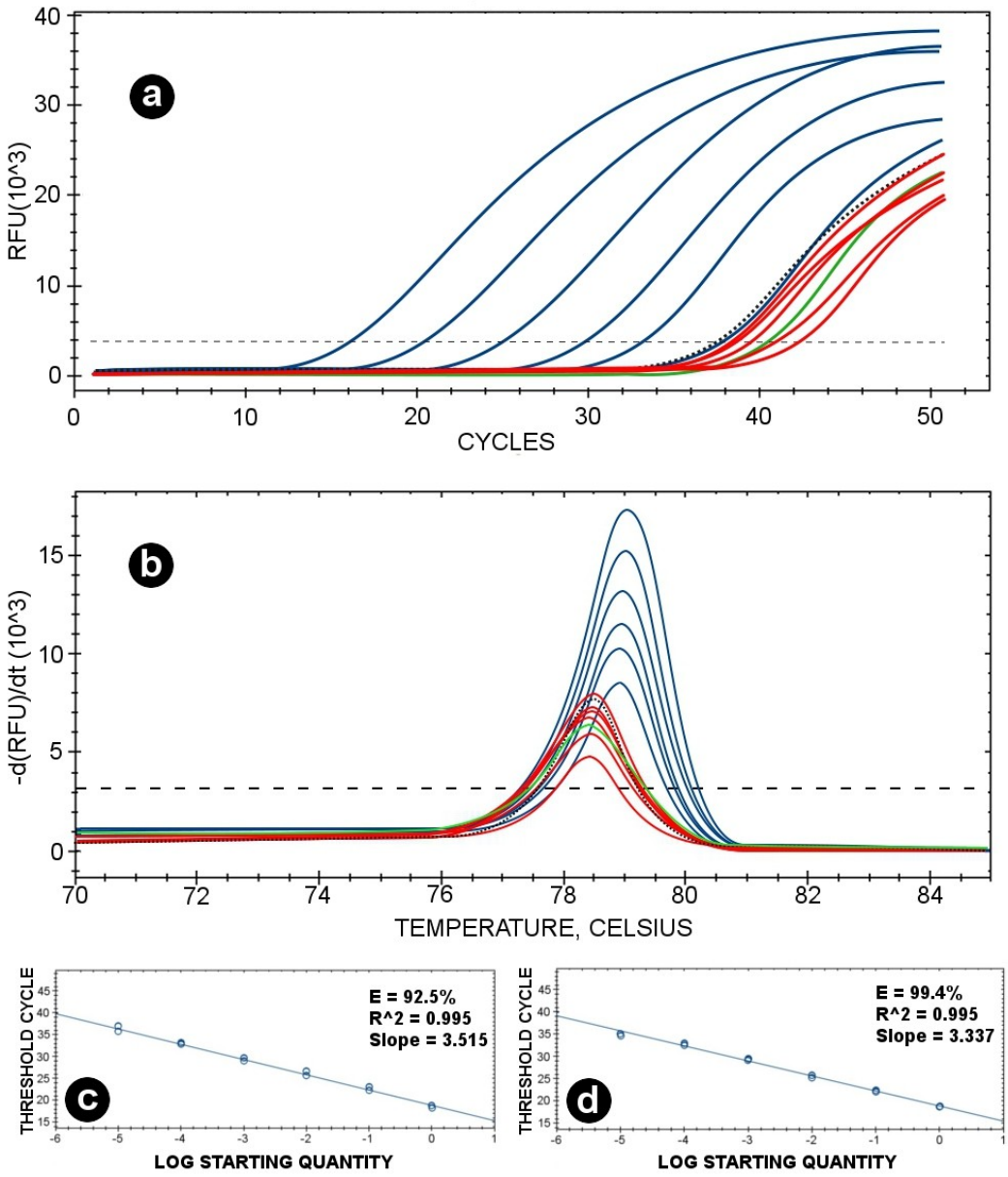

Figure 2. Quantitative Real Time PCR on tenfold serial dilutions of WCSB DNA using the primer pair LeptoRTF/LeptoRTR, shown as **(a)** amplification and **(b)** melting plots. Mean replicate values are depicted. Blue solid lines: WCSB amplification profiles, obtained with 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg of WCSB DNA as template, obtained from laboratory reared specimens [from left to right (a), from top to bottom (b)]. Red solid lines: amplification profiles obtained using as template 50 ng DNA from *E. impressithorax*, *E. parens*, *P. prasina*, *D. mendacella*, and *P. validirostris* [from left to right (a), from top to bottom (b)]. Dotted black line: negative control (DNA-free sterile distilled water). Dashed black line: baseline fluorescence. RFU: Relative Fluorescence Units. **(c-d)** Standard curve generated using as template tenfold dilution series (from 100 pg to 1 fg per µl) of (c) WCSB DNA as such and (d) as spiked with 20 ng/reaction of stone pine DNA. R^2 values, slopes and efficiencies are shown.

designed based on the WCSB *COI* sequences already available (table 1). Using WCSB DNA as template, the primers LeptoRTF and LeptoRTR had to give a shorter amplicon (171 bp) in comparison to that produced by the pair LeptoF/LeptoR. The primer pair specificity was evaluated *a priori* by homology searches on EMBL and GenBank databases, using the BLAST algorithm, and no significant matches were found among insect pests.

Then, the specificity of the primer pair LeptoRTF/LeptoRTR was tested in Real Time PCR runs with SsoFast™ EvaGreen® as fluorescent marker. Since SsoFast™ EvaGreen® efficiently binds to the minor grooves of a DNA double-chain as it is forming, this fluorescent dye can bind to all amplicons produced in a PCR run. Therefore, in Real Time PCR a primer pair can provide a reliable and specific signal only when i) the increase in fluorescence produced by an unknown sample is exclusively associated to the amplicon distinctive melting temperature (Tm), and ii) the cycle threshold of an unknown sample is far below that produced by sterile PCR grade water used as negative control.

The melting curves obtained with the primer pair LeptoRTF/LeptoRTR, using as template several tenfold dilutions of target WCSB DNA ranging from 100 pg to 1 fg per µl, are shown in figure 2b. A single melting peak at 79.0 ± 0.1 °C was always observed at the different WCSB DNA concentrations, indicating that the total fluorescent signal was contributed by a WCSB specifically targeted amplification. Conversely, no signals having the WCSB distinctive Tm were ever recorded when, control insect DNAs, stone pine DNA, (both at 20 ng/reaction), and sterile distilled water were used, thus confirming the specificity of LeptoRTF/LeptoRTR towards WCSB DNA (figure 2b). Moreover, the amplicons produced by SsoFast™ EvaGreen® Real Time PCR were visualized by gel electrophoresis. As expected, single bands of the expected sizes of 171 bp were exclusively and specifically generated with the primer pair LeptoRTF/LeptoRTR with WCSB DNA samples, while no aspecific amplification products were ever observed on DNA-free negative controls, on stone pine DNA and on DNAs from control insect species (data not shown).

In figure 2b the sensitivity of the Real Time primer pair LeptoRTF/LeptoRTR is also represented. Increasing amounts of the specific WCSB target DNA corresponded to higher melting peaks having the same Tm, and WCSB DNA as low as 10 fg/µl could be detected. In addition, an increase in cycle threshold (Ct) values was observed in amplification plots obtained with lower amounts of WCSB DNA (figure 2a).

The standard curve for the absolute quantification of WCSB target DNA with the SsoFast™ EvaGreen® Real Time PCR detection method was generated by evaluating the Ct values *versus* the corresponding decimal logarithm of the initial amount of target DNA for each tenfold dilution series (from 100 pg to 1 fg per µl, with 2 µl for 10 µl reaction volume). As shown in figure 2c, this regression analysis showed that the resulting standard curve was linear over a range of six logs (from 100 pg to 1 fg/reaction). Moreover the standard curve revealed a high correlation between Ct and DNA template amounts ($R^2 > 0.99$, $P < 0.05$), and the slope of the standard curve was equivalent to PCR efficiencies of 92.5%. When the same WCSB DNA amounts were also tested as spiked with stone pine DNA (20 ng/reaction), these parameters were similar and even further improved, confirming this detection protocol to be efficient even in the presence of no target DNA as well as at low DNA target concentrations (figure 2d). Moreover, the absence of any reduction in the PCR efficiency shown by spiked WCSB DNA samples rules out the presence of any PCR inhibitor of plant origin, with undoubted advantage for future applications of this SsoFast™ EvaGreen® Real Time PCR detection protocol on WCSB attacked or contaminated stone pine seeds.

Evaluation of the *in planta* performances of the end Point and Real Time PCR detection protocols for WCSB

A total of 51 samples of stone pine seeds fed upon by WCSB were used for DNA extraction. Recorded WCSB activity included feeding punctures lasting 30-90 minutes, sometimes repeated at intervals resulting in more punctures on the same seed sample. In no case, punctures signs were visible on attacked samples. DNA was also obtained from 17 sound stone pine seeds that showed evident external contamination by WCSB feces. These DNAs were used as template both in End Point and in Real Time PCR runs, according to the protocols set up in this study for WCSB specific detection. For comparison, DNAs from WCSB specimens and from control seeds were used as well.

Unfortunately, the primer pair LeptoF-LeptoR failed to detect WCSB DNA into any attacked seed samples in End Point PCR runs. Conversely, successful and WCSB specific amplifications were constantly achieved with DNA from samples contaminated with WCSB excrements (figure 3a). Assuming that this might be due to low amounts of WCSB salivary residues into attacked tissues, the primer pair LeptoRTF/LeptoRTR were used to perform Real Time PCR experiments on DNA template from WCSB attacked and sound stone pine seeds, and the results obtained were definitely more promising. As shown in figure 3c, a specific and distinctive Tm peak at 79.0 ± 0.1 was observed for all WCSB positive samples, including WCSB fed upon and feces contaminated seeds, while uncontaminated sound seeds showed a different Tm value. However, WCSB positive signals obtained from attacked seeds had Cts comparable to those of negative control (*e.g.* 38th cycle) and of sound seeds (figure 3b). This prevented us to assess unequivocally the presence of WCSB DNA into fed upon seed samples. Conversely, seeds stained with WCSB excrements always gave significantly reliable and specific Cts (figure 3b). Differences observed between the Cts of WCSB positive samples may be easily explained according to their highly different content in WCSB DNA. Supposedly, the amount of WCSB residual saliva inside stone pine attacked tissues was negligible, giving extremely low amounts of WCSB DNA that could be around or below the detection limit of our Real Time PCR protocol.

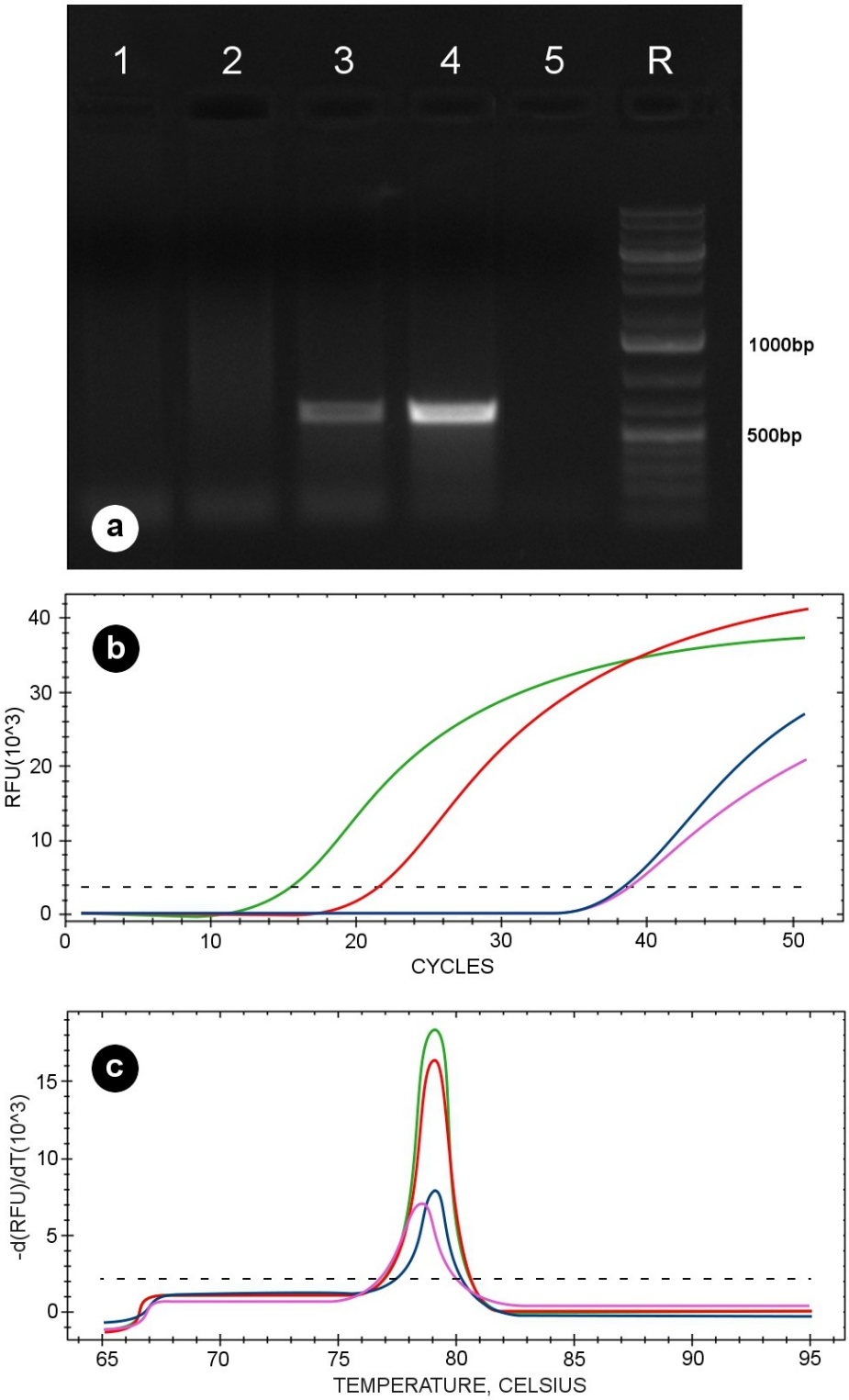

Figure 3. *In planta* tests, using the primer pairs designed in this study. **(a)** Agarose gel electrophoresis of the amplicons obtained with the primer pair LeptoF/LeptoR in End Point PCR runs, carried out on DNA extracted from: (1) stone pine sound seeds; (2) WCSB fed upon stone pine seeds; (3) WCSB feces contaminated stone pine seeds; (4) WCSB pure genomic DNA from laboratory reared specimens (20 ng). (5) Negative control with DNA-free sterile distilled water; (R) Ruler. **(b-c)** Quantitative Real Time PCR on seeds provided to WCSB specimens, shown as (a) amplification and (b) melting plots. Melting curve analysis is shown by plotting fluorescence derivative values *versus* temperature of the amplification products obtained using the LeptoRTF/LeptoRTR primer pair on genomic WCSB DNA (green line) and DNAs extracted from stone pine seed samples: seeds contaminated by WCSB excrements (red line), seeds fed on by the WCSB (blue line), and healthy-looking sound seeds (pink line). Mean replicate values of three independent experiments are depicted. Dashed black line: baseline fluorescence. RFU: Relative Fluorescence Units.

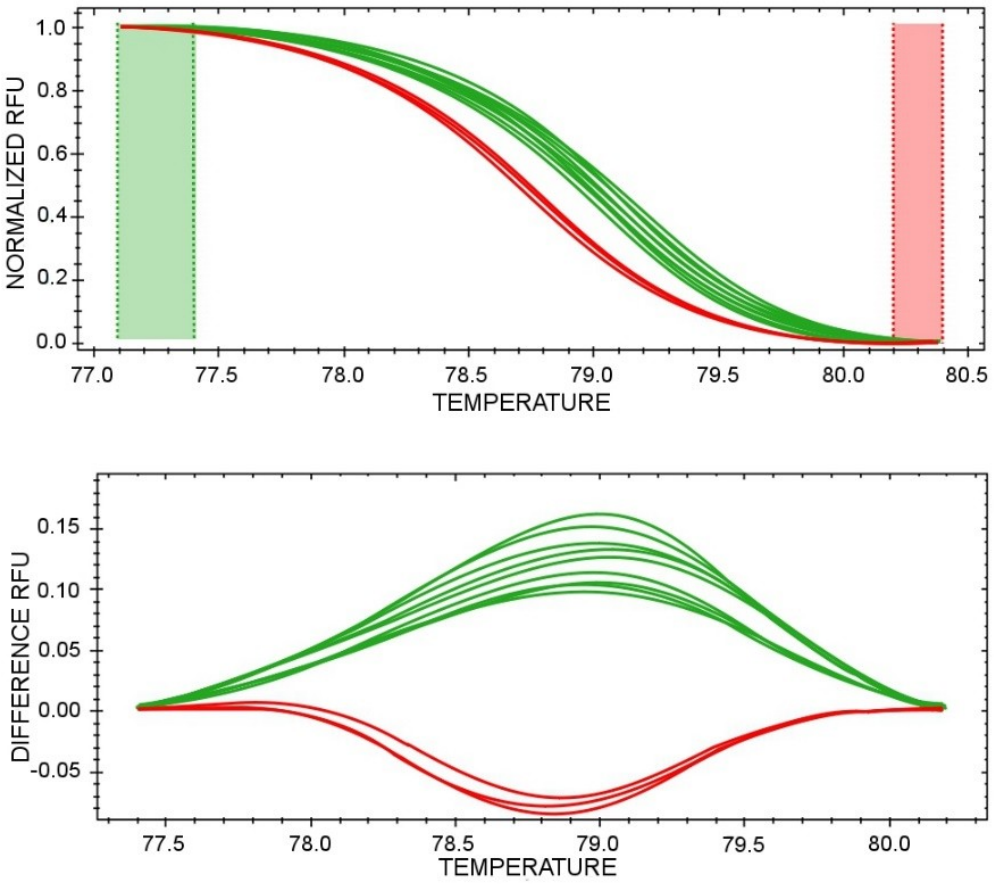

Figure 4. Normalized and difference plots obtained in HRM assay, performed in triplicate on WCSB DNA samples from all sites using the LeptoRTF/LeptoRTR primer pair. Green cluster: Sites A, C, and D; red cluster: Site B. RFU: Relative Fluorescence Units.

WCSB genotyping by HRM analysis

The WCSB specific Real Time PCR protocol was then coupled with HRM analysis, in order to get a preliminary evaluation on its potential as a rapid and inexpensive tool for the genotyping of geographically distant or apparently different WCSB populations. When the DNAs of WCSB specimens from sites A, C and D were used as template (100 pg/µl, with 2 µl for 10 µl reaction volume) in Real Time PCR runs, the WCSB specific and distinctive single melting peak at 79.0 ± 1 °C was always obtained in standard melt analysis (data not shown). On the same samples HRMA experiments were performed, and the data points were now collected every 0.2 °C temperature increments instead of 0.5 °C, enhancing the detection of even subtle melting profile differences. As shown in figure 4, the HRM analysis on both normalized and difference melting curves always distributed the WCSB samples into two independent clusters, readily and consistently resolved. More specifically, WCSB samples from all but one sites (site B) were allocated in a single HRM cluster. These data were in accordance with our sequencing results, which led to the finding of three single nucleotide polymorphisms (SNPs) distinguishing site B specimens when compared with the other WCSB specimens from sites A, C and D. These results further emphasize the potential of our Real Time PCR protocol, which was both highly specific for WCSB as well as able to detect SNP-based variance within this species via HRM analysis, without the prerequisite of sequencing. Such nucleotide variations are otherwise generally observable only via cloning and sequencing of the investigated target DNA, as we had already observed for our WCSB samples, as well as within recently deposited WCSB *COI* sequences (Park *et al*., 2011; Ahn *et al*., 2013; Raupach *et al*., 2014). Based only on these preliminary data, we cannot explain the divergence found for site B WCSB samples when compared to the other Italian specimens, even from the same Region, and *vice versa* to the similarity found for the WCSB specimen from Hungary (site D) and those from Italian sites A and C. More samples should be examined and much more epidemiological and ecological information would be necessary to drawn any scientific conclusion. However, these preliminary data confirm the paramount importance of HRM reliability and its cost-effectiveness for insect genotyping, as it allows highly specific detection of SNPs otherwise discernible only *via* sequencing. Until recently, HRMA has been confined mainly to clinical and diagnostic studies, with few examples in agricultural entomology and plant pathology (Gori *et al.*, 2012; Li *et al*., 2012; Dhami *et al*., 2014). Among the very few examples of HRM analysis applied up to now to insects, it is worth mentioning the studies carried out on the Chagas disease insect vector

and on the members of the *Anopheles gambiae* complex involved in the transmission of malaria disease parasite, to establish and design efficient control and surveillance strategies (Peña *et al*., 2012). Conversely, applying HRM analysis of *COI* gene during field studies on the WCSB could be essential to genotype the populations of this pest for both epidemiological studies and the development of focused control strategies. Although the *COI* gene may not be the best choice for population genomic studies, it could be successfully exploited by HRM analysis to gather preliminary phylogenetic data. Then, to investigate more deeply WCSB population dynamics and structures, more suited markers could be used in the future, such as polymorphic microsatellites (Lesieur *et al*., 2014a).

Conclusion

The rapid, accurate and reliable identification of insects is becoming increasingly important, especially for quarantine pests, as well as for those insects that are vectors of plant viruses and phytoplasmas. The End Point and Real Time PCR protocols developed in this study can be a versatile system of PCR-based tools for the molecular identification of WCSB samples. They can be appropriate for both research and diagnostic purposes, to be chosen accordingly to the available instrumentations and skills of different laboratories as well as to the aims of their work. Furthermore, our WCSB Real-Time PCR detection procedure was shown to have a high analytical sensitivity for the direct assessment of WCSB biological traces directly on plant materials. However, as a consequence of the very low amount of WCSB DNA that can be found on fed upon stone pine seeds, additional research will need to be carried out to further increase the *in planta* sensitivity of our Real Time PCR protocol by the development of a WCSB specific TaqMan® probe. While DNA barcoding may easily find applications in identifying insect species from specimen fragments, this study showed for the first time how DNA-based diagnostic protocols can be successful even when insect excrements or saliva are the only biological traces available. Finally, successful preliminary data were here reported about HRM analysis application for detecting SNPs in insects, with the aim to monitor their spread and distribution, without pursuing the time consuming, as well as expensive, sequencing approach.

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