Multiple incursions and putative species revealed using a mitochondrial and nuclear phylogenetic approach to the *Trogoderma variabile* (Coleoptera: Dermestidae) trapping program in Australia

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Abstract

The Warehouse beetle, *Trogoderma variabile* (Coleoptera: Dermestidae), is an internationally significant invasive pest of packed goods and stored grain. When it was first documented in Australia at Griffith, New South Wales, in 1977, an eradication campaign was initiated. After several years and considerable effort, the eradication campaign was abandoned. To monitor the presence and spread of *T. variabile*, surveys were carried out by government agencies in 1992 and 2002. When survey data was compared, it was concluded that the distribution of morphologically identified *T. variabile* had doubled in most Australian states. Here, we used samples from the 2002 survey to conduct a phylogenetic study using partial sequences of mitochondrial genes Cytochrome oxidase I and Cytochrome B, and the nuclear gene 18S, to examine the distribution and dispersal of *T. variabile* and detect the presence of misidentified species. Based on our molecular results, we show that only 47% of the samples analysed were *T. variabile*, and the remaining were a mixture of six putative species. In addition, *T. variabile* was found in only 78% of the trapping sites. We discuss the importance of correct diagnosis in relation to the eradication campaign.

Keywords: Warehouse beetle, population, cryptic, molecular, eradication, invasive, correct diagnosis

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Introduction

The incursion of economically destructive arthropods into Australia is becoming more frequent due to the increase of international trade in goods, particularly from developing countries (Mack *et al.*, 2000). Once established, invasive pests can cause considerable environmental and economic damage, as they generally proliferate and spread due to factors such as lack of natural enemies and climate or edaphic constraints (Mack *et al.*, 2000). One such invasive arthropod is *Trogoderma variabile* Ballion, the warehouse beetle (Coleoptera: Dermestidae), which infests stored grain and packed goods (Wright, 1994). In Australia, *T. variabile* is regarded as a minor yet persistent pest. However, it is of considerable concern because it could mask the presence of the more damaging and invasive Khapra beetle, *Trogoderma granarium* Everts, due to high morphological similarity between the species (Rees *et al.*, 2003). It has been estimated that an introduction of the Khapra beetle into Australia could cause losses approaching AU$2 billion per annum (Cook, 2003).

When *T. variabile* was first documented in Australia at Griffith, New South Wales, in 1977 and then again in 1979 on the other side of the continent at Morawa, Western Australia, Australian State and Federal agricultural departments attempted to control and eradicate this pest (Hartley & Greening, 1983; Wright, 1994; Rees *et al.*, 2003). After several years, the eradication campaign was terminated when surveys showed that the beetle had spread to surrounding areas (Hartley & Greening, 1983). Although the eradication program was cancelled, surveys have been routinely carried out to monitor the distribution of the pest. One such extensive survey, conducted by the Commonwealth Scientific and Industrial Research Organization (CSIRO) from 2001 to 2003, discovered that, in Victoria and South Australia, the distribution of *T. variabile* had doubled since 1990, suggesting that little progress has been made in limiting its spread (Rees *et al.*, 2003).

In order to understand the apparently uncontrollable dispersal of *T. variabile* throughout Australia, there is a need to better understand the mechanisms underlying their invasion biology. Without a detailed understanding of these mechanisms, efforts to control the dispersal of an organism can be rendered useless (Tsutsui *et al.*, 2000, 2001, 2003; Schutze *et al.*, 2006). Studies on other invasive insect species have shown that molecular markers can provide valuable information about population structure, gene flow and dispersal pathways (Mikac & Clarke, 2006; Mikac & Fitzsimmons, 2010). In turn, this can provide an insight into the dispersal of species and help determine the presence of cryptic species (i.e. species unified under the same taxonomic identity yet different biological species) (Loxdale & Lushai, 1998; Mikac & Clarke, 2006; Mikac & Fitzsimmons, 2010).

In this study, we selected three genes, two mitochondrial and one nuclear, to investigate the population structure and dispersal patterns of *T. variabile* and determine the presence of any cryptic species. The mitochondrial gene Cytochrome oxidase I (*COI*) is frequently used to understand mechanisms underlying invasion biology (Jenkins *et al.*, 2007; Nadel *et al.*, 2010). Therefore, *COI* was selected to resolve patterns of geographical distribution and determine if the dispersal of *T. variabile* throughout Australia has been the result of multiple incursions and/or subsequent human-aided dispersal. Dispersal is likely due to human activities because the distance between grain storage facilities within Australia ranges from

9 to 3760 km, which far exceeds the proposed average dispersal distance for individual *T. variabile* of only 75 m (Campbell & Mullen, 2004). We also wanted to test the possibility that samples identified morphologically as *T. variabile* could in fact be *Trogoderma* species endemic to Australia, as there are currently 60 described endemic species (Booth *et al.*, 1990) and we believe at least another 100, not yet formally described, may exist. While *COI* is well suited for examining intra- and inter-specific variation at both the species and genus level, we also included Cytochrome B (*CYT b*), which evolves more slowly than *COI*. A partial 18S fragment was also included, to help resolve deeper nodes and check for congruent between the mitochondrial and nuclear genomes.

Materials and methods

Collection

One hundred and forty one specimens were collected from 27 grain storage sites throughout Australia (table 1), between 2001 and 2003, using baited sticky flight traps, as described by Rees *et al.* (2003). These were identified using morphological techniques unknown to this study. Ten additional specimens were collected from nine Western Australian sites in 2007 as part of an ongoing trapping program (table 1). These ten samples were the only samples that were verified as *T. variabile* using traditional morphological keys (Szito, 2007) and were subsequently defined as representative *T. variabile* reference samples used for this study. Single specimens of *T. granarium* and *Anthrenus verbasci* Linnaeus, both closely related non-native pest species, were taken from the Department of Agriculture and Food Western Australian insect collection and used as our outgroups.

DNA extraction

The number of specimens used in this study ranged from one to eight per site, depending on the number of specimens collected. DNA was isolated from the whole individual by crushing them in 5% Chelex beads (Biorad) following the methods of (Walsh *et al.*, 1991).

A non-destructive DNA extraction method, ANDE (Castalanelli *et al.*, 2010) (www.ande.com.au), was used to extract DNA from the ten reference *T. variabile* samples collected in Western Australia and the single specimens of *T. granarium* and *A. verbasci*.

Amplification of COI

A semi-nested Polymerase Chain Reaction (PCR) was used to amplify a section of *COI* using the primers UEA 5 (5′ AGTTTTAGCAGGAGCAATTACTAT 3′) and UEA 10 (5′ TCCAATGCACTAATCTGCCATATTA 3′) for the first round of PCR (PCR1) followed by amplification of first round PCR product using the primer combination UEA 7: (5′ TACAGTTGGAATAGACGTTGATAC 3′) and UEA 10 (5′ TCCAATGCACTAATCTGCCATATTA 3′) in a second PCR amplification (PCR2) (Lunt *et al.*, 1996). PCR were carried out in 12.5 µl volumes using 25–50 ng DNA, which was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., PCR1) or 1 µl of PCR product (PCR2), 0.3 µM each primer (PCR1: UAE 5 and UAE 10; PCR2: UAE 7 and UAE 10), 0.2 mM each dNTP, 1 × PCR buffer (New England

Table 1. Details of the number of samples analysed, types of species and the location of the 35 grain storage facilities.

Location	n	B	C	D	E	F	G	T. variabile	State	Latitude	Longitude
Ariah Park	6	0	1	3	0	0	0	2	NSW	−34.332	147.218
Balaklava	6	0	0	0	0	0	0	6	SA	−34.147	138.416
Booroowa	2	0	2	0	0	0	0	0	NSW	−34.482	148.751
Bowman	1	0	0	0	0	0	0	1	NSW	−33.15	151.292
Caracabal	5	0	0	3	0	0	0	2	NSW	−33.795	147.65
Carnanmah*	3	0	0	0	0	0	0	3	WA	−29.69	115.884
Colleambally	4	0	0	3	0	1	0	0	NSW	−34.799	145.785
Coolaman	8	0	0	1	0	0	0	7	NSW	−34.848	147.185
Cowra	7	0	2	3	2	0	0	0	NSW	−33.819	148.658
Crystal Brook	4	0	0	0	0	0	0	4	SA	−33.353	138.205
Dalwallinu*	1	0	0	0	0	0	0	1	WA	−30.249	116.669
Dimboola	8	0	5	0	2	0	0	1	Vic	−36.454	142.028
Garrah	2	0	0	0	0	0	0	2	SA	−35.483	140.3
Geelong	3	0	3	0	0	0	0	0	Vic	−38.149	144.357
Geraldton*	1	0	0	0	0	0	0	1	WA	−28.78	114.613
Griffith	7	0	0	0	0	4	0	3	NSW	−34.282	146.035
Katanning*	1	0	0	0	0	0	0	1	WA	−33.415	117.333
Little desert NP	8	0	8	0	0	0	0	0	Vic	−36.544	141.84
Lockhart	6	0	0	2	0	4	0	0	NSW	−35.215	146.768
Manangatang	8	0	1	0	0	0	0	7	Vic	−35.051	142.882
Narrabri West	3	0	0	0	0	0	0	3	NSW	−30.333	149.767
Nhill	6	0	6	0	0	0	0	0	Vic	−36.333	141.651
Pithara	1	0	0	0	0	0	0	1	WA	−30.389	116.667
Port Adeliade	0	5	4	0	0	0	0	1	SA	−34.846	138.503
Quirindi	1	0	0	0	0	0	0	1	NSW	−31.5	150.683
Rainbow	4	0	1	1	0	1	0	1	Vic	−35.9	141.993
Swan Hill	8	0	0	0	0	0	0	8	Vic	−35.339	143.555
Tamworth	6	0	0	0	0	0	0	6	NSW	−31.091	150.932
The Rock	4	0	0	1	0	1	0	2	NSW	−31.865	149.685
Three Springs*	1	0	0	0	0	0	0	1	WA	−29.536	115.761
Varley	5	0	0	0	0	0	5	0	WA	−32.706	119.641
Wagin*	1	0	0	0	0	0	0	1	WA	−33.187	117.208
West Wyalong	7	0	0	3	2	0	0	2	NSW	−33.932	147.201
Willow Tree	2	0	0	0	0	0	0	2	NSW	−31.649	150.735
Wyalkatchem*	1	0	0	0	0	0	0	1	WA	−31.179	117.382
Total	151	5	33	20	6	11	5	71			

n, total number of samples analysed; *, samples from the 2007 trapping program.

Biolabs, Ipswich, MA, USA), 3 mM MgCl$_2$ and 1.5 U of Taq DNA polymerase (New England Biolabs). PCR1 cycling conditions consisted of an initial denaturation step of 94°C for 3 min, followed by 30 cycles of 94°C for 40 s, 54°C for 1 min 40 s, and 72°C for 60 s, with a final extension temperature of 72°C for 8 min. PCR2 cycling conditions consisted of an initial denaturation step of 94°C for 3 min followed by 40 cycles of 94°C for 40 s, 52°C for 1 min 40 s and 72°C for 60 s, with a final extension temperature of 72°C for 8 mins. Ten percent of the final volume of the PCR2 products were electrophoresed on 2% w/v agarose gel containing 1 × TBE buffer and visualized with SYBR safe (Molecular Probes Inc., Eugene, OR, USA). Unpurified PCR products were sent to Macrogen (Korea) for cleanup and sequencing (see the 'sequencing' section below for further details).

Amplification of Cytochrome B and 18S

Amplification of the *CYT b* gene was performed using PCR primers CB-J-10933 (5′ TATGTACTACCATGAGGA-CAAATATC 3′) and CB-N-11367 (5′ ATTACACCTCCTAATT-TATTAGGAAT 3′; (Simon *et al.*, 1994). The nuclear gene 18S was amplified using PCR primers 18SF2 (5′ TACCACATC-CAAGGAAGG 3′) and 18SR2 (5′ CCTCTAACGTCGCAATAC

3′). Amplification of the *CYT b* and 18S genes was conducted in a reaction mix comprising 1× polymerase buffer (Roche), 3 mM MgCl$_2$, 0.2 µM of each primer and 0.5 U of Faststart Hi Fidelity Taq polymerase (Roche). PCR cycling conditions consisted of 45 cycles at 95°C for 10 s, 45°C for 10 s and 72°C for 30 s. Finally, all PCR products were purified by the addition of 10 U of Exonuclease I (New England Biolabs) and 2.5 U of Antarctic Phosphatase (New England Biolabs) and incubation for 30 min at 37°C, followed by inactivation by heating the reaction to 80°C for 20 min. Twenty percent of the final volume of the PCR products were electrophoresed on 1.5% w/v agarose gels containing 1 × TAE buffer, stained with ethidium bromide and viewed under UV light.

Sequencing

The *COI* amplified products were purified by Macrogen Inc. (Gasan-dong, Korea) using their standard purification method. All amplified products were sequenced by Macrogen Inc. using an Applied Biosystems ABI 3730 48-capillary DNA analyser using Big Dye Terminator Technology according to the manufacturer's protocols (Applied Biosystems, Mulgrave, Victoria, Australia).

Data analysis

Sequences (accession numbers HM243239–HM243470) were edited using CodonCode Aligner 3.0.3 (CodonCode Corporation, Deham, MA, USA) and aligned using a built-in version of ClustalW (Thompson *et al.*, 1994). Paup 4.0 (Swofford, 2003) was used to perform a partition homogeneity test (PHT) to determine the level of congruence between the three genes. The three genes were concatenated, and PAUP 4.0 (Swofford, 2003) was used to generate Parsimony and Maximum Likelihood (ML) gene trees. In addition, PAUP 4.0 (Swofford, 2003) was used to generate a *COI* Parsimony gene tree, which was compared to a Bayesian *COI* gene tree generated by Mr Bayes 3.1.2 (Ronquist & Huelsenbeck, 2003). MEGA 4 (Tamura *et al.*, 2007) was used to calculate net divergences between clades, TCS v1.21 (Clement *et al.*, 2000) for network analysis and an R statistics (R Development Core Team, 2007) function that we designed to plot the distribution of haplotypes. The PHT of the three genes was performed using the parameters hsearch, randomseed=0 and nrep=1000. The Parsimony and ML analysis of the three concatenated genes was calculated under the following conditions: hsearch, addseq=random, nrep=1000, swap=TBR, MaxTrees=1000, with the additional parameters added for the bootstrapping analysis nchuck=5 and chuckscore=1. The ML tree construction used likelihood settings that were selected by Modeltest 3.7 (Posada & Crandall, 1998) from the best-fit model (GTR+I+G) and were: Lset Base=(0.2884, 0.1771, 0.2319); Nst=6; Rmat=(0.1618, 10.5497, 2.0353, 0.3522, 1.6956); rates=gamma; shape=1.8432; and Pinvar=0.6063. Bayesian analysis was performed on the *COI* sequence data using the evolution model 4by4, gen=1,000,000, sample freq=100, sump burnin =2500 and sumt burnin=2500; the bootstrapping values were compared to the Parsimony results. Parsimony analysis of the *COI* data used the same parameters as concatenated gene analysis. *Anthrenus verbasci* was the out group for the generation of all trees. The R statistics program was used to plot the distribution of each clade, as well as the *T. variabile* haplotypes.

Results

Sequence data

The *COI* (620 bp, of which 264 were parsimony-informative) demonstrated 46 haplotypes in 87 individuals, with an average sequence divergence of 16.12% (SE 0.87%). In comparison, the *CYT b* (434 bp of which 171 were parsimony-informative) produced fewer haplotypes (24) in 112 individuals but had a similar average sequence divergence of 14.73% (SE 0.95%). The number of sequences obtained varied between the mitochondrial genes as the amplification of both genes was unsuccessful in some individuals; therefore, each gene was analysed separately. For the *COI* and *CYT b* genes, the polymorphisms resulted in 79 (38%) and 33 (22.9%) non-synonymous changes in the amino acid sequence, respectively. The 18S nuclear fragment length was 518 bp and, as expected, was evolving more slowly than the *COI* and *CYT b* genes, with only seven polymorphic nucleotides in 19 individuals. The average sequence divergence for this gene was only 3.9% (SE 0.17%). No insertions or deletions were observed in any gene fragment.

COI tree

Since the *COI* gene was the most phylogenetically informative fragment (46 haplotypes and 264 polymorphic nucleotides), it was used to generate both Parsimony and Bayesian trees (fig. 1). The *COI* tree revealed nine distinct clades (denoted using a unique letter or reference name) that were separated by deep branch lengths and supported by high net divergence between clades (from 10.72% to 26.11%; table 2). All clades were well supported by Bayesian posterior probability values >0.71. Parsimony bootstrapping values were slightly less supportive for all clades (6–8% less), except the divergence of clade c from d and e, and clade f from g, which were more than 22% different (53% and 77%, respectively). The *T. variabile* reference specimens that we identified using classical morphological techniques formed a distinct well-supported clade with only 47% of the samples. The pairwise divergence between *T. variabile* and the other clades ranged from 18.88 to 25.68% (*ca.* mean 22.88% SE 2.11); the lowest of these values was a comparison with the *T. granarium* reference sample.

Mitochondrial gene analysis

While only the *COI* gene was used to generate the Parsimony and Bayesian trees, both genes were used to examine the intra- and inter-pairwise divergence between clades (table 2). Inter-clade divergence values for *COI* and *CYT b* ranged from 10.72% to 26.11% and 10.61% to 26.11%, respectively. The mean divergence between clades was 1.61%; and, in two cases, *CYT b* was more divergent than *COI* (clades c and g). The mean intra-clade variation for *CYT b* was 0.29%, almost half that of the *COI* value of 0.48%, with *COI* and *CYT b* ranging from 0 to 3.55% and 0 to 3.46%, respectively. Interestingly, *CYT b* intra-clade variation for the *T. variabile* clade was zero (*n*=54), while the divergence for *COI* ranged from 0 to 2.9%. Unfortunately, Tamworth7, which was the most divergent specimen within the *T. variabile* clade (2.9%) was not represented in the *CYT b* dataset.

Congruence between nuclear and mitochondrial genomes

To determine if the nuclear and mitochondrial genomes were congruent, the 18S fragment was amplified from 19 individuals, which represented specimens from each clade identified from the *COI* data. The PHT analysis showed congruence between the nuclear gene 18S and the two mitochondrial genes *COI* and *CYT b*, (*P*=1 and 0.995, respectively). In contrast, the two mitochondrial genes were less congruent with each other but not significantly different (*P*=0.643). With no significant incongruence between the genes trees, the 19 specimens with overlapping sequence data were concatenated to create total evidence Parsimony and ML trees (fig. 2e) for the three genes. The Parsimony tree revealed the same nine distinct clades (depicted using a unique letter or reference name) that were separated by deep branch lengths and supported by high net divergence between clades (from 7.56% to 18.44%; table 3). Most ML bootstrapping values were >84%, apart from the divergence of clade d from e and clade f from g (67% and 59%, respectively). Both the nuclear and mitochondrial genomes supported the nine clades.

The distribution of each clade was visualised on a distribution map (fig. 2). The majority of specimens that were placed in clades d and f (95% and 91%, respectively) were
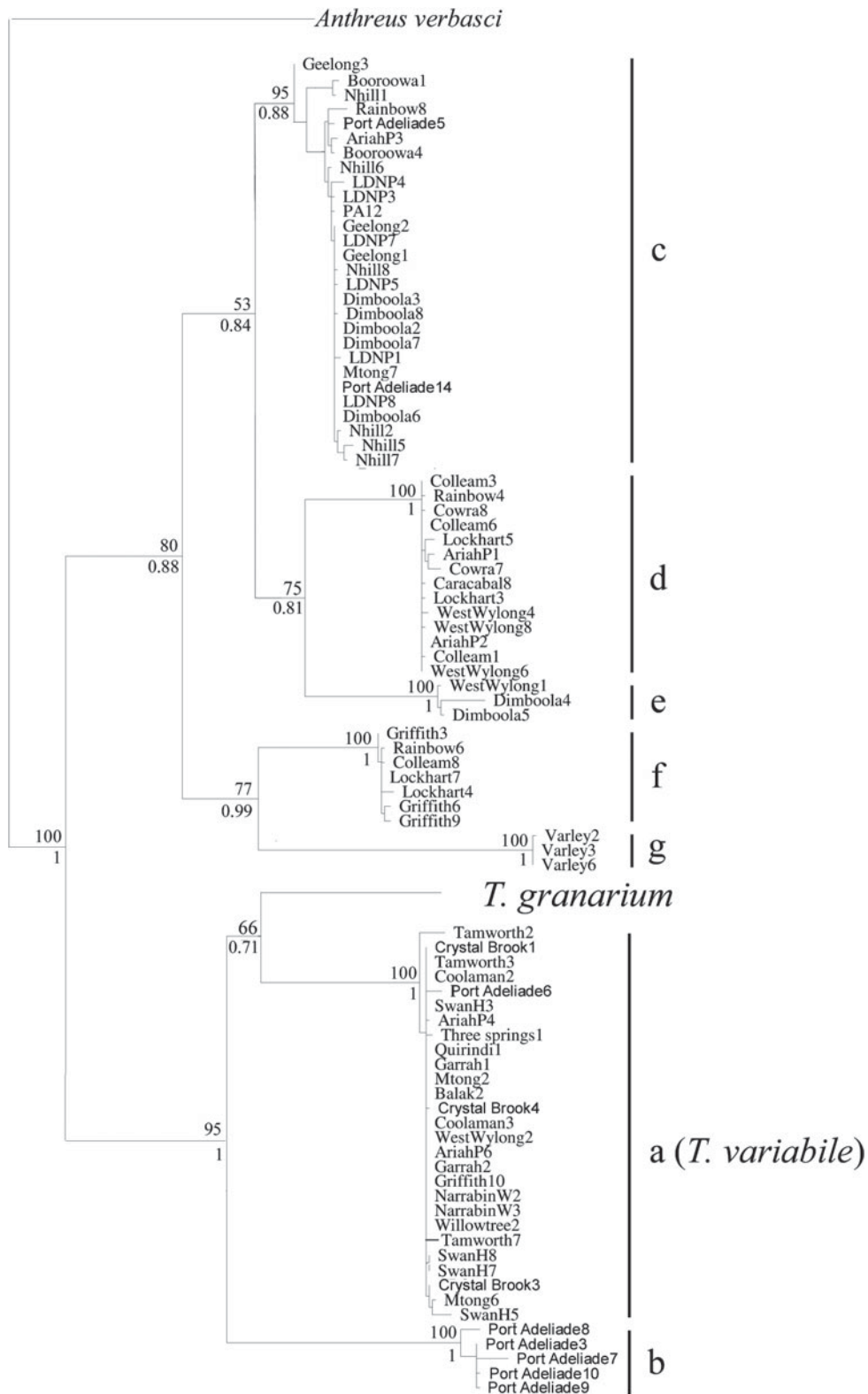
Fig. 1. Cytochrome oxidase I Parsimony tree. The value above the branch represents the parsimony bootstrapping support; the Bayesian bootstrap values are shown below the branch. The *T. variabile* clade shows the divergence of the ten haplotypes, rather than divergence between individuals.

Table 2. Inter- and intra-specific pairwise divergence percentages for *COI* (above the diagonal) and *CYT b* (below the diagonal).

| | | COI Intra | | | | CYT b Inter | | | | | | | |
	n	min	max	mean	A.v.	b	c	d	e	f	g	T.g.	T.v.
n					1	3	23	14	6	7	5	1	54
min					–	0	0	0	0	0	0	–	0
max					–	1.84	3.46	0.69	0.46	0.46	0	–	0
mean					–	0.82	0.62	0.15	0.20	0.23	0	–	0
A.v.	1	–	–	–		26.11	22.6	21.39	20.16	21.3	21.66	20.83	22.58
b	3	0	1.61	0.72	26.11		23.7	23.92	24	22.91	21.35	23.55	22.27
c	25	0	3.55	0.96	23.71	24.57		15.28	13.43	18.43	23.11	13.65	23.11
d	1	0	1.29	0.40	23.14	25.48	10.72		10.61	16.16	20.85	15.68	19.99
e	2	0	0.97	0.48	25.89	22.66	11.34	13.15		15.65	19.93	15.23	19.93
f	4	0	0.65	0.30	24.44	23.43	13.9	16.28	16.21		21.66	15.54	18.76
g	3	0	0.16	0.08	24.52	23.47	14.85	15.69	16.61	12.34		20.61	20.18
T.g.	1	–	–	–	25.65	21.56	21.18	23.43	22.61	23.25	22.04		18.28
T.v.	34	0	2.90	0.40	24.84	21.81	21.59	25.68	23.39	23.22	23.65	18.88	

Left side labels: CYT b Intra (top rows), COI Inter (bottom rows).

A.v., *A. verbasci*; T.g., *T. granarium*; T.v., *T. variabile*.

mainly observed in the southern part of New South Wales, and 73% of clade c was found in Victoria (table 4). Clade b was only observed at Port Adelaide in South Australia, and clade g was only located at Varley in Western Australia. The specimens that clustered with the *T. variabile* reference specimens were found at 78% of the sites sampled (table 1).

Genetic structure of T. variabile *using* COI

Cytochrome oxidase I was the only gene to resolve divergence within the *T. variabile* clade (table 2), revealing nine unique haplotypes (table 5). Network analysis of these haplotypes, using the program TCS v1.21 (Clement *et al.*, 2000), revealed one main haplotype (H4) with four direct descendents (H2, H3, H6 and H8). One haplotype (H5) was distantly related to H4 (missing multiple mutational steps), and three haplotypes (H1, H7 and H9) were not connected in the network because their divergence ranged outside the 95% parsimony limit imposed by the program (fig. 3c). With no connectivity between H1, H7 and H9 and the rest of the haplotypes, the relatedness between these haplotypes could not be determined. Haplotype 4 was found at all the western and eastern states collection sites (where we identified the presence of *T. variabile*; table 4), except for Willow Tree that had only H5. Tamworth and Swan Hill both contained additional haplotypes that showed no connectivity.

Discussion

This study aimed to investigate the population structure of *T. variabile* in Australia to determine what factors may have influenced the unsuccessful outcome of the eradication campaign. The results revealed seven deeply distinct clades in both the all gene Parsimony and ML trees, and in *COI* trees (figs 1 and 2e, respectively) with average pairwise divergence between clades of 18.5% (SE 2.27) and 17.5% (SE 1.87%) for *COI* and *CYT b*, respectively. These data, consistent at both nuclear and mitochondrial gene loci, strongly suggest that seven distinct species were collected, of which only 47% (71)

were clustered with the *T. variabile* reference sample (table 2). Population analysis of those specimens identified as *T. variabile* revealed one main haplotype with several derived haplotypes and three unconnected haplotypes that may be separate incursions. Both misidentification of captured specimens and possible multiple incursions would have a strong negative effect on the success of the eradication campaign.

Of the 153 individuals that were genotyped, only 46.7% could be grouped with the specimens that were morphologically verified as *T. variabile.* Since the specimens were macerated to liberate the DNA, we cannot attempt to match genetic and morphological patterns. However, our data strongly suggests that divergent clades were not *T. variabile*, as large nucleotide percentage differences were observed between clades (tables 2 and 3), implying deep evolutionary divergence and presumptive reproductive isolation. The average inter- and intra-specific divergence for the *COI* locus in this study was 18.5% and 0.48%, respectively. In comparison, the inter- and intra-specific divergence levels for the *COI* loci, previously determined for a multitude of insect pests (Cognato, 2006), are an average of 7.4% (2–24%) and 1.75% (0.077–26%), respectively. The mean inter-clade divergence observed in our study was more than double this previous report, and the intra-clade divergence was only a fraction of that calculated by Cognato (2006), which further supports the presence of a number of deeply divergent cryptic species in the present study. Furthermore, *T. variabile* and *T. granarium*, which can be differentiated using morphological keys, had a *COI* divergence of 18.8% (table 3), the lowest observed between *T. variabile* and any other clade. With further study, morphological characters may be resolved that discriminate between the other cryptic species discovered in this study, making field recognition and management more feasible.

The six putative species, accounting for 53% (*n* = 82) of the individuals analysed, could be attributed to both high trapping yields and the difficulty encountered when identifying samples collected from a trapping program. The traps were placed outside of the grain facilities, possibly to increase the number of *T. variabile* collected (Campbell & Mullen, 2004),
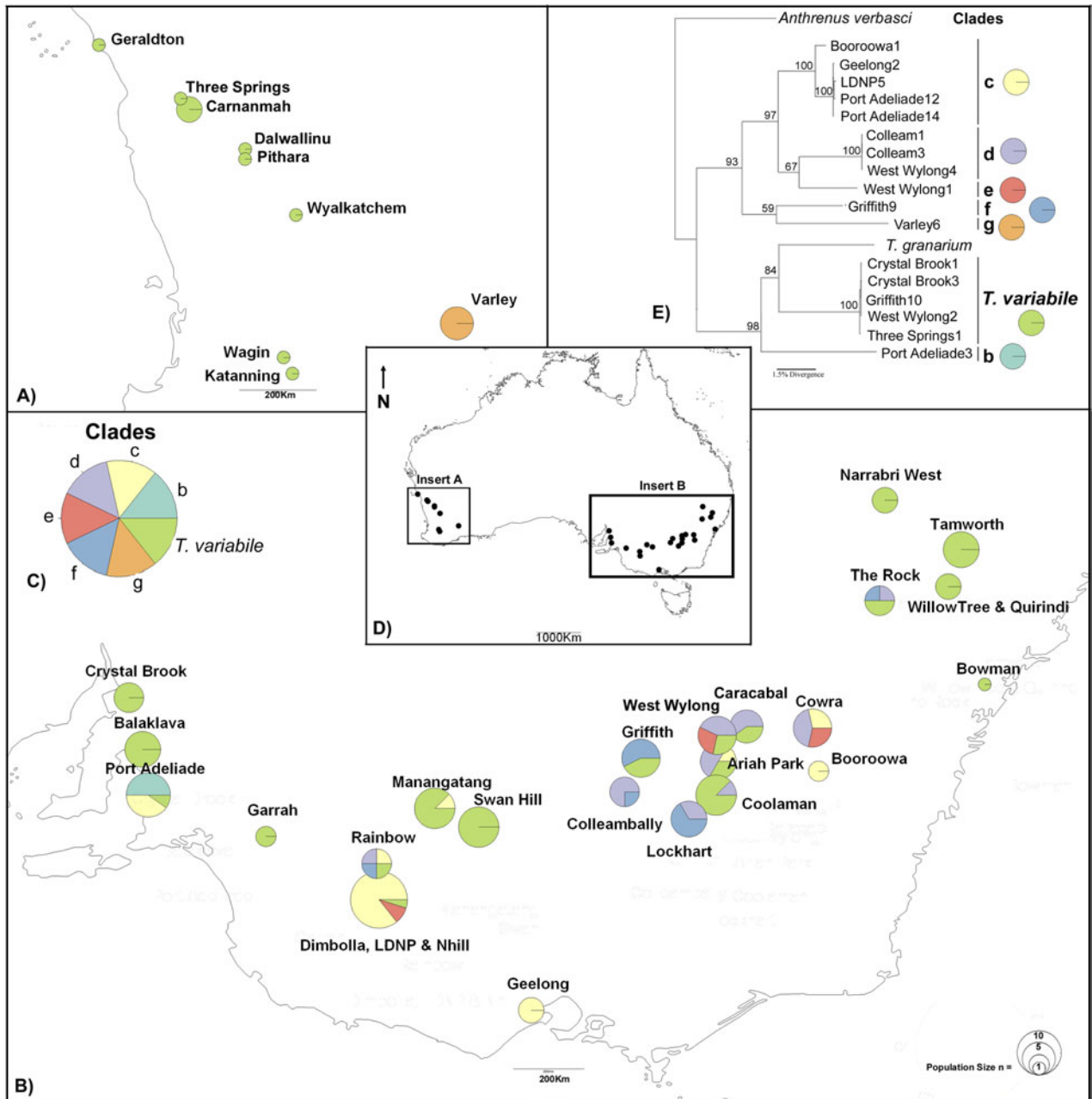
Fig. 2. Distribution of each clade determined by Parsimony and Maximum Likelihood phylogenetic analyses. (a) Spatial distribution of dermestid spp. within Western Australia. (b) Spatial distribution of dermestid species on the east coast of Australia. (c) Clade wheel, each colour representing a clade determined by the phylogenetic trees. (d) Sampling areas within Australia. (e) Consensus Parsimony tree combining the partial sequences of the two mitochondrial gene regions *COI* and *CYT B* and the nuclear 18S gene. The numbers above each of the branches are Maximum Likelihood bootstrap values and each letter depicts a clade. The outgroup was *Anthrenus verbasci*, an exotic pest to Australia. The external branches of the three gene Parsimony tree are labelled with the location of the specimen and the sample number for that site. These labels can be cross-referenced with the sample collection data found in table 1.

but this also potentially increases the number of native Dermestids collected, since some natives have been shown to be attracted to the pheromone used in the traps (unpublished data). The number of specimens collected in each trap was likely to have exceeded a few hundred (unpublished data) making identification particularly difficult, especially when

adopting a quick visual screening approach. Morphological distinction between *T. variabile* and other closely related *Trogoderma* requires dissection and careful microscopic examination of the genitalia (Szito, 2007), not just the examination of external characteristics. Dissection and preparation of the genitalia for examination requires time and skill, and would be

Table 3. Net divergence between the eight clades calculated on the three gene regions (18S, *COI* and *CYT b*).

	A.v	b	c	d	e	f	g	T.g.
A.v.								
b	18.44							
c	15.97	15.17						
d	15.34	15.18	8.35					
e	15.55	14.71	7.56	7.91				
f	15.62	15.76	10.12	10.44	10.06			
g	15.83	15.34	10.27	10.06	10.49	9.08		
T.g.	16.89	14.85	14.17	14.73	13.93	14.5	13.72	
T.v.	17.28	14.47	14.24	15.51	14.07	13.24	13.99	12.93

A.v., *A. verbasci*; T.g., *T. granarium*; T.v., *T. variabile*.
The values are nucleotide percentage differences (%).

Table 4. The distribution of each species per state.

	A. verbasci	B	C	D	E	F	G	*T. variabile*
New South Wales	0	0	15	95	67	91	0	44
South Australia	0	100	12	0	0	0	0	18
Victoria	0	0	73	5	33	9	0	24
Western Australia	100	0	0	0	0	0	100	14
Total Frequency	0.7	3.3	21.7	13.2	3.9	7.2	3.3	46.7

Table 5. Number of *T. variabile* haplotypes and frequency of each haplotype per collection site for the mitochondrial markers Cytochrome oxidase 1 (*CO1*).

Site	State	*n*	H1	H2	H3	H4	H5	H6	H7	H8	H9
Ariah Park	Vic	2				2					
Balaklava	SA	1				1					
Carnanmah	WA	3				3					
Coolaman	Vic	2				2					
Crystal Brook	SA	3		1		1		1			
Dalwallinu	WA	1				1					
Dimboola	Vic	1				1					
Garrah	SA	2				2					
Geraldton	WA	1				1					
Griffith	NSW	1				1					
Katanning	WA	1				1					
Manangatang	Vic	1				1					
Narrabin West	NSW	3				3					
Pithara	WA	1				1					
Port Adeliade	SA	1				1					
Quirindi	NSW	1				1					
Swan Hill	Vic	4	1		1	1				1	
Tamworth	NSW	5				3			1		1
Three_springs	WA	1				1					
Wagin	WA	1				1					
West Wylong	NSW	1				1					
Willow Tree	NSW	1					1				
Wyalkatchem	WA	1				1					
Total		39	1	1	1	31	1	1	1	1	1

impractical to perform on the number of samples collected at each site. If the eradication campaign adopted a similar approach and only examined the external characters, the likelihood of misidentification would be high and potentially result in the management of a non-target species.

In the future, for *Trogoderma* species, we suggest that a non-destructive method, such as the recently developed ANDE (Castalanelli *et al.*, 2010), be used. The availability of such a non-destructive DNA extraction technique when this study was commenced would have allowed us to revisit the samples and identify each specimen using morphological techniques post grouping them into genetic lineages. Two years after the initial Chelex extractions, additional DNA samples (from the same trapping program) were extracted using the non-destructive ANDE method. However, the extracted DNA was subsequently shown to be severely fragmented resulting
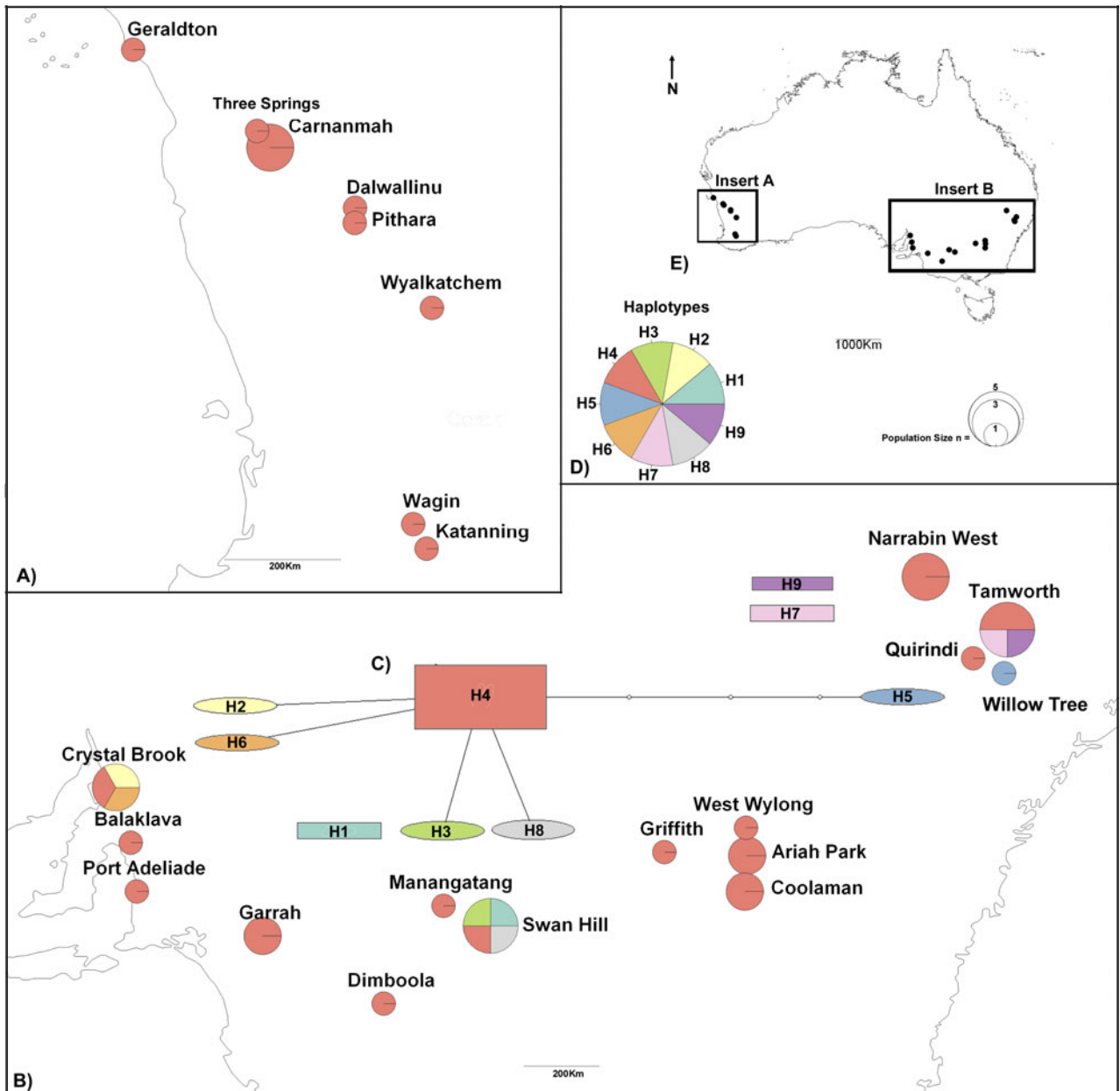
Fig. 3. Spatial distribution of the nine *T. variabile* haplotypes. (a) Spatial distribution of *T. variabile* within Western Australia. (b) Spatial distribution of *T. variabile* on the east coast of Australia. (c) TCS network of the nine haplotypes. (d) Haplotype colour key. (e) Sampling areas within Australia where the presence of *T. variabile* was confirmed.

in unsuccessful amplification. We believe that this was not the result of the ANDE extraction method, since the *T. variabile* reference specimens were also collected using sticky traps, but this time the adhesive was not removed. Rather, the unsuccessful amplification can be attributed to the use of harsh chemicals, such as hexane or limoene used to remove the specimens from the sticky traps (Szito, 2007), combined with long-term storage in ethanol at room temperature. We also observed limited amplification with Chelex samples. Only minimal genetic analysis has been conducted on the native and international dermestids; so until these sequences are

detected again, with accompanying whole specimens, the six new species identified in this work will remain putative.

The genetic data set generated in this study allowed us exploration into possible genetic structure within *T. variabile*. Although sample sizes were low, several interesting trends were observed based upon the distribution and relatedness of haplotypes (table 5, fig. 3). The main *T. variabile* haplotype (H4) was found at all eastern and western Australian sites where *T. variabile* was present, except for Willow Tree in NSW. The distance between each of these sites ranged from 9 to 3760 km; and, considering that *T. variabile* has a limited

dispersal range based on its ecology (Campbell & Mullen, 2004), human-aided transport is the plausible dispersal means. The three non-connected haplotypes observed at Tamworth (NSW) and Swan Hill (Vic) are interesting because they are localised and yet to disperse, suggesting that they are younger – such is the expectation based on coalescent theory (Kingman, 1982; Crandall & Templeton, 1993). However, we are unable to ascertain if this is the result of multiple incursions or a single incursion that contained multiple haplotypes. Increased sampling within Australia and additional samples from international areas would be needed to confirm the multiple incursion events, and additional samples from international areas would be needed to confirm the multiple incursion events.

With increasing international trade, the likelihood of serious pests invading Australia will inevitably increase. When an invasive species is detected, the success of control or eradication is dependent on accurate identification (Walter, 2003; Schutze *et al.*, 2006). This study revealed that 53% of the specimens collected during the 2001 to 2003 *T. variabile* trapping program were misidentified, and only 78% of the sites actually contained *T. variabile*. We believe this to be the result of dealing with several hundred samples per site, as well as the use of a quick screening method to identify *T. variabile*. Without keying out each specimen fully, misidentification is inevitable. Regardless of the method of morphologically based identification adopted in the eradication program, this study strongly implies that the presence of several cryptic species would have thwarted successful eradication or containment of the incursion. Misidentification would have seriously compromised the eradication campaign through the calculation of incorrect estimates of population size and location, with resulting in misdirection of resources (Walter, 2003). Furthermore, analysis of *T. variabile* populations suggested large-scale movement aided by human dispersal (up to approx. 3750 km), but more concerning were the numerous haplotypes that were not derived from the main population. Australia's grain industry should be highly concerned that the Khapra beetle may be allowed to infiltrate our borders under the mistaken identity of *T. variabile*.

Acknowledgements

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