Khapra beetle diagnostics

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Abstract

The khapra beetle, *Trogoderma granarium* Everts, is a serious pest of grains and stored dry food stuffs and is the subject of strict quarantine measures in many countries including Australia. Morphologically the khapra beetle can only be reliably identified by dissection by a limited number of skilled taxonomists. Suspect specimens found in grain products are usually the larvae or larval skins which are difficult to diagnose morphologically. Adult specimens are usually scarce and damaged. Due to their similarity, warehouse beetle (*Trogoderma variabile*) and other native *Trogoderma* spp. could be mistakenly identified as *T. granarium* with market access implicatons or could mask an incursion. Molecular diagnostic protocols have been developed for khapra beetle, but remain largely untested against other species of *Trogoderma*, some also capable of being pests. Western Australia has a broad large, poorly studied native *Trogoderma* fauna, many of which are still undescribed; their estimated number is possibly over 100 species. Occasionally native Australian species can occur in stored commodities. Their identification and at least separation from the pestiferous exotic *Trogoderma* presents a serious problem. The work in this paper has been undertaken in an attempt to distinguish *T. granarium* from Australian native *Trogoderma* and related Dermestid species by both morphological and molecular methods. Dermestid specimens were sourced mainly from a targeted survey around grain silos throughout Australia, using two trap types, inside and outside facilities. Khapra beetle specimens were sourced from different geographical locations around the world.

Keywords: *T. granarium*, PCR, native Australian *Trogoderma*, targeted survey, taxonomy.

Introduction

The khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae) is recognised as one of the world's most destructive pests of grain products and is the subject of strict quarantine measures in many countries. Khapra beetle is listed in the 100 "World's Worst Invasive Alien Species" by the Global Invasive Species Programme (Lowe et al 2000). Plant Health Australia has identified khapra beetle as one of the top 5 biosecurity threats to the Australian Grains Industry. By definition, khapra beetle does not occur in Australia, but there are occasional records of intercepts (Emery et al 2008; Day and White 2016). An incursion could lead to costly control and eradication efforts. Non-khapra beetle countries enforce quarantine restrictions on imported commodities from khapra beetle countries.

There are over 120 described *Trogoderma* spp. worldwide of which four are recognised as stored product pests, including *T. granarium*, *T. glabrum*, *T. inclusum* and *T. variabile* (Banks, 1994). In Australia there are over 50 described native *Trogoderma* species, and many more remain undescribed. None of these are pests but could accidentally get into grain stores and be misidentified. Due to their similarity, *T. variabile*, already in Australia, or native *Trogoderma* spp. could be mistakenly identified as *T. granarium* or could mask an early incursion of *T. granarium*.

Suspected *Trogoderma* specimens found in grain products are usually the larvae or larval skins which are difficult to diagnose morphologically (Banks 1994; Emery et al 1997). Adult specimens are usually scarce and damaged and need expert dissection for identification (EPPO, 2002; IPPC, 2012). Diagnostically the khapra beetle can only be reliably identified by a limited number of skilled taxonomists. Misidentification of *Trogoderma* and related Dermestids has the potential to seriously compromise Australian grain exports (Szito 1997).

The aim of this work was to develop a molecular diagnostic tool that could quickly discriminate between khapra beetle and native Australian *Trogoderma* fauna based on whole specimens or insect fragments.

The approach included a review and modification of a published diagnostic DNA method for khapra beetle (Olson *et al* 2014) as well as review and optimization of in-house protocols.

Thousands of native *Trogoderma* and related Dermestid specimens were used to verify the diagnostic components of this work. Native Australian *Trogoderma* specimens and related Dermestids were sourced from a national *Trogoderma* trapping program conducted throughout Australia between 2009-2011 at targeted sites around grain silos and ports. Khapra beetle material was sourced from overseas collections, of different geographical origin. The molecular approach included conventional PCR, real-time PCR and DNA sequencing methods. DNA was extracted from morphologically verified khapra beetle populations, field collected native Australian *Trogoderma*, warehouse beetle and other related pest Dermestids. Taxonomically verified target specimens were used to data mine for unique DNA sequence profiles.

1. Materials and Methods

Morphology – taxonomic verification of target species

Dermestid material from a national *Trogoderma* trapping program (2009 -2011) was a major resource for the project in terms of diversity of native *Trogoderma*, number of geographic sites and number of specimens (~17,000) in providing a broad range of *Trogoderma* species and *Trogoderma*-like species for DNA-validation work and generation of unique sequence profiles. A targeted trapping approach was used based on previous studies by Wright (1993) and Rees *et al* (2003) and data collected using hand held devices (PDAs) synchronised to desktop server database (Emery et al 2010). The survey involved setting two trap types at >70 seleted sites – both inside and outside grain silos around Australia (Botha et al 2012). The insect traps used in this study were commercially available products – Trece Storgard khapra beetle trap (Barak 2004), and a modified Lepidopteran wet trap (UniTrap) using *Trogoderma*-specific lures (Barak 1989). The survey was conducted between 2009 and 2011, with trap catch material collected on a monthly bassis and identified at the Department of Primary Industries and Regional Development (DPIRD), Western Australia.

Additional Dermestid material was provided by University of Western Australia collaborators (collected from Gnangara area of Western Australia). Ad-hoc specimens, specimens from smaller trapping projects, colony material and curated specimens were also used to build a diverse Dermestid collection for the project.

Khapra beetle specimens from different geographical locations were sourced through international contacts in Spain (colony, established 1956; origin: unknown), Canada (origin: Pakistan), Greece (origin: unknown, possibly Turkey), Germany (origin: Iran) and UK (Centre for Agriculture and Biosciences International (CABI); origin: unkown).

Morphological methodology included specialist insect handling, identification with chain-of-custody labelling for trace-back to collection site, date of collection, trap type etc. Thousands of specimens were pinned, labelled and data-based. Western Australian Department of Primary Industries and Regional Deveopment (DPIRD) taxonomists verified the specimens for the molecular development and verification in this project. Diagnostic image capture (photomontage) of the unique native *Trogoderma* identified was outside the scope of the project, nonetheless, some unique specimens were photomontaged and cross-referenced with specimen ID and DNA sequence codes.

Molecular diagnostics

The methodology included assessment of molecular (real-time PCR) khapra beetle protocols developed in previous Plant Biosecurity Cooperative Research Centre (PBCRC) projects (PBCRC20137,PBCRC60046), as well as testing a published DNA protocol (Olson et al 2014) on an extensive cohort of Australian native *Trogoderma* and khapra specimens from different geographical origin. Optimisation and development of new PCR primers for khapra beetle and

warehouse beetle (*T. variabile*) was also undertaken as part of this study. For DNA extractions and molecular procedures in the DPIRD Diagnostic Laboratory Service (DDLS), insect legs were removed from pinned, labelled adult specimens, or provided as larvae in etOH from multiple, labelled specimens, cross-referenced with DDLS codes for chain-of-custody.

The molecular protocols were tested for accuracy, specificity and reproducibility as outlined by the Australian Subcommittee on Plant Health Diagnostic Standards (SPHDS) instructions for National Diagnostics Protocols. The proposed research was designed to address the "International importance of accredited diagnostic laboratories using accepted diagnostic procedures" as written in the International Standards for Phytosanitary Measures (ISPM 27).

A 'blind-test' challenge using 30 insect specimens, including khapra beetle, warehouse beetle and a selection of related Dermestids (and non-Dermestids) was used to test the rigour of the protocol in a 'real-world scenario'.

Destructive and non-destructive methods for DNA extraction from larvae, adults and skin casts were tested. Below is a summary of molecular methods:

Modified Olson qPCR (Olson et al. 2014) for the detection of *T. granarium* specific mitochondrial 16S ribosomal RNA (16S rRNA) gene.

Conventional Folmer and Simon PCRs (Folmer et al. 1994, Simon et al. 1994) for the universal amplification and sequencing of the mitochondrial COI gene.

Conventional – 16SAr PCR (Simon 1994, Cognato & Volger 2001, Olson et al. 2014) for the amplification and sequencing of arthropod mitochondrial 16S rRNA gene

Universal Arthropod - 16SAr qPCR (Simon 1994, Cognato & Volger 2001, Olson et al. 2014) for the confirmation of successful DNA extraction from arthropod specimens.

Extraction options included:

A. Whole insects – remove 1–2 legs and transfer to a microcentrifuge tube containing 180 µL ATL buffer and 20 µL Proteinase K. Grind the sample using a sterile micropestle.
B. Larvae – a 'core biopsy' taken from the larvae using a fine gauged syringe and transferred to a microcentrifuge tube containing 180 µL ATL buffer and 20 µL Proteinase K.
C. Destructive – if the specimens are not required for further taxonomic work the entire larvae, adult or skin cast (or part thereof) may be homogenised in a microcentrifuge tube containing 180 µL ATL buffer and 20 µL Proteinase K using a sterile micropestle.
D. Non-destructive – place the entire larvae, adult or skin cast in a microcentrifuge tube containing 180 µL ATL buffer and 20 µL Proteinase K (larvae may be 'punctured' with a fine gauge syringe to aid extraction) and incubate at 56°C with gentle agitation for at least 1 hr (can be left overnight).

2. Results

Morphology – taxonomic verification of target species

The trapping program generated more than 17,000 Dermestid specimens, including at least 20 native *Trogoderma* species, which are yet to be formally described. In the project time-frame, 11 different native *Trogoderma* species have been identified, along with thousands of related Dermestid genera. Table 1 provides a summary of the Dermestid species collected and numbers that have been curated. Table 2 provides a summary of the non-dermestid species in the bi-catch trapped.

Tab. 1 Dermestid taxa recorded at grain storages in an Australian Dermestid trapping survey.

Dermestidae	Total numbers
Anthrenocerus	69
Anthrenus	24
Anthrenus verbasci	15
Attagenus	18

Dermestes	9
Dermestes maculatus	2
Orphinus	775
Phradonoma nobile	11
Thaumoglossa	81
Trogoderma (native)	3,793
Trogoderma variabile	12,111
Trogoderma granarium	0

Tab. 2 Non-dermestid Coleopteran taxa recorded at grain silos in an Australian Dermestid trapping survey.

Non Dermestidae
Anobiidae
Bostrichidae
Buprestidae
Carabidae
Chrysomelidae
Coccinellidae
Other Coleoptera
Cucujoidae
Curculionidae
Elateridae
Haliplidae
Hydraeinidae
Hydrophilidae
Laemophloeidae
Melyridae
Mycetophagidae
Nititulidae
Ptinidae
Tenebrionidae
Scarabeaidae
Silvanidae
Staphylindae

Molecular

The qPCR 'road-test'

A total of 1,618 *Trogoderma* and related Dermestid specimens underwent qPCR screening. The majority of the specimens consisted of 2-3 dissected insect legs, with the remaining insect pinned and labelled for reference. The khapra-specific 16S qPCR assay proved successful with a sensitivity of 100% and specificity of 97.20% when tested against the 1,618 specimens, including 61 known khapra isolates and 1,557 endemic beetles (Table 3). The performance of the 16S qPCR assay compared to the gold standard taxonomic identification is presented in Table 4. The performance of the modified Olson qPCR was within the recommended parameters of a validated diagnostic test.

Tab. 3 Total number of specimens tested by the Olson qPCR and the diagnostic sensitivity and specificity of the assay.

Total number of specimens	1,618
Total number of Khapra	61
Sensitivity	100%
Specificity	97.20%

Tab. 4 Confusion matrix detailing the performance of the Olson qPCR assay compared to the gold standard taxonomic identification. TP = true positive, TN = true negative, FP = false positive and FN = false negative.

	Taxonomic ID	
	Khapra	Non-khapra

PCR ID	Khapra	61 (TP)	43 (FP)
	Non-khapra	0 (FN)	1514 (TN)

Confirmatory sequencing

Sequencing of the DNA barcoding COI gene (mitochondrial gene cytochrome oxidase I) revealed >99% sequence homology with *T. granarium* specimens in GenBank. This result means that the qPCR test will rapidly identify positive khapra specimens, which can then be sent off for confirmatory sequencing at a third party laboratory, which is standard practice for NATA accredited Diagnostic Protocols, and current practice in the event of a 'real' incursion.

Molecular blind testing

The khapra beetle qPCR test correctly identified and discriminated khapra beetle specimens in the blind sample set (30 specimens), with no false positives or false negatives, with a results turn-around time of 2 days (non-urgent) (Table 5).

Follow-up sequencing to confirm the preliminary PCR diagnosis was undertaken by a third party facility (AGRF QEII Medical Centre) to simulate the diagnostic process that would occur in the event of a real incursion.

Sequencing results confirmed the positive khapra PCR test results, returning *Trogoderma granarium* partial 16S rRNA gene for all 4 khapra specimens. The four khapra samples were haplotyped as HT1 (Spanish 1956 colony); HT1 (Iran - German colony); HT2 (Pakistan – via Canada); HT2 (Pakistan via Canada).

A neighbour-joining tree for partial mitochondrial 16S rRNA gene sequences based on Olson-defined *Trogoderma* haplotypes was constructed (Fig. 1).

Tab. 5 Multiplex real-time PCR for the detection of *Trogoderma granarium* and *Trogoderma variabile* (in-house assay)

Species No.	Species ID	Species Description	*T. granarium*	*T. variabile*
0001	A1	*Trogoderma variabile*	-	+
0002	A2	Coccinellidae (native)	-	-
0003	A3	*Anthrenus* sp.	-	-
0004	A4	*Sitophilus oryzae*	-	-
0005	A5	*Trogoderma variabile*	-	+
0006	A6	*Anthrenus* sp.	-	-
0007	A7	*Anthrenus verbasci*	-	-
0008	A8	*Tribolium castaneum*	-	-
0009	A9	*Trogoderma* sp. (native)	-	-
0010	A10	*Trogoderma variabile*	-	+
0011	A11	*Anthrenus verbasci*	-	-
0012	A12	*Trogoderma granarium*	+	-
0013	A13	*Rhyzopertha dominica*	-	-
0014	A14	*Trogoderma variabile*	-	-
0015	A15	*Trogoderma variabile*	-	+
0016	A16	*Anthrenus* sp.	-	-
0017	A17	*Oryzaephilus surinamensis*	-	-
0018	A18	*Trogoderma granarium*	+	-
0019	A19	*Trogoderma* sp. (native)	-	-
0020	A20	*Trogoderma variabile*	-	+
0021	A21	*Anthrenus* sp.	-	-
0022	A22	*Trogoderma granarium*	+	-
0023	A23	*Cryptolestes pusillus*	-	-
0024	A24	*Trogoderma* sp. (native)	-	-
0025	A25	*Thaumoglossa* sp.	-	-
0026	A26	*Trogoderma granarium*	+	-
0027	A27	*Anthrenus verbasci*	-	-
0028	A28	*Anthrenus verbasci*	-	-
0029	A29	Coccinellidae (native)	-	-
0030	A30	*Trogoderma variabile*	-	+

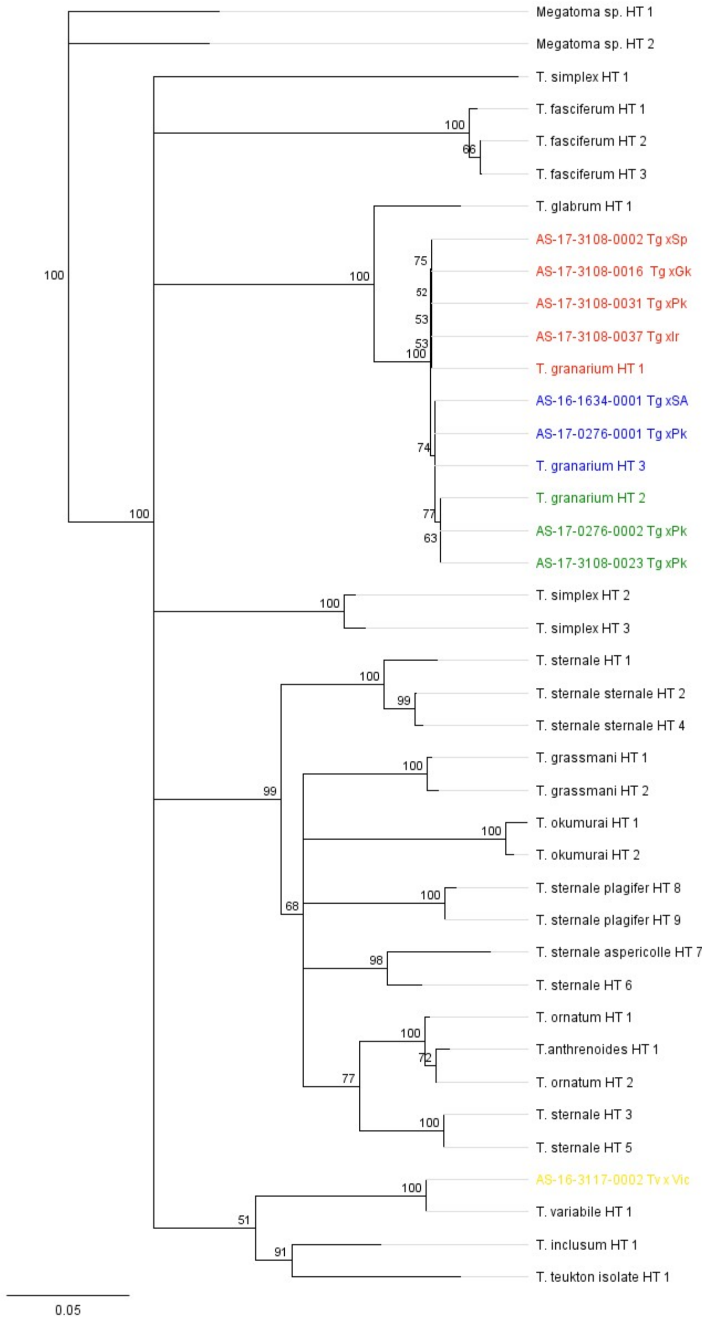

Fig. 1 Neighbour-joining tree for partial mitochondrial 16S rRNA gene sequences based on Olson-defined *Trogoderma* haplotypes (accessed via Genbank, NCBI). DPIRD sequencing results are denoted by laboratory accession numbers in colour (*T. granarium* HT 1 = red, HT 2 = green & HT 3 = blue and *T. variabile* in italics). Genetic distance was computed using the Tamura-Nei method. The tree is rooted to the outgroup species Megatoma sp. HT 1. The number on each node represents bootstrap probability based on 1000 replications.

Discussion

The project work has led to the development of a high-throughput qPCR diagnostic protocol for the species-specific detection of *Trogoderma granarium*. A second qPCR test for *Trogoderma variabile* was also developed. Additional 'universal' conventional (end-point) PCR assays form part of the diagnostic protocol which allow for the confirmation of identification via Sanger sequencing (if required). The protocol includes an optional qPCR method to quality control the DNA extraction process. This protocol is in routine use in our diagnostic DDLS facility, as a high-throughput Dermestid screening test.

As a result of this work, DPIRD has the capacity to undertake high-throughput PCR screening for the exotic khapra beetle which is absent from Australia. The PCR test offers a sensitive and specific quick 'first pass' screen for suspect khapra specimens adding to preparedness and planning options in the event of a pest incursion into Australia.

One of the advantages of the qPCR test is the ability to test insect fragments, damaged specimens and larvae that are almost impossible to identify morphologically. The project work has produced a Dermestid reference collection of more than 17,000 Dermestid specimens. This reference collection includes many previously unknown Australian native *Trogoderma* species and forms a unique and valuable legacy resource. Suspect Dermestid specimens can be tested in-house and their genetic sequences compared with in-house reference material, and against genetic reference profiles in publicly available databases (e.g. BOLD and GenBank). The diagnostic protocol developed for khapra beetle will be submitted shortly to the Subcommittee on Plant Health Diagnostics (SPHDS) in Australia for review as an accredited National Diagnostic Protocol for use throughout Australia (and Internationally). The Australian National Plant Biosecurity Diagnostic Network (NPBDN) publishes diagnostic protocols for priority pests online at:

http://plantbiosecuritydiagnostics.net.au/resource-hub/priority-pest-diagnostic-resources/.

It is anticipated that once approved by SPHDS, the khapra beetle protocol developed in this study will be published on the NPBDN website. The DNA sequences of Australian native *Trogoderma* and related Dermestids will be submitted to an internationally recognised genetic resource website at the conclusion of this study.

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Assessing drivers of maize storage losses in south west Benin using a Fractional Response Model

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Abstract

An assessment of drivers of maize storage losses was undertaken in south west Benin applying the Fractional Response Model on information collected from 400 smallholder maize farmers. Overall, respondents lose on average 10.3% of their harvest during the storage period. The average marginal effect obtained from the fractional response model of storage losses revealed that storage technologies, farmers' post-harvest attitudes, insects damage, the weather conditions and infrastructures played a significant role in the level of storage losses surveyed farmers have experienced. Farmers using bags and plastic containers have respectively reduced their storage losses by 6.7 and 7.8% compared to farmers using cribs. Considering the use of storage protectant, the results indicated that using ash, neem leaves, pepper or lemon lead to an increase of 4.1% of losses relative to