DNA barcoding, species-specific PCR and real-time PCR techniques for the identification of six Tribolium pests of stored products

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Flour beetles of the genus Tribolium Macleay (Coleoptera: Tenebrionidae) are important stored product pests in China and worldwide. They are often found or are intercepted in grain depots, flour mills, and entry-exit ports, etc. Traditionally, Tribolium species are identified according to the morphological characteristics of the adult. However, it is almost impossible to rapidly identify adult fragments and non-adult stages based on external morphological characteristics. Molecular techniques for the rapid and accurate identification of Tribolium species are required, particularly for pest monitoring and the quarantine of stored products pests. Here, we establish DNA barcoding, species-specific PCR, and real-time PCR techniques for the identification of six stored-product pest Tribolium species including T. castaneum, T. confusum, T. destructor, T. madens, T. freemani and T. brevicornis. We detected the mitochondrial DNA cytochrome oxidase subunit I (COI) barcodes for Tribolium from 18 geographic populations and 101 individuals, built a Tribolium DNA barcode library, and designed species-specific primers and TaqMan probes for the above six Tribolium species. The three techniques were applied to identify Tribolium collected from stored samples and samples captured from quarantine ports. The results demonstrated that three techniques were all able to identify the six species of Tribolium both rapidly and accurately.
discriminate *T. destructor* from other species. PCR-RFLP has been developed to distinguish *Tribolium* flour beetles based on a partial 28S rRNA gene sequence. However, there are currently no correlative studies on the molecular identification of *Tribolium* species using DNA barcoding technology, species-specific PCR and real-time PCR.

This study focuses on six *Tribolium* species that are stored-product pests. The aim of the study was to establish molecular techniques to identify *Tribolium* species accurately, rapidly and practically using DNA barcoding, species-specific PCR, and real-time PCR.

### Materials and Methods

#### Tribolium specimens

Cultures of six *Tribolium* species from 101 adult specimens including *T. castaneum*, *T. confusum*, *T. destructor*, *T. madens*, *T. freemani* and *T. brevicornis* were used in this study. Specimens were collected from the P. R. China (Henan, Guangxi, Guangdong, Xinjiang), the Czech Republic (Prague, Kyjov, Herink, Rakovník), France (Bordeaux), Croatia (Osijek), and the United States (Kansas). Original collection locations are shown in Table 1.

### Table 1. Specimens of *Tribolium* used in the study.

| Species   | Collection locality | Accession number |
|-----------|---------------------|------------------|
| *T. destructor* | Prague, Czech Republic | KP892664 |
| *T. brevicornis* | York, the United Kingdom | KP892667 |
| *T. madens* | Kansas, the United States | KP892665 |
| *T. freemani* | Kansas, the United States | KP892666 |
| *T. castaneum* | Henan, P. R. China, Guangxi, P. R. China, Guangdong, P. R. China, Xinjiang, P. R. China, Prague, Czech Republic, Rakovník, Czech Republic, Osijek, Croatia | KP892686, KP892687, KP892688 |
| | | KP892660, KP892681 |
| | | KP892682, KP892683 |
| | | KP892684, KP892685 |
| | | KP892674 |
| | | KP892675 |
| | | KP892677 |
| | | KP892676 |
| | | KP892678, KP892679 |
| *T. confusum* | Prague, Czech Republic, Herink, Czech Republic, Kyjov, Czech Republic, Bordeaux, France, Kansas, the United States | KP892668, KP892669, KP892670, KP892671, KP892672 |

### Table 2. The information of COI gene sequences downloaded from GenBank used in this study.

| Species   | Accession No. |
|-----------|---------------|
| *T. destructor* | FJ743723 |
| *T. brevicornis* | FJ743722 |
| *T. madens* | FJ743721 |
| *T. freemani* | FJ743724 |
| *T. castaneum* | KJ003352, KM207082, KM439779 |
| *T. confusum* | FJ743725, KJ964296 |

### Table 3. The intra- and inter-specific Kimura 2-parameter divergence values (%) of COI gene.

| Species   | Intra | Inter | Inter |
|-----------|-------|-------|-------|
|           | Average | Min | Max | Average | Min | Max |
| *T. destructor* | 0.00 | 0.00 | 0.00 | 20.18 | 18.66 | 21.38 |
| *T. brevicornis* | 0.92 | 0.00 | 0.92 | 20.43 | 18.00 | 22.28 |
| *T. madens* | 0.00 | 0.00 | 0.00 | 19.23 | 18.62 | 20.30 |
| *T. freemani* | 0.00 | 0.00 | 0.00 | 17.89 | 16.34 | 22.28 |
| *T. castaneum* | 0.57 | 0.00 | 1.86 | 19.74 | 16.34 | 21.39 |
| *T. confusum* | 0.04 | 0.00 | 0.15 | 19.69 | 18.00 | 20.46 |
| All       | 0.50 | 0.00 | 1.86 | 19.61 | 16.34 | 22.28 |
listed in Table 1. These laboratory strains were reared in darkness at 27 °C and 75% relative humidity on a diet of powdered wheat germ. Voucher specimens, including adults, larvae and pupae, were kept in 100% ethanol and stored at −80 °C. Detailed specimen information is shown in Table 1.

**DNA extraction, PCR and COI sequencing.** Total genomic DNA at least three *Tribolium* adults from different geographic population was extracted from the thorax of *Tribolium* adults using a commercial TIANamp Genomic DNA kit (TIANGEN, China) according to the manufacturer’s protocol. A pair of universal forward LCO1490 (5′-GGTCAACAAAGCTATTTGG-3′) and reverse HCO2198 (5′-TAAACTTCAAGGGTGCACCAAAAAATCA-3′) primers were used for COI amplification 

15. PCR was performed based on methods by Wang et al.

11 and was modified for half volume reactions containing 12.5 μl MasterMix with loading dye, 10 μl sterilized distilled water, 1.5 μl extracted DNA (approximately 20 ng μL−1), and 0.5 μl forward and reverse primers (10 μM). The PCR protocol included an initial denaturing step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 10 min. The reactions were performed on a Veriti TM 96-well Thermal Cycler (ABI, USA). The amplified DNA fragments were resolved on a 1.0% (w/v) agarose gel (1 × Tris Acetate-EDTA buffer), stained with ethidium bromide and visualized with a UV light (Gel Logic 212 PRO, Carestream Health, Inc.). DNA purification and bidirectional sequencing using the same amplification primers was commercially performed by Sangon Biotech (Shanghai) Co., Ltd. and Beijing Aoke Biotechnology Co., Ltd.

**Sequence assembly and analysis.** Contig Express program was used to produce contigs from the forward and reverse reads of each COI amplicon and correct each read by looking at the chromatogram. Primer sequences from each contig were removed within the Contig Express program also. DNAMAN 7.0.2 software was used for DNA multiple sequences alignment. 110 amplicon sequences were aligned, among which 9 from GenBank and 101 from laboratory. Haplotypes were identified using the DnaSP v.5.1 software16. Pairwise genetic distances for COI genes were computed with the Kimura 2-parameter method (K2P). Neighbour-joining (NJ) phylogenetic trees were constructed in MEGA 6.017, and distance histograms were generated with the online version of automatic barcode gap discovery (ABGD)18. All of the identified haplotypes were submitted to GenBank.

**Specific primer design, selection and sensitivity test.** According to the variability of the partial COI gene sequences from six *Tribolium* species, suitable areas for designing species-specific primers were identified with Bioedit (version 7.2.0), and species-specific primers for *Tribolium* identification were designed with Primer

![Figure 1](https://www.nature.com/scientificreports/) (a,b) The results of *Tribolium* sample analysis by ABGD. (a) Histogram of distances. (b) Automatic partition results for *Tribolium* taxa by ABGD.
Premier 5.0. Primer pairs were evaluated according to eight factors: (1) length between 18 bp to 30 bp; (2) absolute value of Delta G less than 9; (3) 3’-end contains one or more specific bases; (4) no distinct hairpin structure; (5) GC% from 30% to 70%; (6) primers for distinguishing different species; (7) false priming less than 100%; and (8) optimal annealing temperatures. All of the primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Specificity testing with each primer pair in the PCR assays was performed using 18 selected samples (Table 1). PCR amplification in a final reaction volume of 25 μl consisted of 12.5 μl MasterMix with dye, 10.5 μl ddH2O, 0.5 μl specific forward primer, 0.5 μl specific reverse primer and 1 μl template DNA. The PCR cycler conditions used were an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s with a final extension at 72 °C for 10 min. After separation by 1.5% agarose gel electrophoresis and staining in ethidium bromide, the products were confirmed under UV light (Gel Logic 212 PRO, Carestream Health, Inc.) and were sequenced in both directions by Beijing Aoke Biotechnology Co., Ltd.

Sensitivity testing with the selected six Tribolium species-specific primers was determined in PCR runs with a series of samples using decreasing DNA concentrations with the same primer concentration. The DNA concentrations used were 100, 10, 1, 0.1, 0.01 and 0.001 ng μl⁻¹.

TaqMan probe and real-time PCR primer design, selection and sensitivity test. According to the partial COI gene sequences of six Tribolium species, suitable areas for specific primers were identified by Bioedit (version 7.2.0). TaqMan probes and real-time PCR primers were designed with Beacon Designer 8.12. TaqMan probes were evaluated according to seven factors: (1) length between 18 bp and 30 bp; (2) C% more than G%; otherwise complementary use; (3) GC content between 30% and 80%; (4) no G bases at the 5’ end; (5) avoided repeats of the same type of bases, especially four G bases encoded together; (6) probe should be as close as possible to the primers; and (7) no complementary secondary structures or primers.

Real-time PCR primers were evaluated according to six factors: (1) the length of a pair of primers was no more than four bases, with single base primers between 18 bp and 30 bp; (2) GC content between 40% and 60%; (3) no A bases as the first base on the 3’ end; (4) avoided repeat of the same type of base, especially four G bases encoded together; (5) did not use three G or C bases in a row at the 3’ end; (6) no complimentary primers.

Real-time quantitative PCR reactions were processed in 96-well plates in the PCR amplifier (ABI7500) using commercial Premix Ex Taq (rr390A) according to the manufacturer’s protocol. PCR amplification in a final reaction volume of 20 μl contained 10 μl Premix Ex Taq (Probe qPCR, 2X), 0.4 μl specific forward primer, 0.4 μl specific reverse primer, 0.8 μl TaqMan probe, 7.4 μl ddH2O, and 1 μl template DNA. The PCR cycler conditions used were an initial denaturation at 95 °C for 30 s, followed by 35 cycles of 95 °C for 5 s, 60 °C for 34 s and with a final extension at 72 °C for 10 min.

Figure 2. Neighbour-joining phylogenetic tree of Tribolium species based on the COI gene sequences.
Sensitivity testing of the selected TaqMan probes for the six Tribolium species was determined in PCR runs with a series of samples using decreasing DNA concentrations with the same primer concentration. The DNA concentration series was 100, 10, 1, 0.1, 0.01 and 0.001 ng μl⁻¹. Three replicates of each treatment were tested.

Results

DNA barcoding, species-specific PCR and real-time PCR accurately identified six stored-product pest Tribolium species including *T. castaneum*, *T. confusum*, *T. destructor*, *T. madens*, *T. freemani* and *T. brevicornis*.

### Table 4. List of the specific primers of the 6 storage *Tribolium* species.

| Species        | Primers' name | Sequence(5′-3′) bp | Tm(°C) | Product length (bp) |
|----------------|---------------|--------------------|--------|---------------------|
| *T. destructor* | Tde25F20      | ATGACGGAGATAAGTTGGTA 20 | 51.6   | 450                 |
|                | Tde451R24     | ATACGCCGATAATTGATTAGTG 24 | 51.7   |                     |
| *T. brevicornis*| Tbr63F23      | TTCGAGCAGAATTAGGTAATCCC 23 | 55.8   | 354                 |
|                | Tbr394R23     | TTCCTGCTAAATGTAATCTAAAG 23 | 50.7   |                     |
| *T. madens*    | Tma41F22      | GGAACCTCTTTAAGATTATTAG 22 | 49.7   | 490                 |
|                | Tma508R23     | TGATGCTGAATATACACACAGCTCAG 23 | 57.2   |                     |
| *T. freemani*  | Tfr379F22     | CTTAGATTTAAGAATTTTCAGG 22 | 53.3   | 170                 |
|                | Tfr526R23     | GTGAAGAAGTGAGAAAGAAATAGCG 23 | 52.2   |                     |
| *T. castaneum* | Tca33f26      | GAATAGTAGGCACTTCATTAAGACTC 26 | 56.3   | 337                 |
|                | Tca348R24     | CCATGTGCAATGTTTGATGAGAGG 25 | 57.9   |                     |
| *T. confusum*  | Tco261F23     | GGTTCTGCTCAATTTACCTAGTATCTTG 23 | 61.7   | 238                 |
DNA barcoding, genetic divergence and phylogenetic analysis. A 658-bp long region of the mtDNA COI gene was amplified from 101 individual Tribolium beetles (Table 1) using a set of universal COI primers. Alignment of these sequences, and nine additional Tribolium sequences from GenBank (Table 2), revealed six haplotypes were identified for T. castaneum, one haplotype was observed for T. confusum, T. destructor, T. madens, T. freemani and T. brevicornis. The sequences of common haplotype from different geographic populations have been submitted to GenBank, obtain 24 accession number (Table 1). The DNA multiple sequence alignment using DNAMAN 7.0.2 software showed that four bases A, G, C, T average content of these sequences, 30.04%, 22.45%, 16.22%, 31.29% respectively, A + T content 61.33%. Base composition of Tribolium sequences in line with insect mitochondrial genes.

K2P model calculation results using MEGA 6.0 showed that intra- and inter-specific genetic distance exists obvious difference. The inter-specific K2P divergence of the six Tribolium species averaged 19.61%, ranging from 16.34% to 22.28%, such as genetic distance of T. brevicornis and T. freemani reached 22.28%, T. castaneum and T. freemani only 16.34%. The intra-specific K2P divergence ranged from 0.00% to 1.86%, with an average of 0.5% (Table 3). Specimens of T. castaneum were characterized by K2P divergence values up to 2.0%, but less than 2.0%. Intra-specific divergences of other Tribolium species were all less than 1.0%. All inter-specific divergence values were greater than intra-specific values, more than 39 times. A favourable DNA barcode should have a higher divergence among species than within species. In the case of COI, the suggested standard divergence threshold value is ten times (10 ×) the mean intra-specific variation.

The results of applying the ABGD algorithm to the COI data set are presented in Fig. 1. Distance values show a gap between the intra-specific and the inter-specific distances (Fig. 1a). The data set was partitioned into six groups when the prior assumption of maximum intra-specific divergence was set as high as 0.46% (Fig. 1b). The NJ tree grouped the six morphologically identified Tribolium species based on the COI gene sequences as well as the outgroup species P. depressus (GenBank submission number KM450509) and P. subdepressus.
(KM452267) (Fig. 2). The resulting trees showed a clear clade of six Tribolium species distinct from the outgroup clades. There was high bootstrap support (100%) for the terminal branches at the species level.

**Specific primer design, selection and sensitivity test.** One hundred and one partial COI gene sequences (658 bp) from six adult species in the genus Tribolium were used to develop an accurate and fast method for identifying these six Tribolium species (Fig. 3). The specificity tests of the designed primers were performed by uniplex PCR. Six primer pairs were selected for the reliable identification of six Tribolium species.

**Figure 5. Sensitivity tests for six specific primer sets.** (a) T. destructor. (b) T. brevicornis. (c) T. madens. (d) T. freeman. (e) T. castaneum. (f) T. confusum. The concentration of template DNA from lane 1 to lane 6 was 100, 10, 1, 0.1, 0.01, and 0.001 ng μl⁻¹. Lane M: DNA Marker.
| Species       | Primers and Probes | Sequence 5’-3’ | bp | Tm(°C) | Product(bp) |
|--------------|--------------------|---------------|----|--------|-------------|
| T. destructor| TdeF               | CGTACAGAACTAGGAAAC | 18 | 58.1   | 116         |
|              | TdeR               | CCGATATTATAGTATCTATG | 24 | 57.8   |             |
|              | TdeP               | FAM-TTCCTAACGGAATGACCAAT-BHQ | 23 | 65    |             |
| T. brevicornis| TbrF               | GAGCAGTAGCAATTACAG | 18 | 58.9   | 84          |
|              | TbrR               | TTCGGTCGGTTAATATATAG | 21 | 58.7   |             |
|              | TbrP               | FAM-TCACCTCCAGTGTAGCCTG-BHQ | 22 | 69.6   |             |
| T. madens    | TmaF               | TCCTGTTCTCCTAATTGG | 18 | 59.3   | 138         |
|              | TmaR               | GCCCTCTAGTATACGTTGGA | 19 | 59.1   |             |
|              | TmaP               | FAM-AATGTAATTGTCACAGCCATGC-BHQ | 23 | 67.1   |             |
| T. freemani  | TfrF               | CGTAGATTAGGAAATTTCAG | 22 | 61.7   | 169         |
|              | TfrR               | TGAAGAAGTAGAGAAAGAATACG | 22 | 61.9   |             |
|              | TfrP               | FAM-AGCTGGTATCTCATCAATTTCCTG-BHQ | 27 | 69.8   |             |
| T. castaneum | TcaF               | GATCCTCTGTTGATCTTGG | 18 | 58.1   | 183         |
|              | TcaR               | CAGGAAGAATAAGAGAAGAAG | 19 | 57.5   |             |
|              | TcaP               | FAM-TCTGGGAGCCTATATTTCATTACAAC-BHQ | 27 | 66.8   |             |
| T. confusum  | TcoF               | CGGATGAACCTGTGTTACC | 18 | 58.7   | 151         |
|              | TcoR               | GTAGGTCGTATATATACGTTCTG | 22 | 57.3   |             |
|              | TcoP               | FAM-ATCATCTAATGTCAGCCTG-BHQ | 26 | 68.6   |             |

Table 5. List of TaqMan probes and primers of the 6 storage Tribolium species.

Figure 6. Validating species specificity of the TaqMan probe and primer sets for six Tribolium species.  
(a) T. destructor. (b) T. brevicornis. (c) T. madens. (d) T. freemani. (e) T. castaneum. (f) T. confusum.
and are listed in Table 4. These primer pairs were designed to identify different Tribolium species regardless of life stage. The results clearly demonstrated that each primer pair produced a species-specific band without any nonspecific bands (Fig. 4).

Sensitivity of a selected specific primer set for each of the six species was determined using one sample from each species. In all of the species, DNA concentrations of 100, 10, 1, and 0.1 ng μl⁻¹ resulted in strong intensity bands (Fig. 5).

TaqMan probe and real-time PCR primer design, selection and sensitivity test. Six TaqMan probe and primer pairs were selected for the reliable identification of six Tribolium species and are listed in Table 5. These primer and probe sets were designed to identify different Tribolium species regardless of their life stage. The results clearly demonstrated that each primer and probe set produced a species-specific band without any nonspecific bands (Fig. 6).

In the six Tribolium species, template concentrations lower than 0.01 ng μl⁻¹ noticeably decreased the intensity of the visualized bands. Finally, the lower limit for detection was set at 0.01 ng μl⁻¹ for T. destructor and T. freeman and 0.001 ng μl⁻¹ for T. castaneum, T. confusum, T. destructor and T. madens (Fig. 7).

Discussion
Molecular biology techniques have been increasingly applied to species identification. In our study, a series of experiments based on DNA barcoding, species-specific primers were performed to identify six species of the genus Tribolium. The results showed that DNA barcoding technology, species-specific PCR and real-time PCR are useful for the rapid and accurate identification of six Tribolium species. Our data demonstrated that regardless of whether the specimen is an adult, larva or pupa of Tribolium, it is possible to extract sufficient DNA for DNA barcoding, species-specific PCR and real-time PCR for identification of the sample. We speculate that the universal primers for the mtDNA COI gene, the species-specific primers, and the TaqMan probe and primers sets for Tribolium can feasibly identify all of the ontogenetic stages.

The COI gene provides a robust DNA barcode for identifying the six species of stored-product Tribolium with non-overlapping genetic distances between intra- and inter-specific samples (Fig. 1a). The genetic distance between sequences provides an approach for 'DNA barcode' evaluation19. A favourable DNA barcode should have
a higher divergence between species than within species. For COI, the suggested standard divergence threshold value is ten times (10X) the mean intra-specific variation. In this study, the ratio between species is thirty nine times (39X) the within species variation. The NJ tree organized all of the six species determined by morphology by forming robust clades.

The PCR assay with species-specific primers clearly demonstrated that each primer pair produced a species-specific band without any nonspecific bands (Fig. 4). Compared with DNA barcoding technology, species-specific PCR does not require sequencing, only routine laboratory techniques such as DNA extraction, PCR and electrophoresis, so this method is convenient for any quarantine laboratory. The same PCR assay with species-specific primers can be performed within 3 h using unknown Tribolium species DNA.

Six probe and primer sets were designed and selected for the real-time PCR method, which was also able to distinguish the six Tribolium species. Compared with traditional PCR, the real-time PCR method can be assessed directly without melting curve analysis. The amount of fluorescence generated during the reaction directly reflects the number of amplicons in real-time gene copy. However, this method also has drawbacks, e.g., the relatively high price of real-time instruments, reaction kits and TaqMan probes.

Future work should focus on collecting more samples, screening commonly used molecular markers and developing multiplex PCR. In this study, three Tribolium species, T. destructor, T. brevicornis and T. madens, are only distinct populations geographically. Meanwhile, T. parallelus, T. thusa, and especially T. audax Halstead in North America and T. anaphe Hinton in central Africa have not been collected, although attempts to acquire these samples were made. Next, internally transcribed spacer (ITS) can possibly be used for Tribolium species identification, despite the COI gene providing a robust DNA barcode for identifying the six species of Tribolium stored-product pests. Finally, species-specific primers and real-time PCR have the potential to be implemented in multiplex PCR.

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Author Contributions
Z.-H.L. designed this research. T.Z., Y.-J.W. and Z.-H.L. conducted experiments and analysis. T.Z., Y.-J.W., Z.-H.L., W.G., D.L., Z.K., V.S. and G.O. wrote the manuscript. Y.C., F.-J.L. and Y.W. provided their feedback on this research. T.Z., Y.-J.W., Z.-H.L., V.S. and G.O. provided help in reviewing the scientific content and language in this manuscript. All authors reviewed the manuscript.

Additional Information
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