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Rapid and specific detection of *Pentastiridius leporinus* by recombinase polymerase amplification assay

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Research Paper

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Abstract

Pentastiridius leporinus (Hemiptera: Cixiidae) is the main vector of an emerging and fast spreading sugar beet disease, the syndrome 'basses richesses' (SBR), in different European countries. The disease is caused by the γ-3-proteobacterium '*Candidatus* Arsenophonus phytopathogenicus' and the phytoplasma '*Candidatus* Phytoplasma solani' which are exclusively transmitted by planthoppers and can lead to a significant loss of sugar content and yield. Monitoring of this insect vector is important for disease management. However, the morphological identification is time consuming and challenging as two additional cixiid species *Reptalus quinquecostatus* and *Hyalesthes obsoletus* with a very close morphology have been reported in sugar beet fields. Further, identification of females and nymphs of *P. leporinus* at species level based on taxonomic key is not possible. In this study, an isothermal nucleic acid amplification based on recombinase polymerase amplification (RPA) was developed to specifically detect *P. leporinus*. In addition, real-time RPA was developed to detect both adults (male and female) and nymph stages using pure or crude nucleic acid extracts. The sensitivity of the real-time RPA for detection of *P. leporinus* was comparable to real-time PCR, but a shorter time (< 7 min) was required. This is a first report for real-time RPA application for *P. leporinus* detection using crude nucleic acid templates which can be applied for fast and specific detection of this vector in the field.

Introduction

Pentastiridius leporinus (Hemiptera: Auchenorrhyncha: Cixiidae) is the main vector for syndrome 'basses richesses' (SBR) disease in sugar beet (*Beta vulgaris*) which is a fast-spreading disease in Central Europe and causing up to 5% absolute sugar content loss and severe yield reduction of the taproot (Gatineau *et al.*, 2002; Sémétey *et al.*, 2007c; Bressan *et al.*, 2008). The disease was initially reported in eastern France in 1991 (Gatineau *et al.*, 2002) and then further spreaded into sugar beet fields in Germany (Pfitzer *et al.*, 2020; Behrmann *et al.*, 2021) and Switzerland (Mahillon *et al.*, 2022). The causal agents of SBR are the γ-3-proteobacterium '*Candidatus* Arsenophonus phytopathogenicus' (here called: 'SBR proteobacterium') and a phytoplasma from the stolbur group (16SrXII group) '*Candidatus* Phytoplasma solani' which are exclusively transmitted by planthoppers (Sémétey *et al.*, 2007a; Bressan *et al.*, 2008). Among them, *P. leporinus* is the key factor in spreading the SBR disease. This is due to the high population densities, infection rates, the ability to transmit both pathogens and vertical transmission of the SBR proteobacterium to their offspring (Sémétey *et al.*, 2007c; Bressan *et al.*, 2008; Pfitzer *et al.*, 2020; Mahillon *et al.*, 2022). Additionally, this insect vector has been host-shifted from natural host plant, reed (*Phragmites australis*) to sugar beet and other crops including barley (*Hordeum vulgare*) and winter wheat (*Triticum aestivum*) during crop rotations (Holzinger *et al.*, 2003; Bressan *et al.*, 2009b). This phenomenon together with increasing *P. leporinus* populations led to a significant SBR spread in sugar beet (Bressan, 2009; Bressan *et al.*, 2009b; Bressan *et al.*, 2011).

In addition to *P. leporinus*, several Auchenorrhyncha species including two closely related cixiid planthoppers, *Reptalus quinquecostatus* and *Hyalesthes obsoletus*, have been collected in sugar beet fields in France (Sémétey *et al.*, 2007b) and Germany (Pfitzer, 2019). *R. quinquecostatus* has recently undergone a taxonomic revision with a new name, *R. artemisiae* (Emeljanov, 2020). To provide easier connection to the published data regarding this species, we referred to it as *R. quinquecostatus*. *P. leporinus* can be hardly distinguished from *R. quinquecostatus* and *H. obsoletus* by morphological traits. Based on the morphological traits, these species are also closely related (Ceotto and Bourgoin, 2008). They have hyaline or transparent wings and their mesonotum has five keels and the absence of a post-tibial calcar at the hind legs and their fore wings are characterised by a roof-shaped resting position (Holzinger *et al.*,

2003). Identification of *P. leporinus* based on morphological characters is challenging and time consuming (Bertin *et al.*, 2010a). This requires highly skilled and experienced personnel which limits the throughput in practical monitoring. Further, the morphological keys for *P. leporinus* identification at the species level are exclusively described for male adults and thus lacking for female adults and nymphal instars (Holzinger *et al.*, 2003; Biedermann and Niedringhaus, 2004). Further, identification of sticky trap collected insects which is a common method of monitoring the vector spread could be more difficult and error prone for identification based on morphological characters (Bertin *et al.*, 2010b).

Supporting the morphological identification or even as an alternative identification method, molecular methods such as DNA barcoding (Virgilio *et al.*, 2010) have been used for insects. Among these methods, polymerase chain reaction (PCR) is the most widely used (Mullis *et al.*, 1986) to specifically amplify a part of insect genome that can be sequenced and used for identification. However, due to the requirements of a PCR machine, time, and professional operation, it is difficult to apply PCR for on-site detection. In recent years, the advancement and application of isothermal amplification methods have been emerged rapidly. These isothermal amplification methods including loop-mediated isothermal amplification (LAMP) (Notomi *et al.*, 2000), helicase-dependent isothermal DNA amplification (HAD) (Jeong *et al.*, 2009), rolling-circle amplification (RCA) (Ali *et al.*, 2014) and recombinase polymerase amplification (RPA) (Piepenburg *et al.*, 2006, Lobato and O'Sullivan, 2018), which use different enzyme mechanisms and settings. The RPA assay uses three core enzymes including recombinase, single-stranded DNA binding protein, and strand-displacing polymerase (Piepenburg *et al.*, 2006). Due to a simple operation, reaction at 37–42°C and fast amplification speed (5–20 min), RPA is widely used in various fields like the detection of bacteria, fungi, parasites, viruses or drug resistance genes in recent years (Mota *et al.*, 2022; Tan *et al.*, 2022). Additionally, compared to other common isothermal amplification methods (RCA, LAMP, HAD), RPA requires only simple primer design, tolerates certain mismatches in primers and allows multiplex amplification (Tan *et al.*, 2022). However, there are only few examples for application of RPA for the identification of insects. For examples, RPA has been used to identify *Thrips palmi* (Thysanoptera: Thripidae), a vector of tospoviruses. They targeted a conserved region of ITS2 to identify all known intra-specific variants of this insect species (Priti *et al.*, 2021).

The use of mitochondrial gene *cytochrome c oxidase I* (*COI*) was established as the core of a global bioidentification system for animals including insects (Hebert *et al.*, 2003). It was demonstrated that species–level assignments can be obtained by creating comprehensive *COI* profiles (Hebert *et al.*, 2003). This gene also was used for identification and phylogenetic analysis of planthoppers in the genera *Hyalesthes* and *Reptalus* by using species-specific primers (Bressan *et al.*, 2009a; Bertin *et al.*, 2010b). Recently, we have designed specific primers based on *COI* gene to identify and differentiate all life stages of *P. leporinus* from two closely related cixiid species, *R. quinquecostatus* and *H. obsoletus*, in sugar beet using PCR (Pfitzer *et al.*, 2022a). In this study, a rapid and real-time detection of *P. leporinus* was developed based on the RPA assay. The sensitivity and specificity of the RPA assays for detection of both adults and nymphal stages were compared to real-time PCR assay using a simple and quick method for template DNA preparation.

Materials and methods

Planthopper collection from sugar beet fields and morphological identification

The main SBR vector, *P. leporinus*, and two morphologically close planthopper species (*R. quinquecostatus*, and *H. obsoletus*) were field collected with sweep nets during summer 2020 from several locations (Baden-Württemberg, Rhineland-Palatinate, and Saxony) in Germany (Pfitzer *et al.*, 2022b). Morphological identification of the sweep net collected insects was carried out using a stereomicroscope according to the taxonomic key (Biedermann and Niedringhaus, 2004). The family and genus of individual female adult specimens were identified by observation of pronotum, wings, postnotum, mesonotum, and tarsus. Furthermore, the genital structures of male adults were evaluated to allow morphological identification at the species level. Additionally, species identification for both female and male insects was confirmed by sequencing *COI* gene (Pfitzer *et al.*, 2022a). Additionally, nymphal instars of *P. leporinus* were obtained from our local *P. leporinus* rearing on sugar beet (Pfitzer *et al.*, 2022b). Developmental stages of nymphs were determined under a stereomicroscope according to the described key (Pfitzer *et al.*, 2022b) and the third, fourth and fifth nymphal instars were used in this study. We had no access to the nymphal stages of *R. quinquecostatus*, and *H. obsoletus*, therefore, only adults (male and female) were included.

Template DNA preparation

Two methods were used for preparation of template DNA from the insect specimens. Pure DNA from insects (adults or nymphs; fig. S1) was prepared using 'DNeasy Blood & Tissue Kit' (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions and dissolved in 60 µl of Tris acetate buffer (pH = 7). For crude extracts a slightly modified method described by Priti *et al.* (2021) was used. The insects (one adult or one nymph) were individually transferred into 1.5 ml tubes containing 60 µl phosphate-buffered saline (PBS; pH = 7.4) and then crushed with a sterile micropestle. After 10 min incubation at 95°C, samples were centrifuged for 10 min with 13,500 × ***g*** at room temperature. The supernatants were used as a PCR or RPA template. DNA quality and quantity were analysed with a spectrophotometer ('DeNovix DS-110', DeNovix Inc., Wilmington, USA) and via gel electrophoresis (fig. S2).

Primer design and RPA assay

For efficient RPA assay, primers (31 and 32 bp in size) were designed based on the *COI* gene sequence from *P. leporinus* (GenBank accession number ONO94072) following the instruction by TwistAmp® kit (TwistDx Ltd. Cambridge, UK). For this purpose, primer sequences that produced secondary structure, hairpin and primer-primer binding were avoided (Wan Rasni *et al.*, 2022). For the specificity of primers, *COI* sequences of *P. leporinus*, *R. quinquecostatus*, and *H. obsoletus* were obtained from our recent study (Pfitzer *et al.*, 2022a) and NCBI database (National Center for Biotechnology Information, U.S. National Library of Medicine, Rockville Pike, MD, USA). These sequences were aligned using BioEdit software (version 7.2; https://bioedit.software.informer.com/7.2/), then primers with no mismatch to the *COI* gene of *P. leporinus* but with mismatches to the *COI* gene of the closely related species were selected and tested *in silico* on close cixiid species (fig. 1) and species which were reported from sugar beet fields, *P. leporinus, R. quinquecostatus* and *H.*

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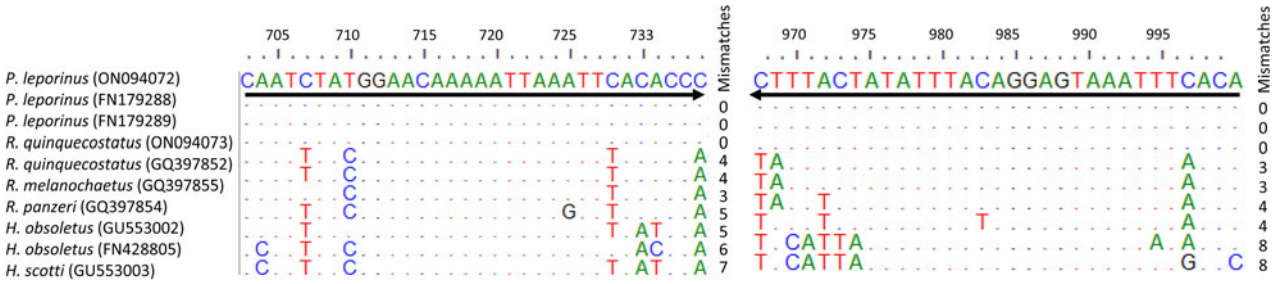

Figure 1. Alignment of the specific primers to the *COI* gene of *Pentastiridius leporinus* and different members of *Hyalesthes* spp. and *Reptalus* spp. The identical nucleotides with the specific primers (black arrows) are shown with dots. The positions of the primers are shown based on the *COI* gene with GenBank accession number ON094072. Number of mismatches between primers and the analysed sequences are shown on the right side of each alignment.

obsoletus (Sémétey *et al*., 2007b; Pfitzer, 2019). Table 1 shows the sequence, GC content and melting temperature of the designed primers.

The specificity of the designed primers was initially tested by PCR and compared to RPA assay. For PCR reactions, 10 µM of each primers (Penta-F and Penta-R) were used in a final volume of 20 µl containing 10 µl 'DreamTaq PCR Master Mix (2X)' (Thermo Fisher Scientific, Waltham, MA, USA), and about 100 ng of DNA templates from *P. leporinus* and two close species, respectively. The PCR conditions were 95°C for 3 min, 30 cycles at 95°C for 30 s, 56°C for 25 s and 72°C for 25 s and a final step at 72°C for 2 min according to the protocol by ThemoFisher Scientific (https://assets.thermofisher.com/TFS-Assets/BID/Reference-Materials/dreamtaq-dna-polymerases-labaid.pdf).

RPA reactions were performed according to the instruction of the TwistAmp® Basic kit (TwistDx Ltd. Cambridge, UK). For each reaction, 10.7 µl of sterile water, 2.4 µl of forward and reverse primers (10 µM; Penta-F and Penta-R), 29.5 µl of rehydration buffer and 2.5 µl of template were mixed. This mixture was added to an enzyme pellet from the kit. All samples were kept on ice during the preparation. To start the reactions, 2.5 µl of magnesium acetate solution (280 mM) was added to each tube. The tubes were briefly vortexed, then centrifuged and incubated in a water bath at 39°C for 5, 10 or 15 min. After the RPA reaction, the products were heated at 80°C for 5 min to inactivate the polymerase. The RPA and PCR products (2 µl) were analysed by gel electrophoresis. The amplified DNA in PCR or RPA assays was sequenced (Microsynth Seqlab GmbH, Göttingen, Germany) and aligned with published sequence data for *COI* gene of *P. leporinus* (Pfitzer *et al*., 2022a) to confirm the specificity of each assay.

The sensitivity of RPA and PCR was tested using ten-fold serial dilutions of crude nucleic acid from *P. leporinus* adults and nymphs. One microliter of each template DNA dilution was amplified in each assay. The RPA and PCR products were analysed by gel electrophoresis.

Real-time PCR and real-time RPA assay

For real-time PCR, each reaction was set up in a 15 µl volume containing 1x iTaq Universal SYBR Green Supermix (BioRad, Hercules, USA), 0.330 µM of each primer (Penta-F and Penta-R) and 1 µl DNA (about 100 ng). The qPCR was carried out in the CFX96 Real Time System C1000 Touch Thermal Cycler (Bio-Rad, Feldkirchen, Germany). The reaction was set with initial denaturation of 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 20 s. Three biological samples were tested for each insect group and each biological sample was analysed with three technical replicates. The threshold cycle (C_T) data was analysed based on Livak and Schmittgen (2001) with some modification. As the aim of this assay was specific detection of *P. leporinus* rather than quantification of *COI* gene, normalisation of data against a house keeping gene was not included. The C_T value is the cycle at which the fluorescence signal reaches a threshold value, so a low number of C_T represents a higher concentration of target gene in each sample. Therefore, an average of 2^{-Ct} was calculated for each sample. Data were statistically analysed with R software (Tukey test, $P < 0.05$).

Real-time RPA assay was performed using the TwistAmp® Basic kit with some modifications. For each reaction, 10.2 µl of iTaq Universal SYBR Green Supermix, 2.4 µl of forward and reverse primers (10 µM, Penta-F and Penta-R), 29.5 µl of rehydration buffer and 3 µl of template (about 200 ng) were mixed. This mixture was added to an enzyme pellet from the kit. Then, 2.5 µl magnesium acetate (280 mM) was added to reach 50 µl volume. This mixture was mixed and divided into two tubes each containing a final 25 µl reaction. This was to have more technical replicates and to scale down the costs for each reaction. The real-time RPA reactions were carried out in the CFX96 Real Time System C1000 Touch Thermal Cycler. The reaction was set at 39°C for 20 min and fluorescence signals were measured after each 40 s. Then, enzyme reaction was stopped at 80°C for 10 s. The melting curve was created by ramping the temperature from 65°C to 95°C by 0.5°C. The fluorescence cycle threshold for SYBR Green was set just above the water control for each platform, so that samples with equal or less fluorescence than the water control were identified as negative (Teoh *et al*., 2015). Three replicates of each variant in at least three independent runs were performed for sensitivity and specificity experiments. In addition, the amplified products were directly run on 1%

Table 1. Primers used for the detection of *Pentastiridius leporinus*.

Name	Sequence	GC content	Tm	Size of product	Reference
Penta-F	caatctatggaacaaaaattaaattcacaccc	31%	57°C	269 bp	This study
Penta-R	tgtgaaatttactcctgtaaatatagtaaag	26%	54°C		Pfitzer *et al*. (2022a)

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agarose gel without any additional purification step (Babujee *et al.*, 2019) and the amplicons were visualised under UV light (312 nm).

The sensitivity of real-time RPA and real-time PCR was tested using a ten-fold serial dilution of crude nucleic acid extracts from individual *P. leporinus* adults and fourth-instar nymphs. To reduce time and cost and also to optimising this assay for the future field application only the crude nucleic acid extracts from individual insects were tested and therefore, the precise measurement of nucleic templates was not possible. Thus, this assay was repeated at least three times to obtain more robust data based on a single insect per sample. One microliter of each template DNA dilution was amplified in both assays using the explained reaction conditions for Real-time RPA assay. The threshold time was plotted against the detected DNA templates.

Results

Specific P. leporinus detection by RPA

The specific primers for *P. leporinus* (fig. 1) based on the sequence of the *COI* gene, produced a specific PCR product of the expected size (269 bp) from adults (female and male) samples using kit purified nucleic acids as a template. No amplicon was detected for the closely related planthoppers, *R. quinquecostatus* and *H. obsoletus* (fig. 2a). The same results were obtained in the RPA assay at 39°C for 15 min (fig. 2b). In addition, shorter reaction times showed clear RPA products to appear as well after 10 minutes. However, *COI* amplification and planthopper detection was not possible as early as 5 min (fig. 2c and S3). The sensitivity of the RPA assay for *P. leporinus* monitoring in crude samples was tested on a 10-fold dilution series. The *COI* gene from *P. leporinus* adults was detectable in dilution range from 1×10^{-1} to 1×10^{-3} for the adults and 1×10^{-1} to 1×10^{-2} for nymphs. Therefore, these specific primers can be used for specific detection of *P. leporinus* by RPA in a short reaction time (10 min).

Real-time PCR for specific P. leporinus *detection*

The real-time PCR assay was used to test specific detection of *P. leporinus* from pure and crude nucleic acids from nymph and adult samples. The *COI* gene was specifically detected in both *P. leporinus* adults and nymphs using specific primers and pure nucleic acid template (fig. 3a). The fluorescence signal intensity for adults (male and female) and nymphal stages (L3, L4, and L5) reflected their body size. Variations of the size of nymphal stages and adults have been described (Pfitzer *et al.*, 2022b). The strongest signals appeared for adults that have the largest size and contain more *COI* templates. For the nymphal stage, L5 has the largest size and showed the strongest fluorescence signals (fig. 3a). This was reflecting the higher DNA concentration in adult samples (250 to 340 ng μl^{-1}) compared with nymphs (65 to 80 ng μl^{-1}) as we intentionally did not optimise the DNA

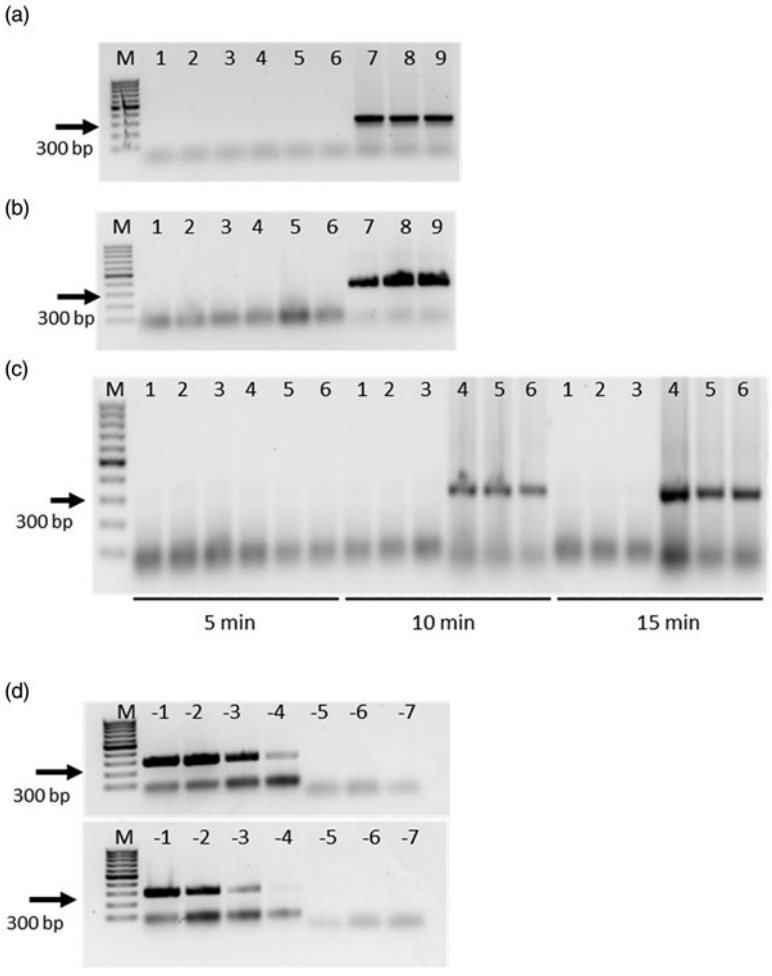

Figure 2. PCR and RPA for specific detection of *Pentastiridius leporinus*. A representative electrophoretic pattern of PCR (a) and RPA (b) products show specific amplification of the *COI* gene of *P. leporinus* (lanes 7 to 9). Lanes 1 to 3 represent *R.* quinquecostatus, and lanes 4 to 6 represent *H. obsoletus* samples. (c) RPA was performed for 5, 10, and 15 min at 39°C using DNA samples of *R.* quinquecostatus (lanes 1 to 3) and *P. leporinus* (lanes 4 to 6) adult samples. (d) RPA assay using 10-fold serial dilutions of crude nucleic acid of adults (top panel) or nymphs (lower panel) shows the sensitivity of this assay for amplification of *COI* gene of *P. leporinus*. RPA products were gel-separated without an additional purification step. The size of amplicons was compared with 100 bp ladder (M).

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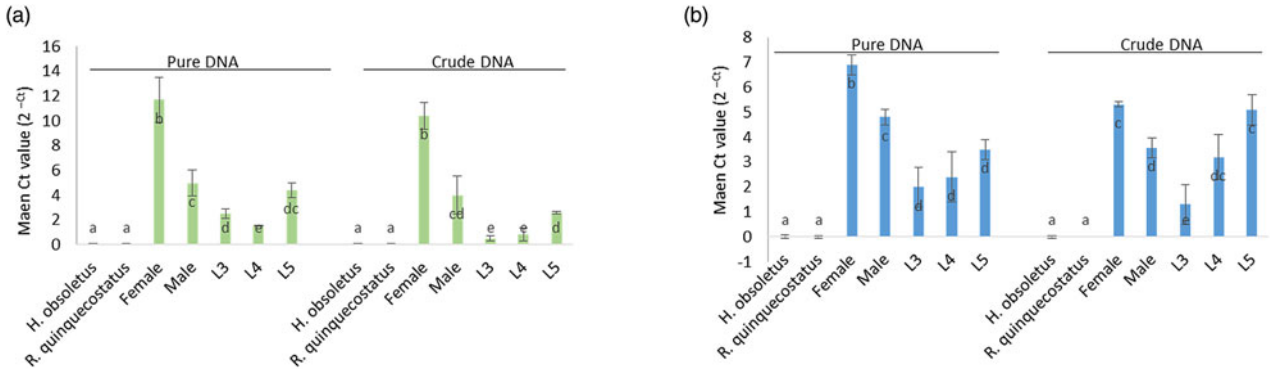

Figure 3. Real-time amplification of the *COI* gene for the specific detection of *Pentastiridius leporinus*. Real-time PCR (a) and real-time RPA (b) show specific amplification of the *COI* gene using pure (kit) or crude nucleic acid of both adults (female and male) and nymphs (L3, L4, and L5) of *P. leporinus* compared with samples of adult *R. quinquecostatus* and *H. obsoletus*. For each treatment three biological replicates were tested, and the average threshold cycle (Ct) is presented. The same letter on the bars means not statistically different ($P < 0.05\%$).

concentration to directly detect and compare all life stages of the insect from individual DNA samples.

In addition, using crude template DNA, a comparable and specific *P. leporinus* detection was possible for adults (female and male) and nymphal stages (L3–L5). For the close cixiid species, *R. quinquecostatus* and *H. obsoletus,* no clear fluorescence signals ($C_T > 32$) were detected (fig. 3a). Therefore, real-time PCR can be used for specific detection of *P. leporinus* adults and nymphs using crude DNA templates.

Optimising the real-time RPA conditions for specific P. leporinus *detection*

Application of real-time RPA based on a SYBR green assay was tested using *P. leporinus* specific primers and the TwistAmp Basic kit. This allows application of real-time RPA for detection of target genes without probe design and extra costs. This assay was initially tested on pure nucleic acids from *P. leporinus* and the two closely related species. Using a constant temperature of 39°C produced fluorescence signals for both *P. leporinus* adults and nymphs after a short incubation time (less than 10 min). However, background fluorescence signals also appeared for the nonspecific targets in both *R. quinquecostatus* and *H. obsoletus* samples after a longer time (more than 10 min) (fig. 4). Testing the real-time RPA products on agarose gel showed a clear amplification of *COI* gene only in *P. leporinus* samples, while no clear amplicons were detected in *R. quinquecostatus* and *H. obsoletus* samples even after 20 min. Thus, real-time RPA specifically amplifies the target *COI* gene exclusively in *P. leporinus* extracts. Based on the fluorescence plot data which included 14 runs and also agarose gel results, the detection threshold of the real-time RPA was established as equal to 10 cycles (= 400 s) and the baseline to 200 relative fluorescence units (RFU) (fig. 4). Samples that produced exponential amplification curves above the threshold were considered as positive for *P. leporinus* and below the threshold were considered as negative. Detection of both adults and nymphal stages was confirmed in this assay (fig. 3b and 4). In addition, this detection threshold was applied to compare real-time RPA for detection of *P. leporinus* using crude nucleic acids from both adults and nymphs. Higher fluorescence signals were observed in pure nucleic acids from adults compare to crude nucleic acid, while fluorescence signals were comparable in both pure and crude nucleic acids of nymphs (fig. 3). Therefore,

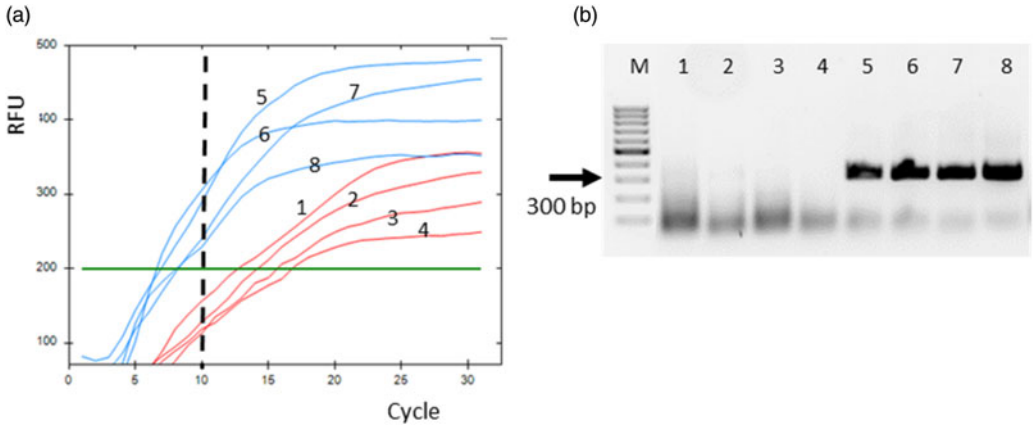

Figure 4. Real-time RPA assay for the specific detection of *Pentastiridius leporinus*. (a) Real-time RPA plot shows strong fluorescence signals for *P. leporinus* (5–8) and weak signals with a delay for *R. quinquecostatus* (1 and 2) and *H. obsoletus* (3 and 4). Time unit of each cycle represents 40 s. The threshold level for reliable *P. leporinus* detection was established as equal to 10 units of time (6.6 min) and baseline of fluorescent signals at 200 relative fluorescence units (RFU) indicated with the horizontal green line. (b) Electrophoretic patterns of real-time RPA products show clear amplification of the *COI* gene in *P. leporinus* samples. There were no amplicons for *R. quinquecostatus* adults and *H. obsoletus* adults. Lane numbers coordinate with panels A. RPA products were gel-separated without additional purification step. The size of amplicons was compared with 100 bp ladder (M).

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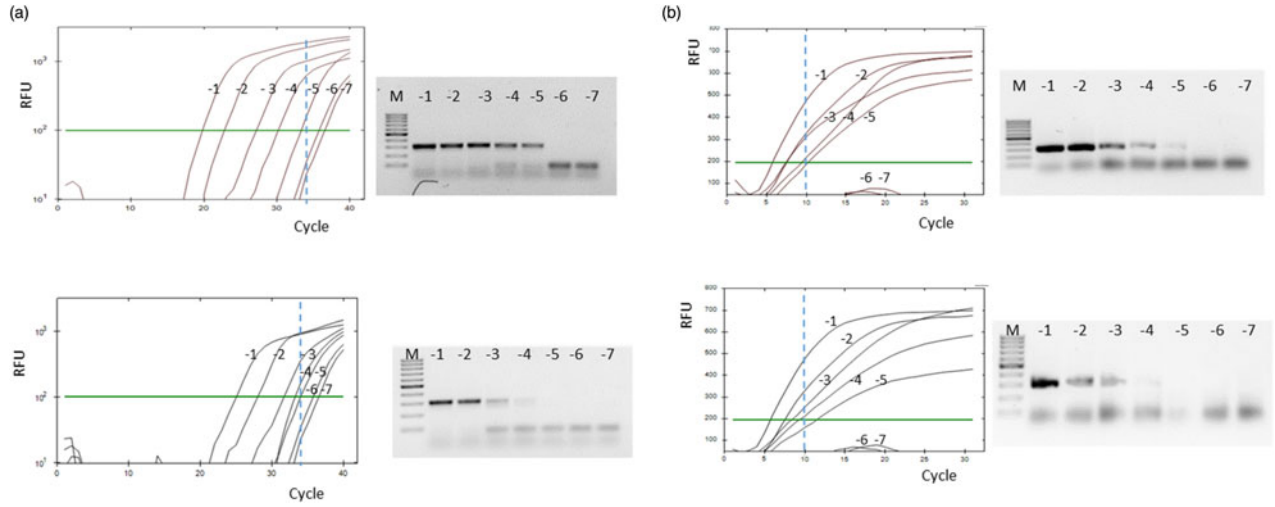

Figure 5. Amplification plots of real-time measurements for 10-fold serial dilutions of template DNA from *P. leporinus*. (a) Real-time PCR and (b) Real-time RPA fluorescence plots for adults (top panel) and nymphs (lower panel) using crude nucleic acid extracts. The threshold level for reliable *P. leporinus* detection was established as equal to 10 units (each unit equal to 40 s) and baseline of fluorescent signals to 200 RFU indicated with a green line. The amplified *COI* gene fragment in each assay was additionally separated on 1% agarose gels. The 100 bp ladder was used as DNA marker (M).

real-time RPA specifically detects *P. leporinus* in both pure and crude nucleic acids of adults and nymphs.

Comparison of sensitivity between real-time PCR and real-time RPA for P. leporinus *detection*

The sensitivity of real-time PCR was compared to real-time RPA using a ten-fold serial dilution of crude nucleic acid of individual *P. leporinus* adults and nymphs. In real-time PCR, *COI* gene from adults was detectable in dilution range from 1×10^{-1} to 1×10^{-4}. However, this range was from 1×10^{-1} to 1×10^{-3} for nymphs (fig. 5a). This indicated that fluorescence signals after 30 cycles are not reliable and adult DNA contains more *COI* templates compared to nymphs. Similarly, in real-time RPA, *COI* gene from adults was detectable in dilution range from 1×10^{-1} to 1×10^{-4} and a lower level of *COI* gene (1×10^{-3} dilution) was detected in nymphs (fig. 5b). The signals after 10 units of time and below 200 RFU were not reliable and considered as no detection.

Discussion

Recently, a PCR method based on *COI* gene was developed to specifically identify and distinguish all life stages of *P. leporinus* from the two closely related planthopper species (Pfitzer *et al.*, 2022a). In this study, we further developed a more rapid assay based on real-time RPA to identify and monitor *P. leporinus* in a short time and without the need for pure DNA extraction and gel electrophoresis.

Isothermal DNA amplification methods such as RPA (Piepenburg *et al.*, 2006) that works under ambient temperature (37–42°C) with short reaction time (5–20 min), considerable sensitivity and specificity, resistance to inhibitors and tolerance for more primer mismatches (Tan *et al.*, 2022), increasingly was used for the detection and monitoring of pathogens (Li *et al.*, 2018; Zhou *et al.*, 2022) and insects (Priti *et al.*, 2021). To detect the RPA products in end-point assays, agarose gel electrophoresis (Tran *et al.*, 2022) and lateral flow (Zhou *et al.*, 2022) have been used. For real-time detection, in most cases an exo-probe which is specific for TwistAmp exo kit has been applied (Li *et al.*, 2018; Babujee *et al.*, 2019). For example, real-time RPA assay was used for the rapid, simple, reliable, and sensitive detection of *Campylobacter jejuni* in food samples (Geng *et al.*, 2019). In addition, RPA reaction can be detected using DNA intercalating fluorophores such as SYBR Green I (Azinheiro *et al.*, 2022). However, due to non-specific interaction with double stranded DNA, this method produces false-positive results (Zou *et al.*, 2020). To avoid such errors, we developed a real-time based RPA which specifically detects the amplified target gene of *P. leporinus* in a short time (about 7 min) and does not require probe design or agarose gel electrophoresis, which can save time and costs.

RPA assays were shown to amplify DNA targets from impure DNA templates and crude extracts from plants (Londoño *et al.*, 2016; Qian *et al.*, 2018; McCoy *et al.*, 2020) and insects (Priti *et al.*, 2021). Most reports have used general extraction buffer (McCoy *et al.*, 2020), Tris-EDTA, CTAB-Tris buffer or Tris-HCl as extraction buffer for template DNA preparation from plant tissue (Kalischuk *et al.*, 2020). In addition, PBS was also used for plant template DNA preparation (Kalischuk *et al.*, 2020). We have recently shown that PBS extracts from *P. leporinus* are suitable for the specific PCR-based detection of this insect vector (Pfitzer *et al.*, 2022a). In this study, the PBS extracted samples were used for specific detection of both adults and nymphal stages of *P. leporinus* using real-time PCR and real-time RPA. In both assays, PBS extracts showed a comparable sensitivity of detection for both adults and nymphal stages. A ten-fold dilution of nucleic acids up to 10^{-6} for adult samples and up to 10^{-5} for nymph samples was still detectable in real-time PCR and real-time RPA assays, respectively. This confirms the sensitivity of real-time RPA for detection of *P. leporinus* even from a low concentration of nucleic acid samples which can be applied for detection of this insect vector from a mixture of cixiid planthoppers. An excellent correlation between real-time PCR and real-time RPA assays for detection of Human mastadenoviruses (HAdVs) (Wu *et al.*, 2019), small ruminant morbillivirus (SRMV) (Li *et al.*, 2018) and *C. jejuni* in food samples (Geng *et al.*, 2019) supports our results. Figure 3 shows that in the real-time PCR assay, the fluorescence intensity for both pure and crude nucleic acid were

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comparable in adult samples. However, a lower level of fluorescence signals was observed in nymphal crude samples. In the real-time RPA assay, the fluorescence intensity for both pure and crude nucleic acid were comparable in both adult and nymph samples. This may reflect the resistance to inhibitors by RPA assays compared to real-time PCR.

Rapid detection (ca 7 min) of *P. leporinus* using real-time RPA is the main advantage over real-time PCR. Similarly, the analysis time for monitoring food samples for *C. jejuni* by real-time RPA was reduced from 60 to approximately 13 min and the results were as reliable as those of the real-time PCR assay (Geng *et al.*, 2019). According to the kit instruction and published reports the optimised temperature is between 37 and 42°C for 15–20 min reaction time (Londoño *et al.*, 2016; McCoy *et al.*, 2020; Londono *et al.*, 2021). However, at lower temperature as low as 25°C, RPA produced amplicons only at a longer incubation time (Wang *et al.*, 2020). In contrast, the incubation time could be shortened to 5 min when the reaction temperature was 42°C (Lee *et al.*, 2020; Kim *et al.*, 2022). In this study, non-target insects produced weak fluorescence signals and non-specific curves with a clear delay. In both assays, the amplicon products were tested on agarose gel and showed no detectable products, which confirm the specificity of both assays. Such non-specific curves were also observed in non-target nematode samples (Subbotin, 2019) and *Fusarium oxysporum* detection using real-time RPA assays (Zou *et al.*, 2020). Therefore, a defined threshold for the reaction period and RFU for real-time RPA assays need to be optimised during assay development to avoid false positive results. In conclusion, the developed real-time RPA allows a rapid and sensitive assay for specific detection of adults and nymphal stages of *P. leporinus*. This method can be further developed for field application and monitoring the main SBR vector in a short time (under 7 min). Such real-time RPA approach was successfully applied in the field for the rapid (under 15 min) and specific detection of *Phytophthora sp.* in various crops using lyophilised reagents (McCoy *et al.*, 2020) and for rapid and real-time detection of *Xanthomonas gardneri* and *X. euvesicatoria* the causal agents of bacterial spots in tomato plants in fields (Strayer-Scherer, 2019).

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