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Rapid discrimination of the common species of the stored product pest *Liposcelis* (Psocoptera: Liposcelididae) from China and the Czech Republic, based on PCR-RFLP analysis

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Abstract. Psocids of the genus *Liposcelis* (Psocoptera: Liposcelididae) are stored product pests that are difficult to identify morphologically. A molecular method based on Restriction Fragment Length Polymorphism (RFLP) of the PCR-amplified 16S rDNA gene was developed for the rapid discrimination of four common species (*L. bostrychophila*, *L. entomophila*, *L. decolor*, and *L. paeta*). Different developmental stages and populations (P.R. China and Czech Republic) were tested. One DNA fragment of about 500 bp in length was amplified from genomic DNA and the fragment was then digested using the restriction endonuclease *Dra*I. Identification of the relevant banding pattern allowed all the developmental stages and both sexes to be discriminated in the species tested. The banding patterns of *L. entomophila* from all populations were identical, while the relevant restriction digests and sequence analysis confirmed that the Chinese and Czech populations of *L. bostrychophila*, *L. decolor*, and *L. paeta* differed. In conclusion, PCR-RFLP with one pair of primers (16Sar and 16Sbr) and one restrictive endonuclease, *Dra*I, proved a reliable method for rapidly discriminating the *Liposcelis* species tested.

INTRODUCTION

In the last decade, psocids (booklice) have become economically important pests of stored commodities (Ding et al., 2002; Beckett & Morton, 2003; Kučerová et al., 2003; Nayak et al., 2003; Stejskal et al., 2003; Nayak, 2006). Species of *Liposcelis* have frequently been captured during quarantine inspections at ports in China. The genus *Liposcelis* Motschulsky (Psocoptera: Liposcelididae) contains 123 species (Li, 2002; Lienhard & Smithers, 2002; Wang et al., 2006), of which several are of economic importance. *Liposcelis bostrychophila* Badonnel, *L. entomophila* (Enderlein), *L. decolor* (Pearman) and *L. paeta* Pearman are the most commonly occurring species of *Liposcelis* in stored products such as grain, animal specimens and archives (Turner, 1994; Baz & Monserrat, 1999; Li et al., 1999; Li, 2001; Rees, 2004).

Correct identification of psocid species is a prerequisite for the establishment of an effective pest management program. Traditional morphological identification of *Liposcelis* adults (Lienhard, 1990; Bai & Cao, 1997; Li et al., 1999; Lienhard & Smithers, 2002) or of the immature stages (Kučerová, 2002) is difficult and requires specialists. Methods for rapidly identifying booklice are unavailable. Molecular techniques based on DNA analysis offer a viable and economical alternative method of identification (Muraji & Nakahara, 2001). Different DNA and Polymerase Chain Reaction (PCR)-based molecular markers have been developed for insect diagnostic purposes, including Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphisms (RFLP), satellite DNA, and Inter-Simple Sequence Repeats (ISSR) (Li, 2007). The nucleotide sequences of *Liposcelis* have been analyzed in order to infer phylogenetic relationships, geographic origin, population genetics or gene flow (Cruickshank et al., 2001; Barker et al., 2003; Johnson & Mockford, 2003; Yoshizawa & Johnson, 2003; Mikac, 2006, 2007; Mikac & Clarke, 2006). As a result, more than 60 sequences have been submitted to international DNA sequence databases, such as the DNA Data Bank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL), and GenBank. Using these *Liposcelis* DNA sequences, we have analyzed a rapid discrimination method for *Liposcelis* from China based primarily on PCR-RFLP (Qin et al., 2007).

In this study, the above discrimination investigations are extended by extracting genetic DNA from single individuals and using PCR-RFLP to discriminate between four species of *Liposcelis* originating from the People's Republic of China (P.R. China) and Czech Republic (CZ), *L. bostrychophila*, *L. entomophila*, *L. decolor*, and *L. paeta*.

MATERIAL AND METHODS

Insect collection and culture

Liposcelis bostrychophila, *L. entomophila*, *L. decolor*, and *L. paeta* were collected in storehouses in the P.R. China and CZ

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(12 geographical populations – Table 1). Adult identification was confirmed by C. Lienhard (Museum of Natural History, Geneva, Switzerland). The rearing diet consisted of whole-wheat flour: skimmed milk powder: yeast powder in a ratio of 10 : 1 : 1 (Ding et al., 2001). The insects were kept at 27–28.5°C and 75–80% RH. Males, females, and nymphs of each species and geographical population (20 individuals of each species and developmental stage) were subjected to PCR-RFLP. In the parthenogenetic *L. bostrychophila*, females and nymphs were tested. The PCR products from a single individual of each of the species *L. bostrychophila*, *L. decolor*, and *L. paeta* was additionally analyzed by sequencing. All psocids were killed in 100% ethanol and their DNA extracted immediately.

DNA extraction

Genomic DNA was extracted from each geographical population of each species using the method of Gong et al. (2002), which is a slight modification based on the method of cetyltrimethylammonium bromide (CTAB).

PCR amplification

16S rDNA was amplified using the primers 16Sar and 16Sbr (Simon et al., 1994): the 16Sar primer has the sequence 5'-GCCTGTTTAACAAAAACAT-3', and the 16Sbr primer has the sequence 5'-CCGGTCTGAACTCAGATCACGT-3'. PCR was then carried out in a 20 µl reaction volume containing 2.0 µl of 10 × reaction buffer, 2.0 µl (25 mM) $MgCl_2$, 0.5 µl (2.5 mM) dNTPs, 1 µl (5 µM) of each primer (two primers), 1 µl (1U/µl) *Taq* DNA polymerase (TianGen, Beijing, China), 1 µl (50–100 ng) template DNA, and 11.5 µl double distilled water (ddH_2O). The thermal cycling conditions were: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 45 s, extension at 72°C for 45 s and a final extension step at 72°C for 10 min. After amplification, 5 µL of the PCR products were subjected to electrophoresis alongside a DNA marker.

Restriction endonuclease digestion and sequencing

Restriction endonuclease digestion was performed in a 20 µl reaction volume containing 10 µl of the target DNA (PCR product), 2 µl restriction buffer, 1 µl restriction endonuclease (*Dra*I) and 7 µl ddH_2O. The digest was incubated at 37°C for 6 h. A 3.5%–4.0% (w/v) agarose gel was made from 1 × TBE buffer, and 8 µl of the digested PCR product was subjected to electrophoresis alongside a DNA marker.

This sequencing method was used on the following species. Genomic DNA of *Liposcelis bostrychophila*, *L. decolor*, and *L. paeta* was extracted from a single individual of each species and the 16S rDNA gene amplified using the 16Sar and 16Sbr primers. The amplified products were then separated on a 1.0% (w/v) agarose gel (1 × TAE), and the bands (stained with ethidium bromide) were excised. The agarose gel slice containing the DNA band of interest was placed in a centrifuge tube. The DNA was then purified and cloned, then the bacterial liquid culture was used as a template for nucleotide sequencing (AuGCT Biotechnology Synthesis Lab, Beijing, China). DNAMAN software was used for restriction analysis of the six sequences.

RESULTS

PCR analysis

All of the PCR products were nearly the same size (~500 bp). The PCR products were not influenced by species, geographical population, stage of development or sex.

TABLE 1. *Liposcelis* species and populations used in this study (populations were coded by combining species names with acronyms of collection countries and sites).

Population	Location collected
*L. entomophila*_HB-P.R.China	Hubei, P.R. China
*L. entomophila*_CQ-P.R.China	Chongqing, P.R. China
*L. entomophila*_P-CZ	Central Bohemia, Czech Rep.
*L. bostrychophila*_GX-P.R.China	Guangxi, P.R. China
*L. bostrychophila*_CQ-P.R.China	Chongqing, P.R. China
*L. bostrychophila*_P-CZ	Central Bohemia, Czech Rep.
*L. decolor*_CQ-P.R.China	Chongqing, P.R. China
*L. decolor*_HN-P.R.China	Henan, P.R. China
*L. decolor*_P-CZ	Central Bohemia, Czech Rep.
*L. paeta*_ZJ-P.R.China	Zhejiang, P.R. China
*L. paeta*_JX-P.R.China	Jiangxi, P.R. China
*L. paeta*_P-CZ	Central Bohemia, Czech Rep.

RFLP and sequencing analysis

The PCR products could be digested at specific regions. Though different *Liposcelis* species could be visually distinguished according to their electrophoresis patterns, digestion result of *Liposcelis* of the same species was not influenced by individuality, sex or developmental stage.

Samples from the P.R. China

16S rDNA of four species of *Liposcelis* were successfully cut into distinct visible bands. Four bands (~300 bp, 120 bp, 70 bp, and 50 bp) were visible in the 16S rDNA of *L. entomophila*; two clear bands of different sizes (~350 bp and 100 bp; ~350 bp, and 200 bp; ~300 bp and 220 bp) were seen in the 16S rDNA of *L. bostrychophila*, *L. decolor*, and *L. paeta,* respectively. These results demonstrate that restriction endonuclease digestion was not affected by the different geographical populations, stages of development or sex of the four common species of the stored product pest *Liposcelis* from the P.R. China (Fig. 1).

Samples from the CZ and comparison with those from the P.R. China

The repeated experiments on the CZ samples produced identical results, and were not influenced by stage of development or sex. 16S rDNA of *L. entomophila* was excised into four visible bands (~300 bp, 120 bp, 70 bp and 50 bp), which were identical with those result obtained for the P.R. China samples. For the other three *Liposcelis* species, i.e. *L. bostrychophila, L. decolor*, and *L. paeta,* the sizes of these fragments differed from those the samples from the P.R. China (Fig. 2). Therefore, the different visible bands observed after restriction digestion of 16S rDNA were further analyzed and confirmed by sequencing. The results showed that the sequences from the same species exhibited a high level similarity, with limited genetic mutations. Fig. 3 shows the sequence lengths and digestion sites. For *L. bostrychophila*, the 16S rDNA of the sample from the P.R. China had 3 digestion sites and 4 fragments of 326 bp, 100 bp, 34 bp, and 33 bp,

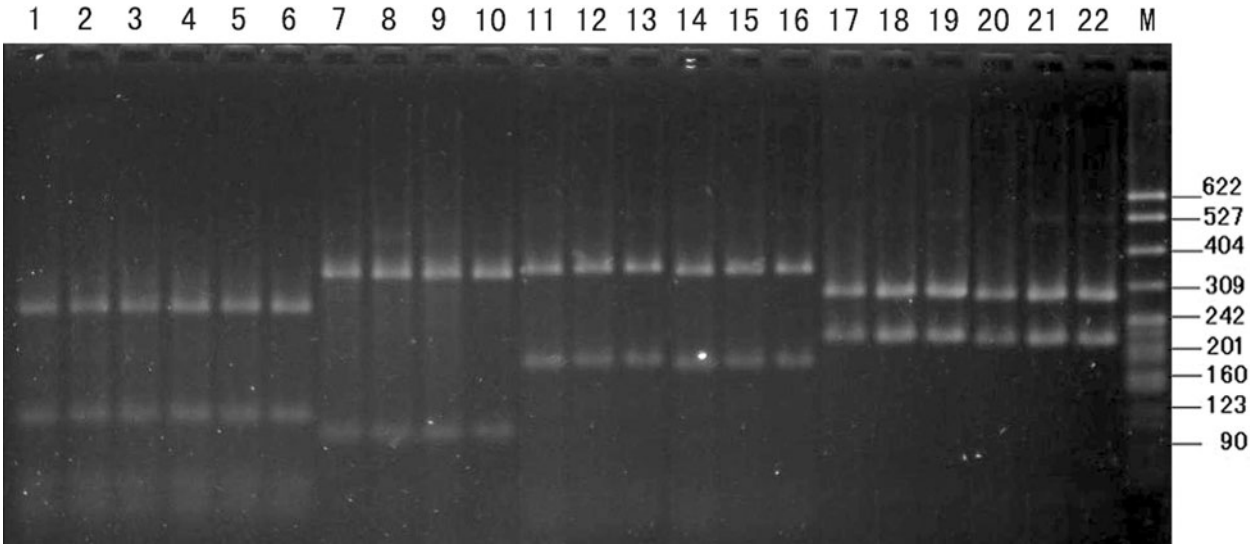

Fig. 1. Electrophoresis of *Dra*I-digested PCR products from the 16S rDNA of four common species of stored *Liposcelis* from the P.R. China. Lanes: 1–3 – female, male, and nymphs of *L. entomophila*_HB-P.R.China; 4–6 – female, male, and nymphs of *L. entomophila*_CQ-P.R.China; 7–8 – female and nymphs of *L. bostrychophila*_GX-P.R.China; 9–10 – female and nymphs of *L. bostrychophila*_CQ-P.R.China; 11–13 – female, male, and nymphs of *L. decolor*_CQ-P.R.China; 14–16 – female, male, and nymphs of *L. decolor*_HN-P.R.China; 17–19 – female, male, and nymphs of *L. paeta*_ZJ-P.R.China; 20–22 – female, male, and nymphs of *L. paeta*_JX-P.R.China; M – molecular size marker (bp).

and the 16S rDNA of the sample from CZ had 2 digestion sites and 3 fragments of 225 bp, 192 bp, and 72 bp. The 16S rDNA of *L. decolor* from the P.R. China and CZ displayed 1 digestion site and 2 fragments (338 bp and 167 bp; 337 bp and 168 bp, respectively). For *L. paeta*, the 16S rDNA of the sample from the P.R. China had only 1 digestion site, and 2 fragments of 279 bp, and 201 bp, but that of the sample from the CZ had 3 digestion sites and 4 fragments of 200 bp, 184 bp, 62 bp and 33 bp. The sequencing results showed that the mutation points in 16S rDNA were consistent with the banding patterns of the electrophoresis map, except for some small fragments that could not be detected by agarose gel electrophoresis.

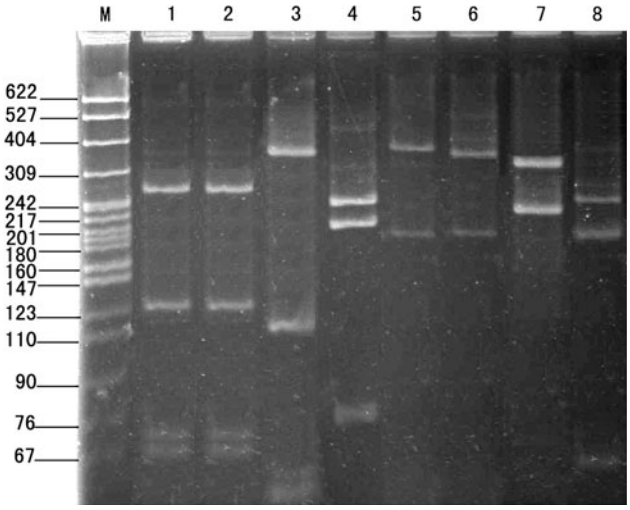

Fig. 2. Electrophoresis of *Dra*I-digested PCR products of 16S rDNA from four common species of *Liposcelis* from the P.R. China and CZ. Lanes: M – molecular size marker (bp); 1 – female of *L. entomophila*_HB-P.R.China; 2 – female of *L. entomophila*_P-CZ; 3 – female of *L. bostrychophila*_GX-P.R. China; 4 – female of *L. bostrychophila*_P-CZ; 5 – female of *L. decolor*_CQ-P.R.China; 6 – female of *L. decolor*_P-CZ; 7 – female of *L. paeta*_ZJ-P.R. China; 8 – female of *L. paeta*_P-CZ.

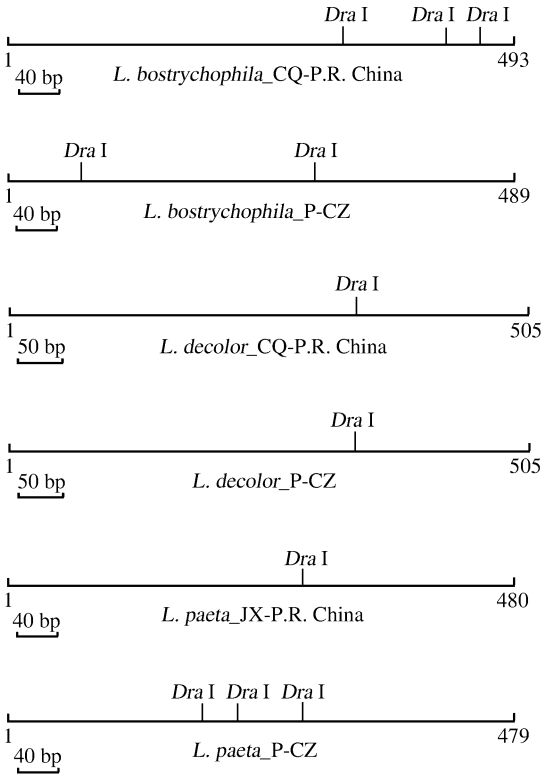

Fig. 3. Restrictive endonuclease sites of six samples produced by sequencing.

DISCUSSION

In this report, we show that PCR-RFLP based on 16S rDNA is an efficient way of rapidly discriminating four storage pest psocids from the P.R. China and CZ. One pair of primers 16Sar/Sbr and one restrictive endonuclease, *Dra*I, are sufficient to discriminate between the *Liposcelis* species tested. The whole process from extraction of genomic DNA to the formation of a restriction map takes only 12h. This rapid molecular method can be used by plant quarantine offices to identify the most commonly occurring species of *Liposcelis* infesting stored products.

The results of this study indicate that the PCR-RFLP method is able to identify *L. entomophila* using the same digestion pattern of 16S rDNA, regardless of differences between geographical populations, stages of development or sex of the individuals in the samples. In comparison, *L. bostrychophila*, *L. decolor*, and *L. paeta* were well discriminated, but the numbers or sizes of the 16S rDNA band differed, even for the same species. For each species of these three psocids, the 16S rDNA of the P.R. China and CZ populations showed different stable electrophoresis patterns. Further, the sequence analysis indicated that the points of mutation in the 16S rDNA are consistent with the electrophoresis patterns. This enabled us to conclude that there are intraspecific genetic differences between the P.R. China and CZ populations of *L. bostrychophila*, *L. decolor*, and *L. paeta*. Further worldwide geographical studies are required in order to draw general conclusions regarding intra-specific genetic differences in *Liposcelis*.

Recently, molecular methods have been used to detect natural populations of Diptera, Coleoptera, Hymenoptera, and Lepidoptera (Ratcliffe et al., 2002; Loxdale & MacDonald, 2004; Gómez-Zurita et al., 2005; Li, 2007). The common methods used are RAPD analysis, RFLP patterns, Amplified Fragment Length Polymorphism (AFLP) molecular markers, species-specific primers, and ISSR. Of these methods, PCR-RFLP analysis of molecular markers is regarded as an easy, fast and reliable method (Muraji & Nakahara, 2002) for identifying closely related species that have an ambiguous taxonomic status, similar insect species that are difficult to identify using morphological characteristics and the immature stages of insects (Sperling et al., 1994; Tuda et al., 1995; Litjens et al., 2001; Zapata et al., 2007). Since different genetic mutations occur in DNA sequences in species during evolution, it is possible to find different points of discrimination using restriction endonucleases. Based on this study, it may be concluded that the PCR-RFLP method with one pair of primers, 16Sar/Sbr, and one restrictive endonuclease, *Dra*I, is a convenient and reliable way to rapidly discriminate between the four common species of stored product psocids. This method can detect the species of *Liposcelis* studied not only the adults, but also the immature stages. Moreover it confirmed that this method is able to reveal some intra-specific differences associated with the geographical distribution of these species. Further molecular research on other psocid species and their geographical strains on a worldwide scale is needed in the future.

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