ONLINE RESOURCES

Molecular identification of *Tribolium castaneum* and *T. confusum* (Coleoptera: Tenebrionidae) using PCR-RFLP analysis

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Introduction

The red flour beetle, *Tribolium castaneum* (Herbst), and the confused flour beetle, *T. confusum* (Jacquelin du Val) (Coleoptera: Tenebrionidae), are sympatric and morphologically similar stored-product pests, and the latter is named because of confusion over its identity as it is so similar to the former (Walter 1990). They are two cosmopolitan pests in flour mills and wherever cereal products and other dried foods are processed or stored, and rank among the most important pests inhabiting grain processing plants and storage facilities (Campbell *et al*. 2004). Fumigants and other chemical insecticides are widely used to protect stored commodities from infestations and contamination. Previous studies showed that *T. castaneum* was more susceptible of two species to pyrethrin aerosol, hydroprene and pyriproxifen (Arthur 2001, 2008; Arthur and Hoernemann 2004; Arthur *et al.* 2009; Sutton *et al.* 2011). Also, studies showed that immature stages (larvae and pupae) of *T. castaneum* and *T. confusum* were more susceptible compared to mature stage (adults) (Arthur 2008; Arthur and Fontenot 2012). Therefore, the insecticidal efficacy was influenced by species and life stages (larvae, pupae and adults), and the differential susceptibility between species and life stages should be taken into account when insecticides are being used to control both species in storage facilities (Arthur 2001). To reduce the use of insecticide, to increase resistance development and improve the level of pest management, it is important for a pest manager to identify them. However, *T. castaneum* and *T. confusum* are indistinguishable in the egg, larval and pupal stages, and difficult to distinguish in adult stage without professional training. Therefore, it is necessary to develop a useful alternative method to identify the two sibling flour beetle species.

DNA-based molecular markers have been used for species identification, including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphisms (RFLP), and simple sequence repeats (SSR), and their use represents a valuable addition or alternative to traditional phenotypic identification methods. Also, DNA amplification by polymerase chain reaction (PCR), followed by estimation of genetic divergences from the sequence data, has been used to discriminate between species. One such promising method is RFLP analysis of PCR products (PCR-RFLP), and it is a modification of the original RFLP method combined with PCR technique. The PCR-RFLP method has been used for species identification in many taxa, including insects (Szalanski *et al*. 2003; Thyssen *et al*. 2005; Rugman-Jones *et al*. 2006; Qin *et al*. 2008; Wang *et al*. 2009).

28S Ribosomal DNA (28S rDNA) has proved to be the suitable and informative region of nuclear DNA for species delimitation and phylogenetic relationships among species (Ohnishi *et al*. 2003; Hasegawa and Kasuya 2006; Angelini and Jockusch 2008; Holznagel *et al*. 2010; Cepeda *et al*. 2012). In this study, by using PCR we amplified a fragment of 28S rDNA in *T. castaneum* and *T. confusum* and analysed their genetic variation, followed by a digestion with species-specific restriction endonucleases in order to identify them.

Materials and methods

Beetle collection

In July 2011, adult *T. castaneum* and *T. confusum* were collected from wheat mills at five locations in China (a total of 10 populations): Huhhot in northern China; Xi'an in central China; Chengdu in southern China; and Liaocheng and Xuzhou in eastern China. To minimize the possibility of collecting identical populations, the minimum distance

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between two sites was 500 km; the maximum distance was 1800 km. For each population, we randomly sampled 10 individuals and they were identified based on their morphological characteristics using the taxonomic key of Bousquet (1990).

DNA extraction

One hundred adults of the two species were individually ground into fine powder with a mortar and pestle, and total DNA was extracted from a single beetle according to the DNA isolation procedure of Ming and Wang (2006). After air-drying DNA, it was resuspended in 20 μL sterile double-distilled water.

PCR amplification and sequencing

The partial 28S rDNA was amplified using the universal primers D2-3665F (5′-AGAGAGAGTTCAAGAGTACGTG-3′) and D5-4749R (5′-GTTACACACTCCTTAGCGGA-3′) (Angelini and Jockusch 2008). The primers were synthesized by Generay Biotech (Shanghai, China). PCR amplification was carried out in a 30 μL reaction volume containing 2× PCR reaction mix (Tiangen, Beijing, China), 0.4 μM of each primer, 0.6 U *Taq* DNA polymerase (Tiangen), 1.3 μL of DNA extraction, and 11 μL of ultrapure water in a Genius thermal cycler (Bioer, Hangzhou, China). The thermal cycling profile was as follows: 94°C for 5 min followed by 33 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 45 s and extension at 72°C for 1 min, with a final extension for 10 min at 72°C. The PCR products were electrophoresed on a 0.8% agarose gel, stained with ethidium bromide (0.5 μg/mL), and visualized under a UV transilluminator in a LG2020 gel documentation system (LongGene, Hangzhou, China). Fifty PCR product samples of each species were sent to the Invitrogen Trading DNA Sequencing Facility (Shanghai, China) for sequencing.

Genetic differentiation analysis

28S rDNA sequences of *T. castaneum* and *T. confusum* were aligned using ClustalX ver. 2.0 (Larkin *et al*. 2007). Based on the aligned sequences, interspecific and intraspecific genetic variations and genetic distances for *T. castaneum* and *T. confusum* were calculated using MEGA 5.05 (Tamura *et al*. 2011).

Restriction site analysis and restriction digestion of PCR products

To establish species-specific restriction sites profile for 28S rDNA sequences of *T. castaneum* and *T. confusum*, restriction site analysis was made using BioXM program (ver. 2.6; http://www.bio-soft.net/format/bioxm.htm). According to the restriction analysis data, *Pvu*I endonuclease with unique sites was chosen for restriction digestion. Digestion of PCR products for the partial 28S rDNA from both species were carried out using *Pvu*I (Fermentas, Burlington, USA) separately in a 30 μL reaction mixture containing 10× buffer R, 5 μL of unpurified PCR products, and 15 units of *Pvu*I endonuclease (10 U/μL) at 37°C for 12 h. A 2 μL sample of the reactant was run on a 0.8% agarose gel and visualized as described above.

Results

Analysis of genetic differentiation

Two haplotypes in the 28S rDNA region were detected among the 100 individuals of *T. castaneum* and *T. confusum*, and there was one haplotype per species. Two sequences have been deposited in GenBank DNA databases (accession no. JX412253 and JX412254). Based on the alignment of 28S rDNA sequences of *T. castaneum* and *T. confusum*, there were 1072 nucleotide sites including gaps, with 991

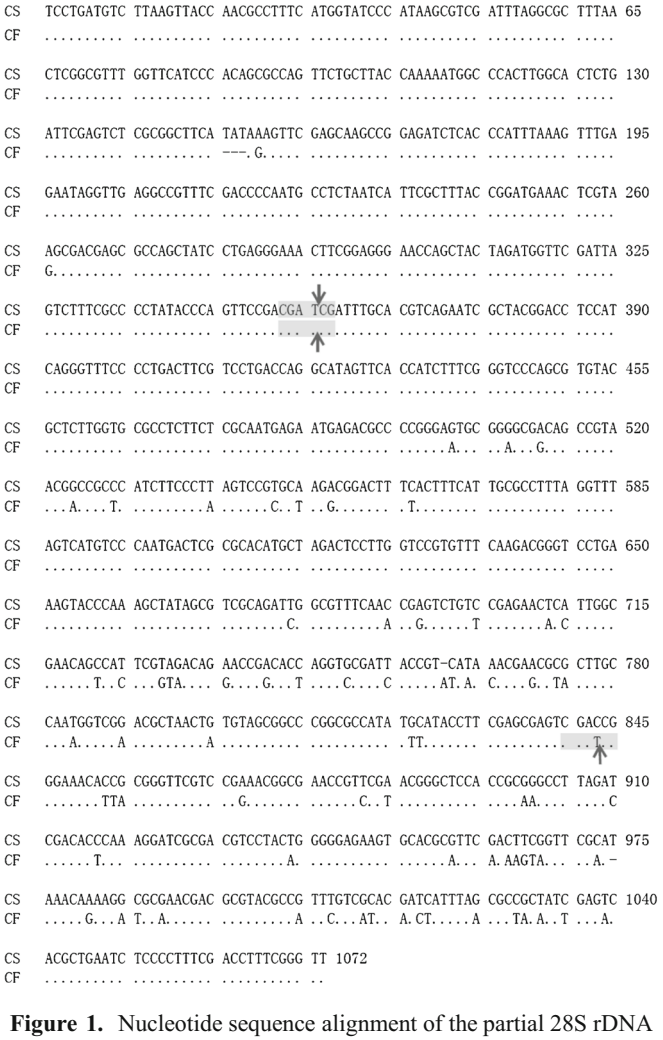

Figure 1. Nucleotide sequence alignment of the partial 28S rDNA of *T. castaneum* and *T. confusum*. The dots indicate same base as in the upper line and hyphens indicate alignment gaps. The shading and arrows indicate the restriction recognition sequences and cutting sites of *Pvu*I. The numbers on the right denote base sites that are relative to the consensus sequence aligned here.

Table 1. Statistics for nucleotide substitutions in 28S rDNA sequences of *T. castaneum* and *T. confusum*.

Transitions (ti)		Transversions (tv)				
A–G	C–T	A–C	A–T	C–G	G–T	Ti/tv ratio
30	26	5	11	1	3	2.80

conserved sites and 76 variable sites (figure 1). The nucleotide substitutions between 28S rDNA sequences of *Tribolium* beetle species consisted of 56 transitions and 20 transversions, and the transition/transversion (ti/tv) ratio was very high (table 1). The interspecific genetic distance of *T. castaneum* and *T. confusum* was 0.076. By contrast, there were no intraspecific variations in both species, and both intraspecific genetic distances were 0.

Restriction digestion and agarose gel electrophoresis analysis

The amplified 28S rDNA sequences were initially analysed with nine restriction endonucleases to show potential site variation and produce different-sized restriction fragment patterns (table 2). Among the restriction profiles of the PCR products, 28S rDNA digestion with *Pvu*I produced the most different restriction fragment patterns for *T. castaneum* (two fragments) and *T. confusum* (three fragments) (table 2). When amplified 28S rDNA PCR products of both species were digested independently with the restriction enzyme *Pvu*I, the fragment band patterns matched the expected results (figure 2).

Discussion

Judged by the three important criteria for evaluating a target DNA region, i.e. high success rate of PCR amplification, suitable intraspecific and interspecific variations, and differentially fragmented band patterns after restriction digestion of PCR products, our study suggests the adoption of the partial 28S rDNA as PCR-RFLP markers of identifying *T. castaneum* and *T. confusum*.

In this study, 28S rDNA can be amplified easily by PCR with a universal primer even from small quantities of DNA, and this high success rate of amplification may be due to the high copy number of rDNA in genome. Also, as can be seen in the gel image, not only the amplification bands of 28S rDNA were bright but there were no nonspecific amplification bands along with the desired band (data not shown). The internal transcribed spacer (ITS) region of rDNA is known to be a variable, species-specific region, and Nowaczyk *et al*. (2009) used PCR amplification of this region with species-specific primers to detect *T. confusum*. For the species-specific primer technique, not only does it require DNA sequence information for candidate species but a failure to amplify may be due to technical reasons or sample quality rather than the absence of the species-specific sequence resulting in false negatives. This might be avoided by the 28S rDNA PCR-RFLP method because universal primer was used. If no PCR amplification with universal primers took place, the sample would be characterized as unidentifiable.

After sequencing this 1072 bp 28S rDNA region there appeared to be 76 species-specific differences present. Compared to the interspecific variation, none of the samples used in this study demonstrated intraspecific variation in both *Tribolium* species. Therefore, this 28S rDNA has adequate variations among species and is conserved within species, exhibiting a high species resolving power. Because of no intraspecific variation, there is a minimum likelihood that restriction enzymes will cleave DNA at the same place in two *Tribolium* species. This is important as variation at these sites might lead to false species identification. Particularly when using PCR-RFLP analysis for attempts at species identification, intraspecific variation has to be taken into account, and the 28S rDNA region may have a more reliable diagnostic PCR-RFLP marker relative to the ITS region.

Nine restriction enzymes showed interspecific restriction-site variation of the partial 28S rDNA region in *T. castaneum* and *T. confusum* (table 2). Of these nine, *Bfr*BI, *Bsp*EI, *Eag*I, *Nde*I, *Nsi*I, *Ppu*10I and *Sal*I were expected to have cutting sites in one species but no cutting sites in the other after digestion of PCR products, and they were not selected to distinguish the two species because a failure to digest

Table 2. Estimated fragment size (base pairs) for restriction digests of partial 28S rDNA region in *T. castaneum* and *T. confusum*.

	Restriction enzymes								
Beetle species	*Bfr*BI	*Bsp*EI	*Bst*BI	*Eag*I	*Nde*I	*Nsi*I	*Ppu*10I	*Pvu*I	*Sal*I
T. castaneum	821, 250	1071*	881, 190	549, 521	817, 254	823, 248	819, 252	715, 356	837, 234
T. confusum	1069*	861, 208	967, 102	1069*	1069*	1069*	1069*	487, 353, 229	1069*

*Not digested.

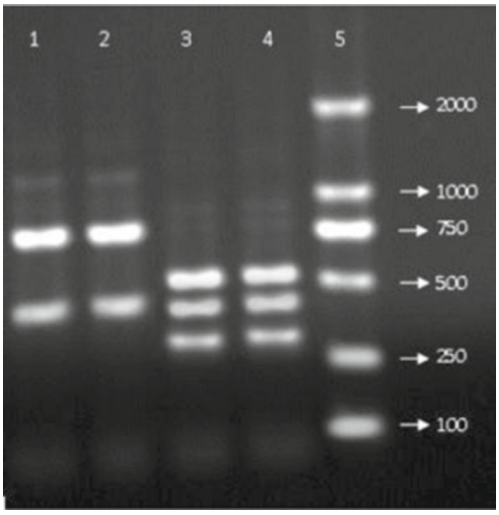

Figure 2. *Pvu*I digest of the partial 28S rDNA. Lanes 1 and 2, *T. castaneum*; lanes 3 and 4, *T. confusum*; lane 5, DNA molecular mass standard (numbers represent the number of base pairs in the molecular standard).

PCR products (i.e., false negative) may be due to technical reasons rather than the absence of cutting sites. *Bst*BI and *Pvu*I were expected to have cutting sites in both species, and they could be selected to distinguish the two species without false negative. However, *Bst*BI produced the similar restriction fragment patterns (the number and size of fragments) for *T. castaneum* and *T. confusum*. Only *Pvu*I produced the different restriction fragment patterns for *T. castaneum* and *T. confusum*. Therefore, *Pvu*I was selected to achieve the species-specific pattern for distinguishing the two species. The recognition site of *Pvu*I was CGATCG, and the amplified 28S rDNA fragments *of T. castaneum* and *T. confusum* had one and two recognition sites, respectively (figure 1). If point mutations occurred within these recognition sites, the observed restriction patterns of 28S rDNA digestion with *Pvu*I of *T. castaneum* and *T. confusum* should be changed. However, this can be avoided due to the high conservation of 28S rDNA region because none of the samples used in this study demonstrated intraspecific variation in both *Tribolium* species. In addition, PCR-RFLP assay successfully differentiated adult individuals of *T. castaneum* and *T. confusum* but the method can be used with larvae and pupae because genome DNA did not vary between adults and immature forms.

In conclusion, the PCR-RFLP assay developed in this study using PCR amplification with a universal primer pair and restriction enzyme digestion of PCR products has proven to be a useful method for the identification of *T. castaneum* and *T. confusum*.

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