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ORIGINAL CONTRIBUTION

Rapid diagnosis of the invasive wax scale, *Ceroplastes rusci* Linnaeus (Hemiptera: Coccoidea: Coccidae) using nested PCR

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Keywords

Ceroplastes rusci, COI, nested PCR, quarantine pests, specific primers

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Abstract

The fig wax scale, *Ceroplastes rusci* (Linnaeus) (Hemiptera: Coccoidea: Coccidae), is an invasive fruit pest of Afrotropical origin and potentially could become a serious threat to commercial fruit crops in China. *C. rusci* is difficult to identify owing to the shortage of easily distinguishable morphological characters. A rapid, accurate and reliable method to identify *C. rusci* in quarantine work is needed to detect further spread. In the present study, we describe a nested PCR method for the molecular identification of *C. rusci*. The nested PCR primers were designed based on variations in the barcode region of COI sequences between *C. rusci* and five other *Ceroplastes* species. A 200-bp fragment was successfully amplified from 96 *C. rusci* individuals of seven geographical populations in China and Vietnam, and 13 individuals of two populations in Italy (the type country for *C. rusci*). These provided diagnostic bands that were not observed in any of five other *Ceroplastes* species widely distributed in China, namely, *C. ceriferus* (Fabricius), *C. floridensis* Comstock, *C. japonicus* Green, *C. pseudoceriferus* Green and *C. rubens* Maskell. Sensitivity tests revealed that diagnostic bands were generated even with a DNA template concentration of ~1.5×10^{-5} ng/μl, and with average DNA template concentrations for adult females, single first-instar nymphs and eggs of 14.7, 6.3 and 3.0 ng/μl, respectively. Our study demonstrates that the molecular diagnosis of *C. rusci* using nested PCR is rapid and accurate and shows potential in plant quarantine programmes.

Introduction

The fig wax scale *Ceroplastes rusci* (Linnaeus) (Hemiptera: Coccidae) is a well known pest of figs (*Ficus* spp.) and attacks a wide range of plants including at least 21 different families (Ben-Dov 1993; Vu et al. 2006). It can cause serious damage to important commercial fruit crops including banana (Marotta 1987), citrus (Inserra 1970), coconut (Chua 1997), grape (Ben-Dov 2012) and mango (Ben-Dov 1993). *C. rusci* individuals suck plant sap, damage plant tissues and secrete sticky honeydew that provides a substrate for mould growth and impairs plant photosynthesis (Ben-Dov and Hodgson 1997). Owing to the serious economic harm to fruit crops, in 2007 it was listed in the Catalogue of Quarantine Pests for Import Plants to the People's Republic of China. *C. rusci* is considered native to the Afrotropical region (Qin et al. 1994, 1998; Hodgson and Peronti 2012), but has now expanded to the Oriental, Neotropical and Palaearctic regions (Ben-Dov 1993). Recently, invasions of *C. rusci* have been detected in Australia (Waterhouse and Sands 2001), India (Kumar 2013) and China (Li and Wu 2013). It is highly likely that *C. rusci* has much a wider distribution in the tropics and the subtropics, but is misidentified as other *Ceroplastes* spp., due to the difficulties of morphological identification (FERA 2010).

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Morphological identification of wax scales is very difficult because of their small size and high degree of similarity. The traditional method to identify *C. rusci* in quarantine work is microscopic examination of slide-mounted specimens, which requires the preservation of the adult female cuticle, proper preparation of specimens and examination by a trained taxonomist. If adult female specimens of a wax scale are absent, identification of nymphs is more difficult, even impossible because taxonomic keys to scale insect species are based on the adult female. Moreover, time-consuming preparation of slides makes it impractical for quarantine work, which requires rapid and accurate identification for timely interception of invasive species.

In contrast to traditional morphological taxonomy, molecular identification is not hindered by phenotypic polymorphism, sex or developmental stage variation of the target species (Zhang et al. 2012). It is easy even for a non-specialist to distinguish between unknown species using molecular identification tools (Rugman-Jones et al. 2006; Asokan et al. 2007; Jiang et al. 2013). With the development of molecular techniques, DNA barcodes have emerged as popular tools for species-level identification of invertebrates (Costa et al. 2007; Mikkelsen et al. 2007) and vertebrates (Hebert et al. 2004; Hajibabaei et al. 2006; Wong et al. 2009). In recent years, DNA barcodes have been used in mealybugs, armoured scales and wax scales (Malausa et al. 2011; Park et al. 2011; Abd-Rabou et al. 2012; Deng et al. 2012), reflecting the COI gene as a marker suitable for identifying scale insects. The process of DNA barcoding requires two basic steps: (1) constructing the DNA barcode library from known species and (2) identifying unknown samples by matching their barcode sequences to the barcode library (Kress and Erickson 2012). When sequences of the known species are absent from the library, misidentification may occur due to interference from similar DNA sequences. Moreover, DNA sequencing involves additional cost and time for quarantine work. Some recent studies have suggested that PCR with species-specific primers could overcome the need of DNA sequencing and accelerate the process of identification (Barcenas et al. 2005; Rugman-Jones et al. 2009; Zhang et al. 2012; Jiang et al. 2013).

In this study, nested PCR with species-specific primers were used to identify *C. rusci*. The species-specific primer set was designed according to variations in COI barcodes among six *Ceroplastes* species, namely, *C. ceriferus* (Fabricius), *C. floridensis* Comstock, *C. japonicus* Green, *C. pseudoceriferus* Green, *C. rubens* Maskell and *C. rusci*. The specificity and sensitivity of the species-specific primers were tested on the basis of nested PCR performance. Our aim was to develop a specific COI primer for rapid and accurate identification of *C. rusci* using nested PCR.

Materials and Methods

Sample collection

In addition to the previously reported sites in China, that is, Maoming and Panzhihua (Li and Wu 2013), fig wax scales were collected from seven other sites in China, Vietnam and Italy (the type country for *C. rusci*). Eggs and first-instar nymphs of *C. rusci* were collected in Menglun, Yunnan. Individuals of *C. rusci* from different sites were preserved in 95% ethanol once found and stored at −20°C until DNA extraction. Other *Ceroplastes* species collected in a previous work (Deng et al. 2012) were included. Detailed information of *Ceroplastes* species used in this work is listed in Supplement Table S1. All individuals were identified according to morphological characters and the taxonomic keys (Hodgson and Peronti 2012). Slide-mounted voucher specimens were deposited in the Insect Collection of Beijing Forestry University.

DNA extraction, PCR and sequencing

Genomic DNA was extracted from single-parasitoid-free adult females, eggs, or first-instar nymphs of *C. rusci* using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. For adult females, 200 μl elution buffer was added to elute the DNA, while 30 μl elution buffer was added for single eggs or first-instar nymphs. The concentration of all DNA templates was assayed by spectrophotometry (NanoDrop2000 spectrophotometer, Thermo Scientific, Wilmington, DE). To estimate average DNA concentration of *C. rusci* in different life stages, at least ten adult female individuals (if possible) per population and six eggs or first-instar nymphs were used (Supplement Table S1).

PCR amplifications were processed in a 50 μl reaction volume including 5 μl DNA template, 5 μl 10× Buffer, 25 mM $MgCl_2$, 2.5 mM dNTP mixture, 10 pmol of each primer, 30 μl ddH_2O and 1 unit of *LA Taq* DNA polymerase (TaKaRa Bio Inc., Dalian, China) in both PCR rounds. Thermal cycles followed Deng et al. (2012). PCR products were detected on 1% agarose, and sequenced bidirectionally using BigDye v3.1 on an ABI3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA).

Specificity of *C. rusci*-specific primers

To test the specificity of *C. rusci*-specific primers, nested PCR were conducted on 96 *C. rusci* individuals and 110 individuals from five other *Ceroplastes* species widely distributed in China (detailed in Supplement Table S1).

In the first round of PCR, 5 μl of extracted DNA was used as template and COI of approximately 760 bp was obtained with the primer set C1-1554F/C1-2342 (Deng et al. 2012). Prior to the second-round PCR, the first PCR products were diluted 200 times. In the nested PCR, a new primer set CI-1752-F (5′-TGAATGTTATTTCCATCTCTT-3′)/CI-1956-R (5′-ATA-GATCAGCAATATAATGTCAAG-3′) was designed to distinguish *C. rusci* from others with 5 μl diluent as template, and approximately 200-bp products were generated. For both rounds of the PCR, double-distilled water was used as a negative control. To assure the accuracy of result, each positive product was sequenced. The specificity test was repeated three times for each sample to ensure the reliability of the specific primer pair. Moreover, *C. rusci* specimens from Italy (the type country for *C. rusci*) were tested based on the methods described as above. The COI sequences of *C. rusci* from Italy were almost identical to those of *C. rusci* from China and Vietnam, except that a substitution of one base pair was found.

Sensitivity of *C. rusci*-specific primer test

To evaluate the sensitivity of the nested PCR primer, a 10-fold dilution series of genomic DNA dissolved in ddH_2O from an adult *C. rusci* specimen (15 ng/μl) was used for amplification. Double-distilled water was used as a negative control. The reaction mixture and thermal cycling conditions were as the PCR described above. The sensitivity test was repeated in triplicate.

Results

DNA quality

The template DNA concentration was 14.7 $\pm$ 4.9 ng/μl (adult female), 6.3 $\pm$ 1.0 ng/μl (first-instar nymph), and 3.0 $\pm$ 0.3 ng/μl (egg), respectively. For all individuals used in this study, a 800-bp fragment was generated using the outer primer pair C1-1554F and C1-2342R, indicating high-quality DNA had been extracted from single adult females, first-instar nymphs and eggs.

Specificity of the nested PCR primers

The specificity of the nested PCR primers was assessed using six isolates of *C. rusci*, one of *C. pseudoceriferus*, and three isolates, each of *C. floridensis*, *C. japonicus*, *C. ceriferus* and *C. rubens* from China and Vietnam (Supplement Table S1). In the first-round PCR, a fragment of 800 bp was amplified from all *Ceroplastes* individuals, suggesting that the primer set was suitable for recovering the COI gene of six *Ceroplastes* species. The second-round PCR produced 200 bp in six isolates of *C. rusci*, which were not detected with the other species (fig. 1). The results of three replicate experiments were consistent, indicating the high specificity and

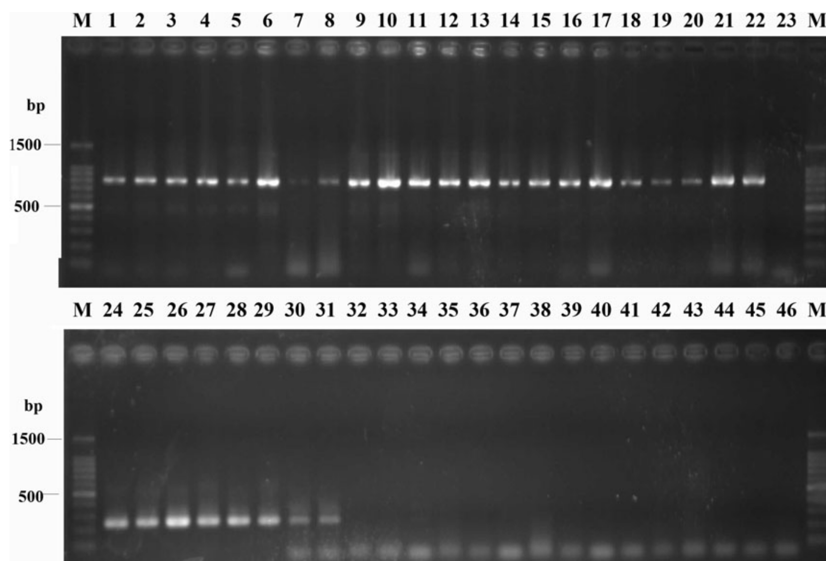

Fig. 1 Specificity test of *Ceroplastes rusci* specific primer. Lanes 1–23: the first round PCR results, lanes 24–46: the second round PCR results (Lanes 1–6 and 24–29: adult females of *C. rusci* from each geographical population; lanes 7 and 30: *C. rusci* egg, lanes 8 and 3: *C. rusci* nymph; lanes 9–11 and 32–34: *C. rubens*; lanes 12–14 and 35–37: *C. ceriferus*; lanes 15–17 and 38–40: *C. japonicus*; lanes 18–19 and 41–42: *C. pseudoceriferus*; lanes 20–22 and 43–45: *C. floridensis*), lane 23 and lane 46: negative control (ddH_2O), and lane M: 100 bp DNA Ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp).

reproducibility of the primer pair. Furthermore, a 200-bp fragment was successfully amplified from Italian specimens using the nested primers (fig. 2), indicating the potential for the rapid and accurate identification of *C. rusci* specimens outside Eastern Asia.

Sensitivity of the nested PCR primers

The sensitivity of the *C. rusci*-specific primer pair was tested with a serial dilution ranging from 15 ng/μl to 1.5×10^{-7} ng/μl. After the first-round PCR, 800-bp bands with the outer primer pair were observed from 15 to 1.5×10^{-3} ng/μl of DNA template (fig. 3a), suggesting that a minimum of 1.5×10^{-3} ng/μl genomic DNA could be detected successfully. When the two rounds of PCR were finished, a 200-bp fragment was generated from 15 ng/μl to 1.5×10^{-5} ng/μl of DNA template (fig. 3b), indicating the detection limit with the inner primer pair was 1.5×10^{-5} ng/μl. These results indicated that nested PCR was nearly 100-fold sensitive compared with conventional PCR. The DNA concentration test results demonstrated that the range of DNA concentration of *C. rusci* (3.0–23.4 ng/μl) exceeded the detection limit (1.5×10^{-5} ng/μl). Thus, no matter the life stage of the *C. rusci* individuals, they could be accurately diagnosed with specific primers using nested PCR.

Discussion

Soft scale insects are serious pests, especially when invasive to a region (Hodges and Hodgson 2010). Miller and Miller (2003) reported that only one of 42 soft-scale species introduced in the USA was not damaging. Of 255 invasive arthropod species introduced into Florida over the last 20 years, only *C. rusci* has been established in Florida (Hodges and Hodgson 2010), indicating that *C. rusci* has strong adaptability and is capable of causing serious economic damage to non-infested areas. As a destructive invasive pest in China, *C. rusci* was first reported on *Ficus microcarpa* in Maoming city of Guangdong Province and on *F. viren* in Panzhihua city of Sichuan Province in 2012 (Li and Wu 2013). Therefore, it is necessary to provide a more rapid and accurate diagnostic tool to identify *C. rusci* for quarantine so as to assist in preventing further invasions of *C. rusci* into non-infested areas of China.

We initially attempted to design a specific primer set for single-step PCR for the purpose of diagnosing *C. rusci* species, but all primers tested failed to amplify the target region, as indicated by unexpected or multiple bands. Meanwhile, it is not easy to exclude the absence of a PCR product results from the lack of DNA template when using one-step PCR to diagnose target species. However, the key advantage of nested PCR is its high sensitivity for the identification of *C. rusci*, here estimated by sensitivity tests to be approximately 100-fold higher than conventional PCR. Hence, the nested PCR approach is excellent for identifying *C. rusci* individuals and has the potential to identify other invasive species.

To guarantee primer specificity, we designed specific primers based on the COI barcode sequences of *C. rusci* and five congeneric species, which have a wide distribution in China and share high sequence similarity (>87%) with *C. rusci*. Considering the COI sequence variation among different geographical populations, seven populations of *C. rusci* were selected to test specificity. None of the individuals of the seven populations failed the test, demonstrating the robust specificity of the nested PCR primers. Furthermore, the detection limit of 1.5×10^{-5} ng/μl template DNA showed high sensitivity in identifying *C. rusci*, which

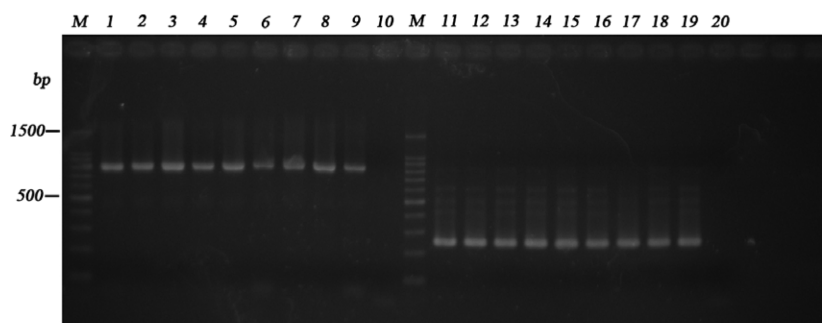

Fig. 2 Specificity test of *Ceroplastes rusci* specimens from Italy. Lanes 1–10: the first-round PCR results, lanes 11–20: the second-round PCR results, lane 10 and lane 20: negative control (ddH_2O), and lane M: 100 bp DNA Ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp). Lanes 1–6 and 11–16 represent *C. rusci* individuals collected from Portici, Italy, and lanes 7–9 and 17–19 represent *C. rusci* individuals collected from Palma Campania, Italy.

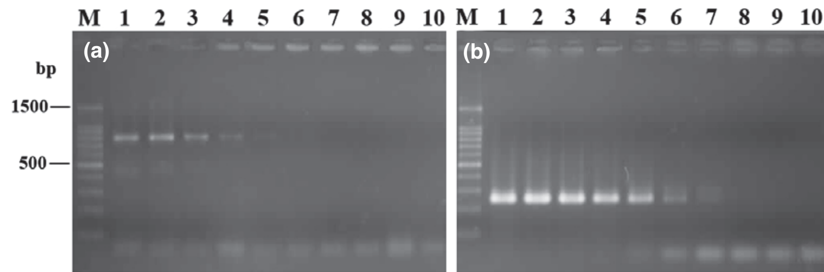

Fig. 3 Sensitivity test for the nested PCR. a. The first round PCR reaction; b. Nested PCR with specific primer set. The template DNA concentrations were as follows: lane 1: 15 ng/μl; lane 2: 1.5 ng/μl; lane 3: 1.5×10^{-1} ng/μl; lane 4: 1.5×10^{-2} ng/μl; lane 5: 1.5×10^{-3} ng/μl; lane 6: 1.5×10^{-4} ng/μl; lane 7: 1.5×10^{-5} ng/μl; lane 8: 1.5×10^{-6} ng/μl; lane 9: 1.5×10^{-7} ng/μl; lane 10: negative control (ddH_2O); lane M: 100 bp DNA Ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp).

means that this method is particularly useful for the identification of all life stages including eggs, nymphs and adult females.

Our study provides a nested PCR method using the specific primer set for diagnosing the presence of *C. rusci*, which is the first simple and robust detection tool for invasive *C. rusci* in quarantine applications. According to the tests performed, our specific primer set was confirmed to be sensitive and specific for the detection of *C. rusci*. The nested PCR assay is a simple approach to help non-taxonomists identify *C. rusci*, especially, when the adult females are absent. Moreover, the method consists of two simple PCR amplifications and can skip the time-consuming step of slide preparation, and will accelerate the process of identification without any further sequencing or restriction digestion of the amplified products. In conclusion, we describe a reliable and efficient method with the potential for the rapid and accurate identification of *C. rusci*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Detailed information of *Ceroplastes* species used in the study