A SCAR-Based Method for Rapid Identification of Four Major Lepidopterous Stored-Product Pests

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ABSTRACT Since Taiwan became a World Trade Organization member in 2002, large quantities of grain have been imported from different countries, and insect pests are frequently intercepted from these imported commodities in quarantine inspection. Because most insects are intercepted as immature forms, morphological identification is problematic; therefore, we developed a DNA identification method based on a sequence-characterized amplified region- polymerase chain reaction (SCAR-PCR). Three sets of multiplex SCAR-PCR mixtures, namely SCAR-I, -II, and -III, were developed with each set composed of four species-specific primer pairs derived from the genomic DNA of four major lepidopterous stored-product pests: *Corcyra cephalonica* (Stainton), *Cadra cautella* (Walker), *Sitotroga cerealella* Oliver, and *Plodia interpunctella* (Hübner). The SCAR-I amplicons of *C. cephalonica*, *C. cautella*, *S. cerealella*, and *P. interpunctella* were 205, 550, 324, 382 bp, respectively, while those of SCAR-II were 341, 565, 261, and 170 bp, and those of SCAR-III were 514, 555, 445, and 299 bp. These multiplex PCR mixtures could sensitively and unambiguously detect and identify in ≈5 h individuals among the four lepidopterous pests intercepted in imported stored-products. In summary, the SCAR-PCR method we developed represents a rapid, sensitive and accurate technique for identifying insect species of stored products in plant quarantine operation.

KEY WORDS stored-product insect, RAPD, SCAR, multiplex PCR, quarantine

Taiwan joined the World Trade Organization in 2002, and since then, the categories and quantities of imported agricultural commodities have increased rapidly. For example, >8 million tons of grain were imported in 2009, and living insect pests were found in 12% of the brown rice samples examined (Yao et al. 2009). To facilitate the inspection of a massive number of samples for plant quarantine purposes, reliable and efficient methods, such as DNA-based identification techniques, are needed to save time and labor.

Each species has its own unique deoxyribonucleic acid (DNA) composition that has been used to distinguish the difference between individuals, species, or even populations for decades; furthermore, after the polymerase chain reaction (PCR) was developed, a variety of PCR-based methods make species identification even more convenient. Commonly, the genes on mitochondrial and genomic DNAs, such as mitochondrial cytochrome oxidase I (COI) and ribosomal internal transcribed spacers (ITS1 or ITS2), respectively, were targeted for this purpose; by amplifying a specific DNA fragment with universal PCR primers and comparing the sequences from different individuals or populations, the species are able to be identified and phylogenetic relationships can be revealed as well (Weller et al. 1992, Folmer et al. 1994, Simons et al. 1994, Kjer 2004).

In addition to the DNA sequence comparison, a variety of relatively simple methods that identify species based on the PCR amplicon profiles (numbers and sizes) from unknown regions of genomic DNA, such as random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (restriction fragment-length polymorphism) (Chen et al. 1992, Gasparich et al. 1995, Orui and Mizukubo 1999), have been developed for insect identification. Williams et al. (1990) first developed RAPD-PCR to analyze genomic DNA of human, corn, soybean, and red bread mold. The major advantage of RAPD-PCR is that no sequence information of the target genome is required, so it has been widely used to identify species or subspecies. RAPD markers can also provide an efficient assay for polymorphisms, which allows rapid identification and isolation of chromosome-specific DNA fragments. For example, Dowdy and McGaughey (1996) used RAPD-PCR to distinguish the *Plodia interpunctella* (Hübner) populations among fields and bins in central and western United States, and Hidayat et al. (1996) used this method to distinguish *Sitophilus oryzae* L. from *Sitophilus zeamais* Motschulsky. However, RAPD-PCR frequently produces unstable and variable amplicons, because of the low annealing temperature of the short random primers (≈10 bp) used (Black 1993, Chiu et al. 2000). Later, Paran and Michelmore (1993) developed a se-

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Table 1. Sources of samples and insect body parts used for DNA extraction in the study

Species	Source of country	Stored product	Body parts used
C. cephalonica	Taiwan	Brown rice	Pupa
	Brazil	Soybean	Larval abdomen
C. cautella	Taiwan	Garlic	Pupa
	America	Soybean	Adult metaleg
	India	Corn	Adult metaleg
	Brazil	Soybean	Larval abdomen
S. cerealella	Taiwan	Paddy rice	Pupa
	India	Corn	Adult metaleg
P. interpunctella	Taiwan	Sorghum	Pupa
	America	Corn	Adult metaleg
	India	Wheat	Adult metaleg
	Cambodia	Corn	Adult metaleg

quence-characterized amplified region (SCAR) technique, which used 18≈25-bp primers, based on a unique RAPD sequence. Because it is possible to perform SCAR under high stringent reaction conditions, its specificity and stability are enhanced. Moreover, a pair of SCAR primers is usually able to specifically amplify only a single amplicon, making gel analysis more accurate. SCAR has been widely used to identify a variety of insect species (Agustí et al. 1999, 2000; Manguin et al. 2002; Kethidi et al. 2003); Lu et al. (2003) also used this method to successfully identify *Cydia pomonella* L. intercepted from imported apples in Taiwan.

Sitotroga cerealella Olivier, *Corcyra cephalonica* (Stainton), *Cadra cautella* (Walker), and *Plodia interpunctella* (Hübner) are four major lepidopterous pests of stored products. While they can be easily distinguished in the adult stage, their identification becomes more difficult in the egg, larval, and pupal stages because of morphological similarities. In this study, we developed several pairs of species-specific SCAR primers and further combined them in multiplex PCR reactions to rapidly identify these four species of pests in imported stored products.

Materials and Methods

Insect Stocks. The experimental insects were laboratory colonies collected from various places in Taiwan: *C. cephalonica* from rice mills at Wufeng, *C. cautella* from garlic storehouses at Dounan, *S. cerealella* from rough rice storehouses at Wufeng, and *P. interpunctella* from sorghum storehouses at Kinmen. The colonies were fed on wheat flakes in 2-liter glass containers in dark environmental chambers at 30°C and 70% RH. Additional individuals of each species were obtained from quarantined shipments of various grains as shown in Table 1.

RAPD-PCR and DNA Sequencing. Total DNA from a single insect was extracted with a Tissue & Cell Genomic DNA Purification kit (GeneMark, Tainan, Taiwan) following the manufacturer's instructions. Because of the small size of eggs, DNA was extracted with an Easy DNA High Speed Extraction Tissue kit (Fisher Biotec, Wembley, WA, Australia).

RAPD-PCR reactions were carried out in 20-μl mixture containing 60–630 ng of template DNA, 2X PCR Master Mix (including 2U SuperTherm DNA polymerase; JMR, Kent, United Kingdom), and 25 pmoles of random primers (Bio Basic Inc., Markham, Ontario, Canada). A parallel reaction without template DNA was performed as the negative control. PCR amplification was performed with a GeneAmp PCR System 9700 (Applied Biosystems Inc., Foster City, CA) as follows: 94°C for 5 min; 45 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min; and final extension at 72°C for 20 min. Five micrograms of PCR products were analyzed in 1.4% agarose gels. To confirm the consistency of amplification patterns, triplicate reactions were performed using different sources of DNA from each species.

The species-specific and reproducible PCR amplicons were gel excised and purified with a Micro-Elute DNA Clean/Extraction kit (GeneMark). The purified DNA fragments were ligated to the vector pCR II-TOPO (Invitrogen, Carlsbad, CA) and transformed into DH5α competent cells (GeneMark) and sequenced by Tri-I Biotech. Inc. (Taipei, Taiwan) using an ABI 3730 DNA autosequencer.

SCAR-PCR Primer Designing and Screening. Using Vector NTI 9.0 (Invitrogen), SCAR-PCR primers were designed based on the above-mentioned species-specific and reproducible RAPD-PCR sequences. For testing the specificity of the SCAR-PCR primers, 0.3–3 μg of template DNA was added in a PCR tube containing a 20-μl mixture of 10 μl of 2X PCR Master Mix (including 2U SuperTherm DNA polymerase) and 10 pmoles of designed primers. The PCR amplification was started with 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 1 min, and ended with 72°C for 5 min. The specificity of the amplicons was examined by gel electrophoresis as described above.

Multiplex PCR and Test of Sensitivity. Four pairs of SCAR primers for each species were mixed and multiplex PCR amplification was carried out in a 20-μl reaction mixture containing 0.3–3 μg of template DNA, 1X QIAGEN Multiplex PCR Master Mix (containing HotStarTaq DNA polymerase) (QIAGEN, Valencia, CA) and 10 pmoles of primer mix. The conditions for multiplex PCR and analysis of the resulting products were the same as those described above.

To test the sensitivity of the multiplex PCR, DNA from eggs of *S. cerealella* was serially diluted $5–4 \times 10^4$ fold, while pupal DNA was diluted $40–8 \times 10^5$ fold. PCR amplification conditions and analyses were the same as described above. The lowest detectable dilution was considered the threshold for sensitivity of the multiplex PCR.

Results

To obtain species-specific primers for identification of *C. cephalonica, C. cautella, S. cerealella,* and *P. interpunctella,* we first screened *ca.* 300 various random primers by RAPD-PCR, with four of them, S31, S64, S128, and S232, selected for further development of SCAR primers.

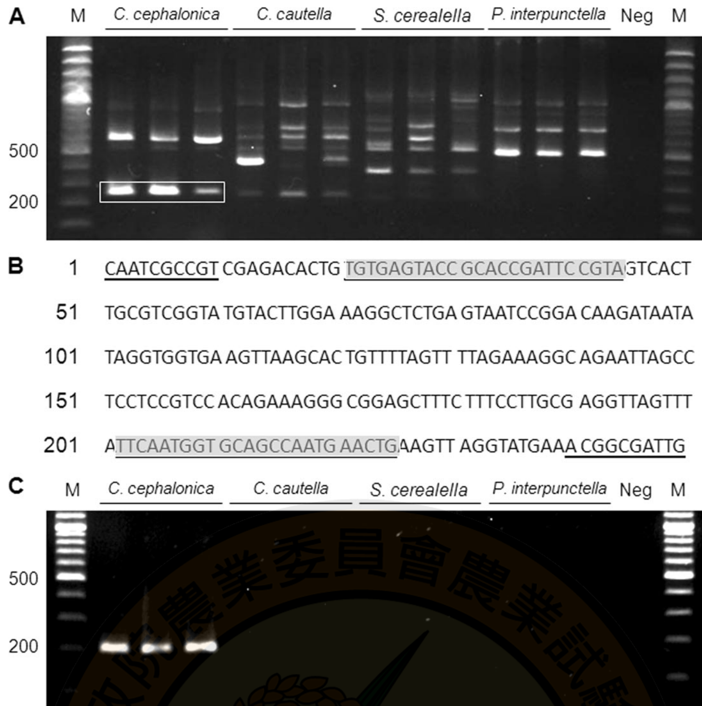

Fig. 1. Screening for a species-specific SCAR primer for *C. cephalonica.* (A) RAPD-PCR profiles of the four examined insect species amplified with the S31 RAPD primer. The specific amplicons are boxed. (B) Sequence of the specific DNA fragment, with the sequence of the S-31 primer underlined, and the sequences of the SCAR-PCR primer pair marked with double underlines and shaded regions. (C) SCAR-PCR profiles generated using the *C. cephalonica* specific primer pair. Neg, negative control; M, 100-bp DNA markers.

Using the S31 primer (5′-CAATCGCCGT-3′) as an example of how we screened the PCR primers, a unique DNA segment (≈300 bp) was specifically amplified in the three individuals of *C. cephalonica* but not in the other three species (Fig. 1A, boxed). Subsequent sequencing confirmed that the actual size of this amplicon was 249 bp (Fig. 1B). Based on this DNA sequence, several pairs of PCR primers were designed and screened, with one of these primer pairs, RM-S31N/RM-S31C, was shown to be able to specifically amplify a 205-bp amplicon only in *C. cephalonica* DNA (Fig. 1C). Therefore, we designated it as the specific to primer pair for *C. cephalonica.*

Following the same procedures, we further screened for species-specific primer pairs to *C. cautella, S. cerealella,* and *P. interpunctella* DNAs, respectively. As shown in Table 2-I, primers S232, S128, and S64 uniquely amplified 520-, 544-, and 521-bp amplicons to *C. cautella, S. cerealella,* and *P. interpunctella,* respectively. Based on these known sequences, SCAR primer pairs, named AM-S232 F/AM-S232R, AGM-S128 F/AGM-S128R, and IMM-S64 F/IMM-S64R, with amplicons of 250-, 324-, and 382-bp, respectively, were subsequently obtained (Table 3, SCAR-I). These amplicons have >50-bp difference in size and can be identified easily on the electrophoretic profiles (Fig. 2).

Table 2. Sequences of random primers used in the RAPD-PCR for identification of four lepidopterous stored product insects

Group	Primer name	Primer sequences	Major DNA fragment size	Species distinguished
I	S31	5′-CAATCGCCGT-3′	249	*C. cephalonica*
	S232	5′-ACCCCCCACT-3′	520	*C. cautella*
	S128	5′-GGGATATCGG-3′	544	*S. cerealella*
	S64	5′-CCGCATCTAC-3′	521	*P. interpunctella*
II	S18	5′-CCACAGCAGT-3′	433	*C. cephalonica*
	S237	5′-ACCGGCTTGT-3′	718	*C. cautella*
	S136	5′-GGAGTACTGG-3′	299	*S. cerealella*
	S19	5′-ACCCCCGAAG-3′	449	*P. interpunctella*
III	S23	5′-AGTCAGCCAC-3′	668	*C. cephalonica*
	S39	5′-CAAACGTCGG-3′	777	*C. cautella*
	S23	5′-AGTCAGCCAC-3′	855	*S. cerealella*
	S31	5′-CAATCGCCGT-3′	519	*P. interpunctella*

Table 3. Sequences of oligonucleotide primers used in SCAR-PCR for identification of four lepidopterous stored product insects

Group	Primer name[a]	Primer sequences	Size (bp)	Species distinguished
SCAR-I	RM-S31F	5′-TGTGAGTACCGCACCGATTCCGTA-3′	205	*C. cephalonica*
	RM-S31R	5′-CAGTTCATTGGCTGCACCATTGAA-3′		
	AM-S232F	5′-TTGACTGCCACTATGATTGAGCGG-3′	250	*C. cautella*
	AM-S232R	5′-TGCCCACCGCAAACATTACACAAG-3′		
	AGM-S128F	5′-TCACCAGTCGCGATGAAGC-3′	324	*S. cerealella*
	AGM-S128R	5′-ACCAGAAATCGTTCATCGTGATCG-3′		
	IMM-S64F	5′-CCAGGTTTGATCCACGCTCAGGTG-3′	382	*P. interpunctella*
	IMM-S64R	5′-GGCTTTGTTTGGTGAGTCGTTGAC-3′		
SCAR-II	RM-S18F	5′-GCAGCATTCTGCGTGTTAGTGCATGT-3′	341	*C. cephalonica*
	RM-S18R	5′-ATGTTTTCCTCCGTAACGGACGTTG-3′		
	AM-S237F	5′-AAAGCTTGCGGTTTCGAGATGCGT-3′	565	*C. cautella*
	AM-S237R	5′-GTGAAACGCGTGCTTTACTTCCGA-3′		
	AGM-S136F	5′-AGTGGGTGTATGTATGTACCTACCTCTGCC-3′	261	*S. cerealella*
	AGM-S136R	5′-ATGTGTGATTGGAGAAACGCTGTG-3′		
	IMM-S19F	5′-CATAGATGCCTAGTTGTGCCTATCAGGTTG-3′	170	*P. interpunctella*
	IMM-S19R	5′-CCGGAGTGCCACTCAGTGAAAATGGAATTA-3′		
SCAR-III	RM-S23F	5′-GCCGTGAAATACCCGAATAGACCG-3′	514	*C. cephalonica*
	RM-S23R	5′-AGGCGATAAGCAGGATTAATGCGT-3′		
	AM-S39F	5′-AGGCGCATTGAAATCTTGAACTTC-3′	555	*C. cautella*
	AM-S39R	5′-TTGCTATTGAGGGGAGATTCGTGG-3′		
	AGM-S23F	5′-CCAGTGTCTGGGCACTGGGATGACATCAT-3′	445	*S. cerealella*
	AGM-S23R	5′-CGGCATAGGTGGCCCAATGATTACA-3′		
	IMM-S31F	5′-GACTCGGGTTTCAGAGCATCACAA-3′	299	*P. interpunctella*
	IMM-S31R	5′-TCGCCGTTGTATCGCAGTAATTGA-3′		

[a] F, forward; R, reverse.

With the same approach, a total of three random primer sets, that is, groups 1, 2, and 3, respectively, each containing four primers that could specifically amplify DNA from this species (Table 2) were adopted to screen species-specific SCAR primer pairs. The final SCAR primer pairs along with their sequences and amplicon sizes are shown in Table 3.

To make identification easier and faster, a multiplex PCR was developed with all of the above species-specific primers mixed in groups and evaluated for their species-specificity and reproducibility. Three sets of multiplex PCRs have been developed in the current study with each set composed of four primer pairs that are able to accurately amplify a single amplicon from the corresponding DNA of each of the four insect species. The first set of primer pairs (SCAR-I) generated amplicons of a 205-, 250-, 324-, or 382-bp amplicon from DNAs corresponding to *C.*

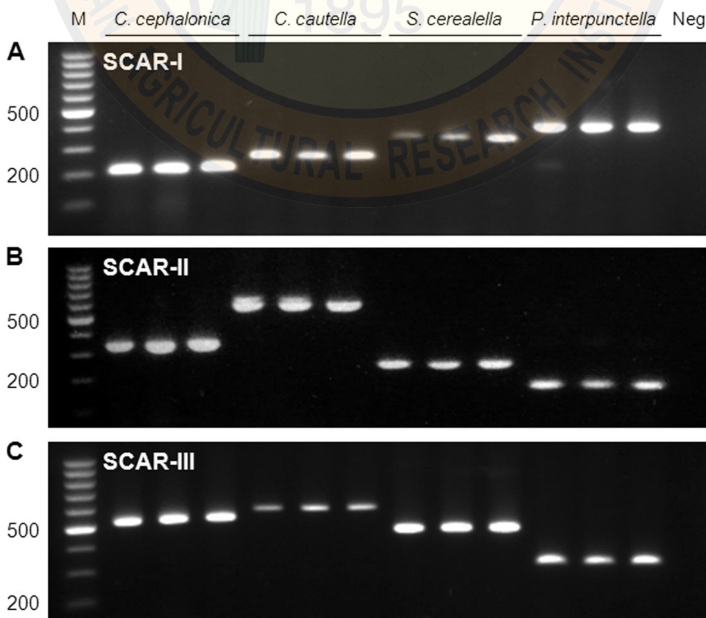

Fig. 2. Electrophoretic profiles of the multiplex SCAR-PCR products. Each SCAR profile was generated with the mixture of four species-specific SCAR-PCR primer pairs for identification of *C. cephalonica*, *C. cautella*, *S. cerealella*, and *P. interpunctella*, respectively.

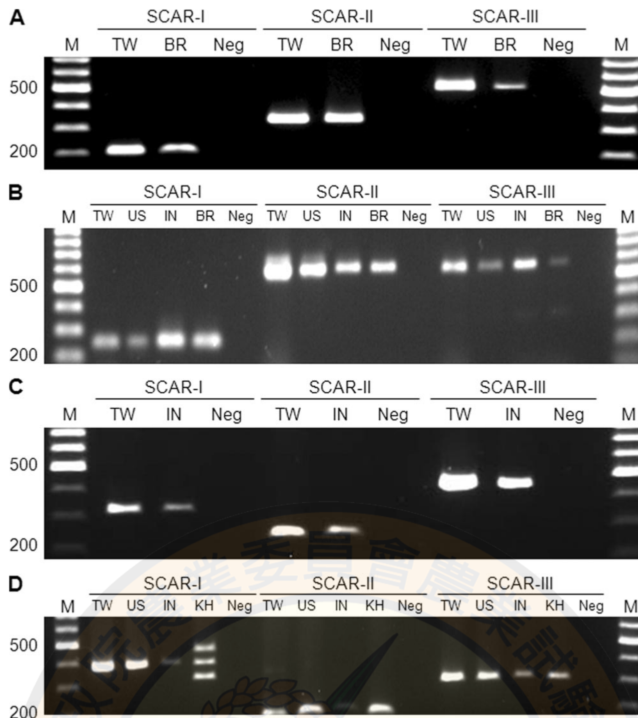

Fig. 3. Multiplex SCAR-PCR profiles of specimens collected from the stored products imported from several countries. (A) *C. cephalonica*; (B) *C. cautella*; (C) *S. cerealella*; (D) *P. interpunctella*. TW, Taiwan; BR, Brazil; US, United States of America; IN, India; KH, Cambodia. The molecular size standard (M) is 100-bp DNA markers.

cephalonica, *C. cautella*, *S. cerealella*, and *P. interpunctella*, respectively (Fig. 2A). The second set of primer pairs (SCAR-II) generated amplicons of 341-, 565-, 261-, or 170-bp (Fig. 2B), while the third set of primer pairs (SCAR-III) generated amplicons of 514-, 555-, 445-, or 299-bp (Fig. 2C). These results showed that the designed SCAR primer pairs were reliable and could be applied to identify unambiguously the four insect species.

For validation, SCAR-I, -II, and -III were used to examine the lepidopterous pests intercepted from stored-product samples (Table 1). The same amplification profiles were obtained in native and foreign (or imported) samples of *C. cephalonica*, *C. cautella*, and *S. cerealella* (Fig. 3A–C). The lone exception was *P. interpunctella*, which had multiple amplicons in the sample from Cambodia (Fig. 3D).

S. cerealella is the smallest of the four insects. To test the sensitivity of the multiplex PCR, serial dilutions of DNA from eggs and pupae of this species were amplified with primer set SCAR-I. The results showed that *S. cerealella* species-specific amplicon marker was still detectable at dilutions of 1:500 and 1:4 $\times$ 10^5 from DNA stocks of a single egg and a single pupa, respectively (Fig. 4). These results indicate that this method is very sensitive for detecting the presence of minute quantities of DNA, even from a single egg.

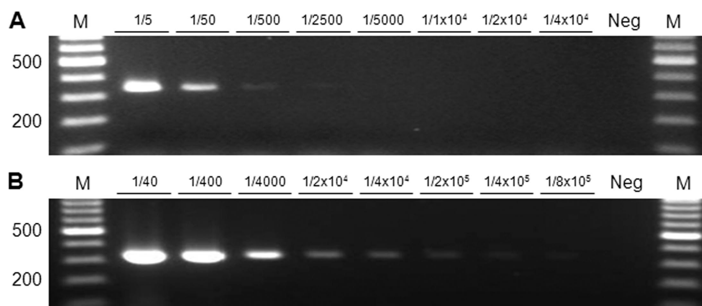

Fig. 4. Relative sensitivity of the multiplex SCAR-PCR. The DNAs of *S. cerealella* egg (A) and pupa (B) serially diluted and PCR performed using the SCAR-I primer set. The molecular size standard (M) is 100-bp DNA markers.

Discussion

To protect local agriculture, all countries implement strict quarantine measures on imported agricultural commodities to prevent the invasion of exotic pests. In this respect, it is essential to develop rapid and accurate methods to diagnose and identify the species of invasive insect pests. In this study, we sought to develop a quick and easy molecular diagnostic method for four common species of lepidopterous pests frequently found in stored-product imports.

Using short primers usually 10 oligonucleotides in length, RAPD-PCR can only be carried out at low temperature (around 36°C), a condition that frequently produces complicated and variable amplicons because of the presence of diverse annealing targets (Dowdy and McGaughey 1996, Laroche et al. 1996). In contrast, SCAR primers, usually much longer (18–25 bp) than RAPD primers, allow for PCR analysis under more stringent conditions; therefore, their amplicons are more specific, reproducible and easily recognized (Zou et al. 2000). Our results are in full agreement with this characteristic.

Considerable work on molecular identification of stored product insect species has been performed. Dowdy and McGaughey (1996) used the RAPD technique to analyze six populations of *P. interpunctella* in the western and central United States. Nowaczyk et al. (2009) developed a molecular technique for the identification of *Tribolium castaneum* (Herbst) and *T. confusum* Jacquelin du Val; Hidayat et al. (1996) used the RAPD primer *UBC-431* to amplify a DNA segment from *Sitophilus oryzae* L. and *S. zeamais* Motschulsky individually, while Peng et al. (2003) further developed a species-specific primer for the amplification of nuclear ribosomal DNA (nrDNA) to distinguish *S. oryzae* from *S. zeamais.* Until now, however, no molecular identification of *S. cerealella, C. cephalonica,* and *C. cautella* has been reported. Our results showed that three sets of SCAR primer pairs could accurately identify almost all of the tested quarantine samples. However, the exotic *P. interpunctella* from Cambodia showed more than one band when identified with SCAR-I (Fig. 3D), indicating the occurrence of intraspecies differences between the populations of different countries. Although the sequences similar to the extra bands were not found in NCBI Database, we confirmed that these specimen from Cambodia are correct species by comparison of the COI sequences with known *P. interpunctella* (Hebert et al. 2003; GenBank GU096546).

In the current study, we have for the first time used the SCAR methods to develop three multiplex PCR primer sets, each composed of four SCAR primer pairs that specifically identify *C. cephalonica, C. cautella, S. cerealella,* and *P. interpunctell,* when present simultaneously, with no interference of amplicons (Fig. 2). Moreover, the sensitivity for the detection was high, for instance the lowest detectable levels were 1:500 and $1{:}4 \times 10^5$ dilutions of DNA stocks from a single *S. cerealella* egg and pupa, respectively, indicating that only a very small amount of DNA from any stage of an insect is sufficient for accurate and reliable species identification.

Although molecular biology provides many tools for identification of insect pests, for plant quarantine inspection, determining the practical DNA markers for repaid and accurate diagnosis and identification remains the greatest concern. RFLPs required identification of appropriate restriction sites and specific genes, which may not often available. DNA barcoding has the advantage that the targeted sequences are from known regions of the genome, which aids evolutionary studies (Gleeson et al. 2000, Ball et al. 2005, Scheffer et al. 2006, Greenstone et al. 2011), but it costs more time and money for the sequencing in every individual. Furthermore, universal primers of multiplex PCR from specific gene might be interfered greater than the SCAR-PCR primers designed from difference sources of the genome. The random regions of genes amplified in SCAR-PCR have its own disadvantage and advantage. The main disadvantage is that the evolutionary meaning of the random genes is uncertain, while the advantages include spending less time and money for identification, and searching for the species-specific primers are easier. The technique developed here for lepidopterous insects should be useful for the identification of other insect pests in stored products. In conclusion, SCAR-PCR, with further development and modification, is expected to facilitate and ensure successful plant quarantine in an ever-increasing global trade of agricultural commodities.

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