

Molecular phylogeny supports the paraphyletic nature of the genus *Trogoderma* (Coleoptera: Dermestidae) collected in the Australasian ecozone

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Abstract

To date, a molecular phylogenetic approach has not been used to investigate the evolutionary structure of *Trogoderma* and closely related genera. Using two mitochondrial genes, Cytochrome Oxidase I and Cytochrome B, and the nuclear gene, *18S*, the reported polyphyletic positioning of *Trogoderma* was examined. Paraphyly in *Trogoderma* was observed, with one Australian *Trogoderma* species reconciled as sister to all Dermestidae and the *Anthrenocerus* genus deeply nested within the Australian *Trogoderma* clade. In addition, time to most recent common ancestor for a number of Dermestidae was calculated. Based on these estimations, the Dermestidae origin exceeded 175 million years, placing the origins of this family in Pangaea.

Keywords: systematic, paraphyly, *18S* rDNA, *COI*, Cytochrome b, molecular phylogeny

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Introduction

The family Dermestidae is a group of mostly xerophilic, necrophagic beetles that scavenge for dried, proteinaceous materials, such as dried fish, meat and hides (Peacock, 1993; Kiselyova & McHugh, 2006; Zhantiev, 2009). This type of material is consumed mainly during the larval stages, whereas the majority of adults feed on plant material, generally nectar and pollen, to help with egg maturation (Kiselyova &

McHugh, 2006; Zhantiev, 2009). Exceptions do occur; for example, some species of *Trogoderma* can complete their entire lifecycle on plant material, making them pests of stored products (Hinton, 1945; Mroczkowski, 1968).

Of particular interest to this study is the subfamily Anthreninae and, within it, the tribe Megatomini. This tribe is widespread, with most of the 27 extant genera occurring in the Palearctic (11), Australian (9) and Oriental regions (4: Mroczkowski, 1968; Peacock, 1993). One of the largest genera within the Megatomini tribe is the *Trogoderma* genus, containing approximately 130 described species (Hava, 2003). *Trogoderma* species are geographically widely distributed and found in most zoogeographical regions defined by Mroczkowski (1968), excluding Antarctica and Ethiopian

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regions. The majority of species inhabit Australia (52 species not taking into account the proposed synonymies: A. Szito, personal communications) and the Neotropics (30 species: Mroczkowski, 1968). Dispersal routes for *Trogoderma* have been proposed to have followed a similar path to mammals, migrating from North America to South America and into Australia at about 30 to 65 million years ago (MA) (Kiselyova & McHugh, 2006). This scenario does not explain how several *Trogoderma* species come to exist in the Indian and Palaearctic regions, of which *Trogoderma granarium* Everts and *Trogoderma variabile* Ballion are major pests of stored grains (Hinton, 1945; Lowe *et al.*, 2000). Interestingly, the native Australian *Trogoderma* species are not pests of stored products, yet some species belonging to other Megatomini tribes, such as *Anthrenocerus australis* Hope and *Orphinus fulvipes* Guérin-Méneville, have been reported as minor pests to stored products other than grain (Hinton, 1945).

The latest phylogenetic study on the dermestid family, using larval morphological characteristics, was conducted by Kiselyova & McHugh (2006). The authors observed different *Trogoderma* species scattered throughout the crown of the tree and concluded that the genus *Trogoderma* was polyphyletic, but were unable to ascertain whether this was a result of inadequate larval data or the incomplete state of dermestid taxonomy (Kiselyova & McHugh, 2006). Furthermore, in three instances, the relationship between *Trogoderma* and closely related genera was unable to be resolved, leading to multifurcation at a number of nodes (referred to as polytomy). Multifurcation can occur when there is insufficient data (referred to as 'soft' polytomy) or when real biological events, such as rapid radiation, occur ('hard' or true polytomy: Maddison, 1989; Humphries & Winker, 2010). If a group of species resulted from a rapid radiation event, then polytomy may not be resolved no matter how much data is included in the study.

As the dermestid fossil record is poor (Mroczkowski, 1968; Kiselyova & McHugh, 2006), a molecular clock in conjunction with the biogeographical distribution of several dermestid genera was used to investigate the origins of a few genera within Dermestidae. Since few molecular studies have been performed on this family, molecular clock data generated by Hunt *et al.* (2007) was used as a basis for this study. Dermestids belong to the series Bostrichiformia (Crowson, 1981), whose origins have been estimated at 219 MA (Hunt *et al.*, 2007). Assuming the Dermestid ancestors fed on angiosperm pollen, it is plausible to suggest that their origins are later than the oldest angiosperm pollen fossils which have been dated at 130 MA (Brenner, 1996). However, molecular data suggests that the radiation of angiosperms was somewhat earlier, 190 MA (Sanderson & Doyle, 2001). Beal & Zhantiev (2001) did suggest that the Dermestidae origins were Pangean when a new species of *Egidyella*, only thought to occur in the deserts of central Asia, was found inhabiting the sand dunes of California. Therefore, on the balance of current available evidence, it is possible that the Dermestidae origins are Pangean (>175 MA), and thus that the Megatomini tribe is later than this period.

The objective of this study was to evaluate the existing morphologically based polyphyletic positioning of *Trogoderma* (in species both exotic and endemic to Australia) using a molecular phylogenetic approach. The second objective was elucidation of the molecular relationships between six related genera: *Anthrenocerus* Arrow, *Anthrenus* Müller, *Attagenus* Latreille, *Dermestes* Linnaeus, *Orphinus* Motschulsky and

Trogoderma Dejean, in order to better understand evolutionary processes that may have led to their current biogeographical distributions, specifically, to investigate the relationship between the pest *Trogoderma* species and the Australian native *Trogoderma*. To study these relationships, three genes were selected. Cytochrome Oxidase I (*COI*), is frequently used for species identification (DNA barcoding), phylogeographical and phylogeny studies of closely related species (Hebert *et al.*, 2003; Foottit *et al.*, 2008; deWaard *et al.*, 2009; Castalanelli *et al.*, 2011), thus being well-suited for examining intra- and inter-specific variation at both the species and genus level. We also included mitochondrial Cytochrome b (*CYT b*), which evolves more slowly than *COI* (Castresana, 2001) to inform intermediate tree nodes. The entire 18S nuclear gene, a slowly evolving gene normally used in higher classification studies (Hwang *et al.*, 2000), was selected, as it might be needed to resolve deep polyphyletic structuring inferred in the earlier morphology based study by Kiselyova & McHugh (2006). The 18S gene included the expansion segments (also known as variable domains) that have been shown to help resolve genera within insects (Hwang *et al.*, 2000; Gillespie *et al.*, 2008). Highly conserved and expansion segments within the nuclear gene, as well as faster evolving mitochondrial genes, were included in order to resolve the structure across the tree.

Materials and methods

Samples

More than 3000 dermestid specimens were collected during the Australian spring and summer (September to February) for three seasons from 2007 to 2010. Specimens were collected by either sweep netting or using a pheromone based trap. The traps were a Gypsy Moth bell trap (APTIV Inc) baited with a Khapra beetle kairomone attractant lure (Trécé Inc). The traps were hung from low-lying tree branches (<1 m from the ground) then filled with 20% propylene glycol. Every two months, the propylene glycol was transferred into plastic bottles and a new pheromone lure was added to the trap. A sieve with a 1 mm gauge was used to remove the specimens from the propylene glycol. The specimens were then rinsed with sterile water, followed by a 70% ethanol rinse and, finally, stored in 95% ethanol at -20°C. One specimen each of *T. variabile*, *T. granarium*, *Anthrenocerus*, *Attagenus* and *A. verbasci* were obtained from the Department of Agriculture and Food Western Australian insect collection.

Morphological identification

Due to the large number of specimens, the first step towards identification was to separate specimens into morphospecies, groups that shared similar morphological features. These features included club size and number, setae colour, body shape and the antenna cavity, or 'fossa', either open or closed at the posterior end (fig. 1). One to four specimens were taken from each group and used in the subsequent study (table 1). Due to the absence of morphological keys for most of the Australian native Megatomini, all the specimens were identified to genus level only, using descriptors outlined by Peacock (1993) and Booth *et al.* (1990). Specimens of exotic *T. granarium*, *T. variabile* and *A. verbasci* were identified to species level using the morphological keys of Peacock (1993).

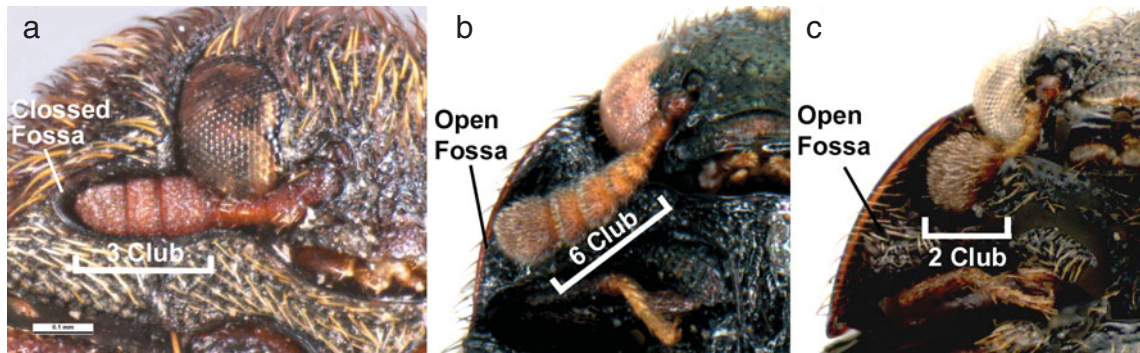


Fig. 1. Photographs of (a) *Anthrenocerus*, (b) *Trogoderma* (sample 107) and (c) *Orphinius* (sample 116), highlighting the open and closed fossa, and antenna clubs. These morphological features are used to differentiate these genera. The *Anthrenocerus* photograph is courtesy of the Pest and Disease Image Library (PaDIL; <http://www.padil.gov.au/>).

DNA extraction

A non-destructive DNA extraction method, ANDE (Castalaneli *et al.*, 2010) (www.andecom.au) was used to extract DNA from all the specimens used in this study. These samples have been stored in the Department of Agriculture and Food's Insect Collection.

Amplification

Amplification of the *COI*, *CYT b* and *18S* genes was performed using primer pairs C1-J-2183 and TL2-N-3014, CB-J-10933 and CB-N-11367 (Simon *et al.*, 1994), four pairs of *18S* primers, and the *T. granarium* primers Tg-J-Cytb and Tg-N-Cytb (table 2). PCR volume was 25 μ l, including 2.5 μ l of ANDE extracted DNA (1:10 dilution DNA:water), which was the equivalent of 20 to 25 ng μ l⁻¹. The reaction mix comprised of 1 \times polymerase buffer (Roche), 1.8 mM MgCl₂, 0.2 μ M of each primer, 200 μ M of each dNTP and 0.5 U of Faststart Hi Fidelity Taq polymerase (Roche). Due to the size of the *18S* fragment (~1850 bp) it was amplified in four sections. The four sections were amplified using primers that were designed using Primer 3 (<http://frodo.wi.mit.edu/primer3/>) to target conserved regions of previously published beetle *18S* sequences (GENBANK accession numbers: AY748111, AY748105, AY748103, EF213875, EF213892, EF362981). Annealing temperatures and extension times varied as shown in table 2. The thermocycler conditions were: 95°C for 10 min; 40 cycles of: 95°C for 30 s, annealing for 30 s and 72°C for extension, with a single final extension period of 72°C for 5 min. Quality and quantity of the amplified PCR products was determined by agarose gel. The concentration of agarose gel was 1.5% w/v and was made up with 1 \times TAE buffer. Electrophoresis of the agarose gel was carried out for 1 h at 60 volts, then stained with ethidium bromide and examined under UV light. PCR products were purified by the addition of 10 U of Exonuclease I (NEB) and 2.5 U of Antarctic Phosphatase (NEB) and incubation for 30 min at 37°C followed by inactivation by heating the reaction to 80°C for 20 min. Purified DNA was stored at -20°C until required.

Sequencing

Sequencing of the amplified genes was carried out by Macrogen Inc (Seoul, Korea) using an Applied Biosystems ABI 3730 48-capillary DNA analyser using Big Dye Terminator

Technology according to the manufacturer's protocols (Applied Biosystems).

Data analyses

All sequences were edited using CodonCode Aligner 3.0.3 (CodonCode Corporation), and the sequences associated with each locus were aligned using the built-in version of MUSCLE (Edgar, 2004). MEGA 4 (Tamura *et al.*, 2007) was used to calculate the Maximum Likelihood (ML) distances between individuals. The model used was Tamura-Nei, as it takes into account different substitution rates between nucleotides and corrects for multiple hits. The ML distance values were plotted against the number of transition and transversion to examine the levels of saturation at each codon position. The phylogenetic signal of each mitochondrial gene and the *18S* were evaluated in PAUP 4.0 (Swofford, 2003) using the Partition Homogeneity Test (PHT). The parameters include hsearch, randomseed=0 and nrep=1000.

To examine the polyphyletic positioning of *Trogoderma*, the sequences from all three genes were concatenated to create an all-gene dataset. The Dermestidae lineage was rooted with *Tribolium castaneum* Herbst, of the series Cucujiformia, for both Parsimony and Bayesian analysis. Bayesian trees were generated using Mr Bayes 3.1.2 (Ronquist & Huelsenbeck, 2003) following the evolution model of GTR+I+ Γ which was determined by jModelTester using both AIC and BIC (Posada, 2008). The mitochondrial protein-coding genes (*COI* and *CYT b*) were divided into six partitions, each partition corresponding to the first, second and third codon positions, and the *18S* was not partitioned. The program was run for gen=40,000,000, sample freq=1000, sump burnin=25,000 and sumt burnin=25,000. PAUP 4.0 was also used to generate Parsimony trees to compare against the Bayesian trees. Due to saturation at the third codon position, several approaches were adopted to generate the best tree. First, for each gene, the first, second and third codon positions were analysed separately; then, each tree was compared with majority rule to determine which position was forcing particular tree topologies. Second, total evidence trees were generated whereby we either weighted the first position at two or weighted the transition and transversions (1:2). The Parsimony trees were calculated under the following conditions: hsearch, addseq=random, nrep=10,000, swap=TBR, MaxTrees=Auto, with the additional parameters nchuck=5 and chuckscore=1 added for the bootstrapping analysis.

Table 1. Specimens used in the phylogenetic study, including collection method, location and genetic loci analysed.

ID	Genus	Species	Origin	Collection sites		Collection	Lat	Long	COI	CYT <i>b</i>	18S
				State	Region						
0	<i>Anthrenus</i>	<i>verbasci</i>	Palearctic	WA	Australia	Sweep	-31.983	115.8619	HQ419076	HQ419112	HQ419149
72	<i>Anthrenocerus</i>		Australia	WA	Australia	Sweep	-33.8386	117.1511		HQ419142	JF808096
73	<i>Anthrenocerus</i>		Australia	WA	Australia	Sweep	-33.8386	117.1511	HQ419106	HQ419143	HQ419172
80	<i>Attagenus</i>		Unknown	WA	Australia	Sweep	-32.108	115.8658	HQ419107		HQ419173
96	<i>Trogoderma</i>		Australia	WA	Australia	Wet trap	-31.983	115.8619	HQ419108	HQ419145	HQ419174
97	<i>Trogoderma</i>		Australia	Qld	Australia	Wet trap	-27.4	153.0667	HQ419109	HQ419146	HQ419175
103*	<i>Trogoderma</i>	<i>unknown</i>	Unknown						HQ419080	HQ419151	HQ419151
105	<i>Trogoderma</i>		Australia		Australia	Wet trap	-41.26	147.5825	HQ419081		JF808095
107	<i>Trogoderma</i>		Australia	ACT	Australia	Wet trap	-35.1627	149.064	HQ419083	HQ419115	HQ419152
116	<i>Orphinus</i>		Australia	Qld	Australia	Wet trap	-27.4	153.0667	HQ419085	HQ419118	HQ419153
124*	<i>Trogoderma</i>	<i>granarium</i>	India		Sri Lanka				HQ419090	HQ419123	HQ419157
132	<i>Trogoderma</i>		Australia	WA	Australia	Sweep	-32.3259	118.9491	HQ419091	HQ419125	HQ419159
134	<i>Trogoderma</i>		Australia	WA	Australia	Sweep	-32.3259	118.9491	HQ419092	HQ419126	HQ419160
137	<i>Trogoderma</i>		Australia	WA	Australia	Sweep	-34.3193	118.7918		HQ419128	HQ419161
138	<i>Trogoderma</i>		Australia	WA	Australia	Sweep	-34.3193	118.7918	HQ419093	HQ419129	HQ419162
142	<i>Trogoderma</i>		Australia	WA	Australia	Sweep	-33.3965	119.9092	HQ419094	HQ419130	HQ419163
157	<i>Trogoderma</i>		Australia	WA	Australia	Wet trap	-34.4069	117.9218	HQ419098	HQ419133	HQ419166
168	<i>Trogoderma</i>		Australia	WA	Australia	Wet trap				HQ419136	HQ419167
170	<i>Trogoderma</i>		Australia	WA	Australia	Wet trap			HQ419100	HQ419137	HQ419168
184*	<i>Trogoderma</i>	<i>variabile</i>	Australia	Tasmania					HQ419103	HQ419139	HQ419169
	<i>Attagenus</i>	<i>polio</i>							DQ221954		AY748110
	<i>Dermestes</i>	<i>laniarius</i>							EF213947		EF213892
	<i>Dermestes</i>	<i>murinus</i>							EF213932		EF213875
	<i>Tribolium</i>	<i>castaneum</i>							DQ222030		AJ312413
	<i>Trinodes</i>	<i>rufescens</i>							EF213946		EF213891

* Note: Quarantine intercepts are made at the border in Australia and exact origins overseas is uncertain.

Table 2. PCR primers used to amplify the mitochondrial and nuclear genes.

Primer no.	Genome	Target gene	Primer name	Primer sequence 5'-3'	Anneal temp. (°C)	Ext. time (s)	Exp. size (bp)	Reference
1	Nuclear	<i>18S</i>	18SF1 18SR1	TGCTTGCTCAAAGATTAAG GTATCGTTATTTTTTCGTCAC	45.1	30	500	This study
2	Nuclear	<i>18S</i>	18SF2 18SR2	TACCACATCCAAGGAAGG CCTCTAACGTCGCAATAC	45.1	30	500	This study
3	Nuclear	<i>18S</i>	18SF3 18SR3	TCTATTTTGTGGTTTTTCG AGAACATCTAAGGGCATC	45.1	30	600	This study
4	Nuclear	<i>18S</i>	18SF4 18SR4	CTTCTAGCCGCACGAGAT CCTTGTTACGACTTTTACTTCC	45.1	30	400	This study
5	mtDNA	<i>COI</i>	C1-J-2183 TL-2 – 3014	GGATCACCTGATATAGCATTYCC CAACATTTATTTTGATTTTTTGG	43.7	30	800	(Simon <i>et al.</i> , 1994)
6	mtDNA	<i>CYT b</i>	CB-J-10933 CB-N-11367	TATGTACTACCATGAGGACAAATATC ATTACACCTCCTAATTTATTAGGAAT	43.7	60	500	(Simon <i>et al.</i> , 1994)
7	mtDNA	<i>CYT b</i> <i>T. granarium</i>	Tg-J-Cytb Tg-N-Cytb	GCTAATGGAGCCTCATTCTTC TGAATTGGCGTAACTAATGGGT	43.7	60	500	This study

Table 3. Loci information including variable sites, pairwise divergence (%) values and transition/transversion ratios.

Gene	Codon position	Conserved	Informative sites	ML distance (Tamura-Nei)
<i>COI</i>	All	366	323	0.12–0.35 (0.26)
	1	157	73	
	2	201	33	
	3	8	217	
<i>CYT b</i>	All	217	188	0.25–0.46 (0.3)
	1	92	43	
	2	120	16	
	3	5	129	
<i>18S</i>		1761–1777	78	0.002–0.038 (0.019)

To estimate time to most recent common ancestor (TMRCA), Bayesian evolutionary analysis by sampling trees (BEAST: Drummond & Rambaut, 2007) was used. This allows for the nodes within the phylogenetic tree to be dated, which may help determine origins. The same concatenated dataset that was used to generate the Parsimony and Bayesian trees was used. The Dermestidae lineage was rooted with *Tribolium castaneum* Herbst, of the series Cucujiformia. The root height parameters were set to the following: prior distribution normal, mean 235 MA (std dev 10 MA). These dates correspond to origins of the series Cucujiformia, with the low boundary incorporating the origins of Bostrichiformia (series of Dermestidae: Hunt *et al.*, 2007). The *Dermestes* and *Trinodius* samples were clustered and the root height parameters were set to the following: prior distribution lognormal mean=2, SD=0.5, and offset lower bounds of 65 MA, which corresponds to the earliest fossil record (Cockerell, 1917). Using the strict clock, both GTR and HKY evolution models were evaluated, $\text{gen} = 50,000,000$ and $\text{log} = 500$. Tree Annotator v1.5.4 was used to generate the consensus tree, using the following parameters: burnin at 10,000, posterior probability 0.95 and mean heights (Drummond & Rambaut, 2007).

Results

Mitochondrial sequence variation

The 743bp of the mitochondrial *COI* was highly polymorphic, with 323 parsimony-informative nucleotide sites

from 23 individuals. The average sequence divergence, calculated using ML distance, was 0.26 and the highest level of variation between Dermestid sequences was 0.35 (table 3). Cytochrome b (430bp) was similarly polymorphic with 188 parsimony-informative sites from 17 individuals. Despite both genes having variation occurring at 43% of their sites, *CYTb* was considerably more variable with a divergence range of 0.25 to 0.46, the highest between two *Trogoderma* species collected in Australia (T96 and T134). The polymorphisms observed, within the mitochondrial genes, resulted in 63 (25%) and 34 (23%) non-synonymous changes in the amino acid sequence. No insertions or deletions were observed in the mitochondrial gene fragments. In addition, no stop codons were observed when the sequences were translated, suggesting that there were no NUMTs sequence within the dataset.

Saturation curves

The number of transitions and transversions for each codon position and mtDNA gene were plotted against ML distances to examine sequence saturation (fig. 2). For both genes, transition saturation was evident at the third position, illustrated by the plateauing of the polynomial curve. No transition saturation was observed at the first and second positions, but rather an upward directional curve. Similarly, transversion saturation was not observed at the first and second positions, only at the third positions.

Analyses of 18S

The *18S* ranged from 1839 to 1855bp in length in which there were 78 parsimony-informative sites (table 3) from 26 individuals. These polymorphisms included 134 single nucleotide polymorphisms and 39 indels. As expected, *18S* showed evidence of much slower evolution than *COI* and *CYT b*, with average sequence divergence of only 0.019, in comparison to 0.26 and 0.3, respectively. Limited variation was observed between the variable domains of the *18S*, with only 21 polymorphic sites, of which five were parsimony-informative. Variation within the seven variable domains ranged from two to 46 polymorphic sites. Three variable domains were highly variable (V2, V4 and V7) containing 40, 46 and 44 polymorphic sites, respectively. Sequence length variation was only observed within the V2, V4, V7 and V9

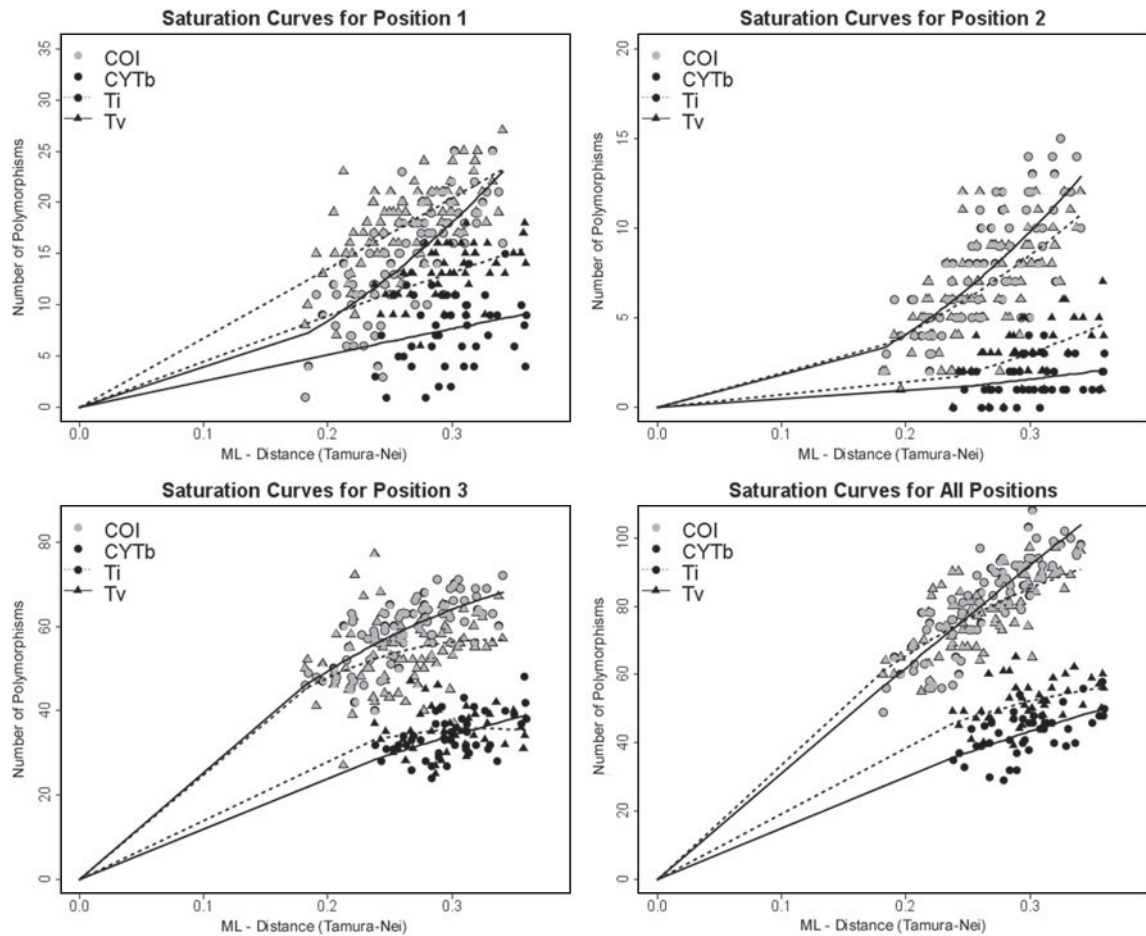


Fig. 2. Saturation Plots for the three codon positions.

domains and was the result of increased sequence length of the *Attagenus* sample and Australian *Trogoderma* sample 96.

Partition homogeneity test

The different number of polymorphic sites observed within all of the variable domains indicated that each domain was under varying evolutionary constraints (Weider et al., 2005). To determine if this may lead to incongruence between the nuclear and mitochondrial genomes, a PHT was performed to compare the variable domains to each other as well as the mitochondrial genes. The PHT revealed that there was no significant incongruence between any of the gene fragments. Therefore, the gene fragments were concatenated and used to generate a total evidence tree.

Bayesian and parsimony trees

The primer pairs did not successfully amplify all specimens; therefore, there are differing numbers of total *COI*, *CYT b* and *18S* sequences available in this dataset. The high levels of variation within the mitochondrial genes suggested possible mismatch in the priming sites as a cause for failure to amplify. Seven pairs of alternative primers were trialled but amplification was refractory. Samples with

missing sequences were included in the analysis, but the sequence data was treated as missing. Maximum likelihood distances (Tamura-Nei) were calculated for the combined *COI* and *CYT b*, and *18S* for the 23 individuals (table 4).

The Bayesian phylogenetic tree identified *Trogoderma* as paraphyletic with respect to *Trinemestes*, *Trinodes*, *Attagenus*, *Anthrenus*, *Orphinus* and *Anthrenocerus* (fig. 3a). The majority of the *Trogoderma* and *Anthrenocerus* species form a well-supported sister group to the *Orphinus* sample (posterior probability 1). The only exception was the Australian *Trogoderma* sample 96 that was sister to the rest of the ingroup. This sample was morphologically re-examined and confirmed to be part of the *Trogoderma* genus. The exotic *Trogoderma* species, originally from the Palaearctic and Indian regions, were strongly supported sister taxa (posterior probability 1.00; fig. 3a). This exotic *Trogoderma* clade was sister to the Australian *Trogoderma* clade and the *Anthrenocerus* samples. The two *Anthrenocerus* samples were nested deep within the Australian *Trogoderma* clade and sister to T170. While the Bayesian analysis was able to resolve all but the deepest internal node, the majority of internal branches within the discordant zone (grey box; fig. 3) were short.

Due to saturation at the third position, each codon position was concatenated with the *18S* and analysed separately using Parsimony (data not shown). All parsimony trees supported the same deep nodes as the Bayesian analysis, but major

Table 4. Maximum Likelihood distances between the 23 individuals that were used to construct the phylogenetic trees; below the diagonal is *COI* and *CYT b* combined, and above the diagonal is the *18S*.

		<i>Dermestes</i>			<i>Attageus</i>			<i>Orphinus</i>	Exotic <i>Trogoderma</i>			Western Australia <i>Trogoderma</i>					Eastern <i>Trogoderma</i>			Anthrenocerus			
		<i>T. castanuem</i>	T96	<i>T. rufescens</i>	<i>D. lanarius</i>	<i>D. murinus</i>	<i>A. unknwon</i>		<i>A. pello</i>	<i>A. verbasci</i>	T103	T184	T124	T142	T134	T138	T132	T170	T157	T97	T105	T107	A72
<i>T. castanuem</i>		0.017	0.028	0.030	0.027	0.032	0.027	0.028	0.032	0.029	0.029	0.029	0.031	0.030	0.030	0.030	0.029	0.029	0.030	0.030	0.030	0.033	0.032
T96	0.31		0.024	0.026	0.021	0.029	0.022	0.029	0.030	0.029	0.028	0.029	0.029	0.028	0.026	0.026	0.027	0.027	0.027	0.027	0.029	0.029	0.030
<i>T. rufescens</i>	0.36	0.34		0.031	0.029	0.034	0.028	0.032	0.038	0.035	0.035	0.035	0.036	0.035	0.036	0.034	0.033	0.035	0.034	0.035	0.035	0.035	0.033
<i>D. lanarius</i>	0.31	0.29	0.30		0.006	0.032	0.027	0.028	0.034	0.033	0.032	0.034	0.032	0.029	0.032	0.031	0.031	0.030	0.032	0.031	0.031	0.034	0.034
<i>D. murinus</i>	0.28	0.29	0.27	0.12		0.029	0.024	0.025	0.030	0.029	0.028	0.030	0.028	0.026	0.029	0.027	0.027	0.028	0.027	0.027	0.030	0.030	0.030
<i>A. Unknown</i>	0.31	0.25	0.31	0.27	0.26		0.011	0.027	0.031	0.029	0.026	0.029	0.030	0.025	0.030	0.027	0.028	0.028	0.027	0.027	0.029	0.029	0.031
<i>A. pello</i>	0.33	0.28	0.35	0.29	0.30	0.21		0.021	0.026	0.026	0.023	0.026	0.027	0.023	0.027	0.023	0.024	0.026	0.024	0.024	0.025	0.026	0.027
<i>A. verbasci</i>	0.32	0.32	0.30	0.30	0.28	0.27	0.29		0.021	0.022	0.022	0.024	0.023	0.023	0.023	0.022	0.023	0.022	0.023	0.024	0.021	0.023	0.023
<i>Orphinus</i>	0.35	0.32	0.34	0.27	0.28	0.24	0.25	0.33		0.009	0.009	0.012	0.015	0.012	0.014	0.009	0.011	0.014	0.012	0.011	0.009	0.012	0.011
T103	0.32	0.34	0.32	0.26	0.28	0.26	0.30	0.32	0.29		0.004	0.004	0.014	0.009	0.013	0.008	0.007	0.012	0.008	0.007	0.009	0.011	0.010
T184	0.31	0.32	0.29	0.26	0.26	0.28	0.29	0.29	0.27	0.24		0.014	0.008	0.013	0.008	0.006	0.012	0.008	0.007	0.008	0.008	0.009	0.009
T124	0.33	0.37	0.34	0.28	0.30	0.30	0.31	0.31	0.28	0.24	0.23		0.013	0.008	0.012	0.005	0.004	0.013	0.005	0.004	0.006	0.009	0.008
T142	0.33	0.35	0.30	0.29	0.31	0.29	0.31	0.35	0.28	0.29	0.26	0.28		0.011	0.001	0.010	0.011	0.004	0.011	0.010	0.011	0.012	0.013
T134	0.37	0.39	0.30	0.28	0.27	0.27	0.32	0.32	0.32	0.29	0.29	0.32	0.28		0.011	0.007	0.007	0.011	0.006	0.008	0.008	0.009	0.012
T138	0.33	0.35	0.33	0.27	0.30	0.27	0.27	0.29	0.28	0.25	0.27	0.27	0.23	0.25		0.009	0.009	0.003	0.009	0.009	0.009	0.011	0.012
T132	0.34	0.33	0.28	0.25	0.24	0.24	0.25	0.30	0.28	0.25	0.25	0.25	0.24	0.26	0.22		0.001	0.011	0.002	0.002	0.002	0.006	0.006
T170	0.31	0.29	0.27	0.27	0.27	0.25	0.22	0.28	0.23	0.26	0.22	0.27	0.27	0.25	0.22	0.23		0.011	0.002	0.002	0.002	0.006	0.006
T157	0.35	0.31	0.31	0.27	0.27	0.26	0.27	0.29	0.24	0.27	0.26	0.28	0.27	0.25	0.24	0.22	0.22		0.011	0.010	0.011	0.012	0.014
T97	0.36	0.31	0.27	0.28	0.24	0.25	0.29	0.30	0.29	0.31	0.27	0.32	0.27	0.29	0.24	0.27	0.24	0.25		0.001	0.004	0.007	0.008
T105	0.31	0.36	0.34	0.28	0.27	0.28	0.32	0.30	0.28	0.28	0.26	0.26	0.26	0.25	0.24	0.23	0.25	0.24	0.22		0.004	0.007	0.008
T107	0.32	0.30	0.30	0.26	0.25	0.26	0.24	0.30	0.26	0.27	0.26	0.31	0.26	0.27	0.23	0.25	0.21	0.27	0.24	0.24		0.007	0.006
A72	0.34	0.37						0.29	0.32	0.30	0.29	0.27	0.30	0.32	0.27	0.26	0.23	0.28	0.26		0.30		0.002
A73	0.33	0.32	0.33	0.25	0.26	0.26	0.28	0.30	0.26	0.29	0.28	0.28	0.27	0.28	0.22	0.24	0.24	0.24	0.26	0.25	0.24	0.15	

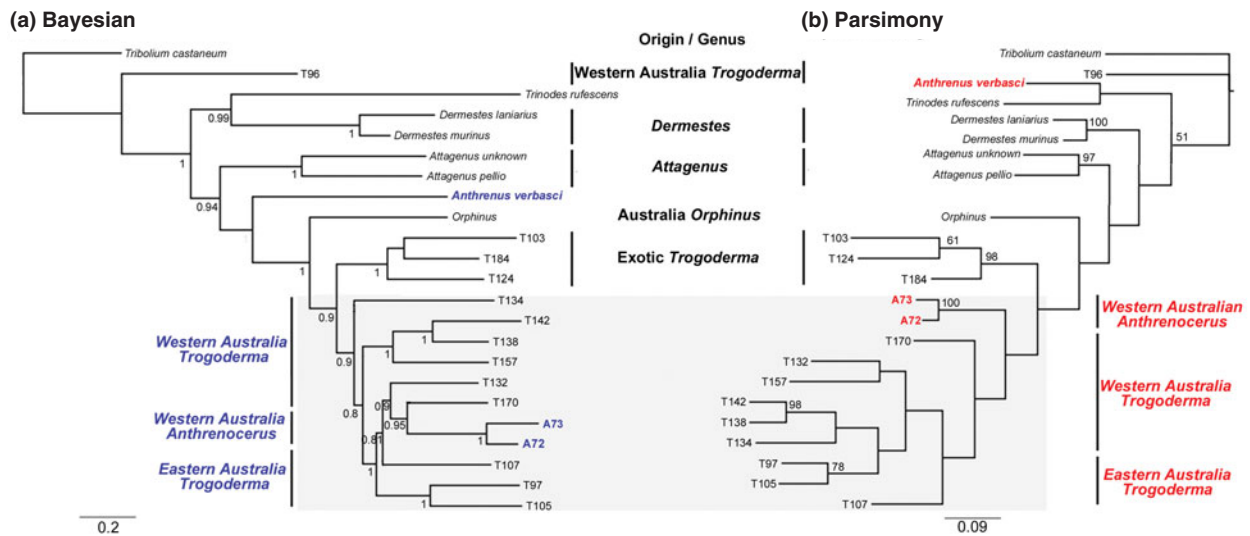


Fig. 3. (a) Bayesian and (b) Parsimony trees based on the combined 18S, COI and CYT *b* datasets. The value below the branch of the Bayesian tree represents the Bayesian posterior probability. The values below the line represent of the Parsimony tree represents bootstrapping values. Nodes with <50% bootstrapping support were not shown on the tree. The outgroup for both trees was *T. castaneum*.

structural change was observed below the *Attagenus* clade and within the discordant zone. The first codon position supported the placement of the exotic *Trogoderma* but positioned *Anthrenocerus* as sister to the Australian *Trogoderma* and clustered *A. verbasci* with *T. rufescens*. The second position nested the exotic *Trogoderma* and *Orphinus* deep within the Australian *Trogoderma* clade. Interestingly, only three polymorphisms between *T. granarium* (T124), T107 and T134 were observed. The placement of *Anthrenocerus* mirrored that of the Bayesian analysis. The third position also placed the exotic *Trogoderma* and *Anthrenocerus* deep within the Australian *Trogoderma* clade. This placement of the exotic *Trogoderma* was rejected as it was neither biologically or biogeographically sensible nor phylogenetically robust.

Each position was reanalysed with the transversions/transitions weighted 2:1. This made minimal difference and the same Parsimony trees were returned. Finally, the first position was weighted twice that of the other positions (2:1). Parsimony trees generated under these parameters generated four equally parsimonious trees, each with a score of 3857 (fig. 3b), which was the closest match to the Bayesian tree (fig. 3a). The main differences were *A. verbasci*, forming a clade with *T. rufescens*, and *Anthrenocerus* clade, which was sister to the Australian *Trogoderma*. Minimal bootstrapping support was generated on each node. Apart from the clades that had bootstrapping support >50%, all samples within the discordant zone shared different common ancestors.

BEAST analysis

Estimates of TMRCA were generated using BEAST (Drummond & Rambaut, 2007). Both GTR and HKY substitution models predicted effective sample sizes (ESS) >200, indicating a moderate degree of support for each node. GTR ESS values were slightly higher and, subsequently, this model was used to estimate TMRCA. The split between the series Bostrichiformia and Cucujiformia was estimated at 237 MA, with the ingroup estimated at 209 MA (fig. 4, table 5). The TMRCA for *Attagenus* was calculated at 194 MA, while the

common ancestor for *A. verbasci* was 181 MA. The mean estimate for the *Orphinus* common ancestor was 157 MA. The proposed common ancestor for exotic *Trogoderma* was 102 MA, while the split from the Australian *Trogoderma* and *Anthrenocerus* was 141 MA. The common ancestors for the Australian *Trogoderma* samples, excluding sample 96, ranged from 128 to 66 MA.

Discussion

Despite discordance between Bayesian and Parsimony approaches, the genus *Trogoderma*, according to this study and excluding *Trogoderma* sample 96, is paraphyletic with respect to *Anthrenocerus*. The current taxonomic nomenclature of the Megatomini tribe, as described by Peacock (1993) and Booth *et al.* (1990), uses two main morphological characteristics to differentiate *Anthrenocerus* and *Trogoderma*. The main morphological characteristics used are the number of antenna segments that form the club and the open or closed fossa. The *Anthrenocerus* genus is identified by the three segmented clubs joined eccentrically. In comparison, *Trogoderma* have three to eight segmented clubs, joined more or less symmetrically along the central axis. However, several species of *Trogoderma*, held in the Department of Agriculture and Food Western Australian insect collection, have well-defined three-segmented clubs that are eccentrically joined. The only definitive difference between these two genera is the closed antennal fossa found in *Anthrenocerus* species. Our study, similar to the morphological study conducted by Kiselyova & McHugh (2006), suggests that *Anthrenocerus* is possibly an Australian *Trogoderma* variant.

The morphological characteristics used to delineate *Trogoderma*, as well as the results presented here, suggest that the body plan of *Trogoderma* has been retained throughout history (parallelism) rather than arising independently (convergent evolution: Wiens *et al.*, 2003). This may give the impression that morphologically similar species are more closely related than they are genetically, a common phenomenon (Bossuyt & Milinkovitch, 2000). The number of

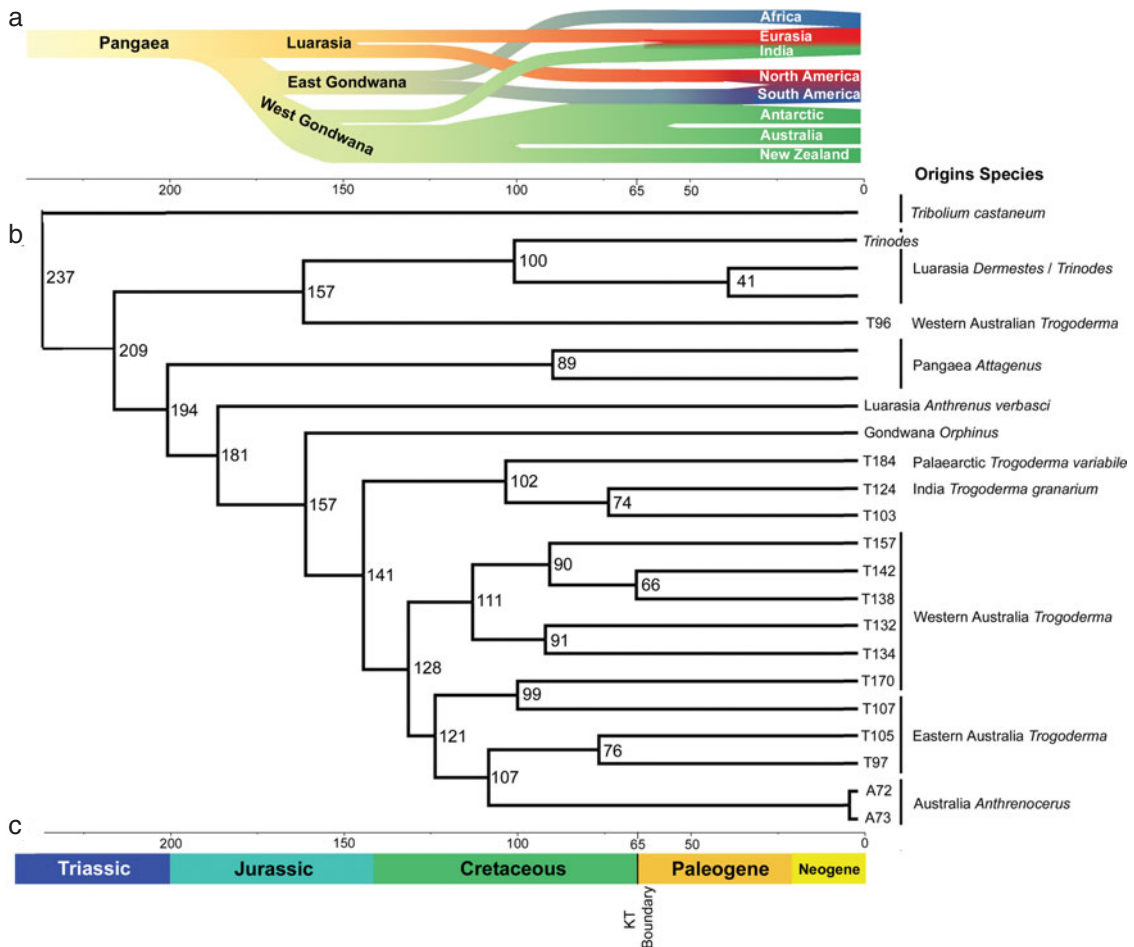


Fig. 4. Phylogenetic history of the Dermestidae genera *Attagenus*, *Anthrenus*, *Anthrenocerus*, *Dermestes*, *Trogoderma* and *Orphinus*. (a) Timeline of the continental breakup and drift from 250 MA to present (based on Li & Powell, 2001). (b) Bayesian tree with estimations of time to most recent common ancestor (MA). Mean time estimates are shown after the node. Scale bars are located above and below the phylogenetic tree and represent millions of years. The outgroup *Tribolium castaneum* is from the superfamily Curcujiformia. (c) The geologic timeline from the Triassic to the present.

morphological characteristics used to delineate between the three *Trogoderma* groups (sample 96, the exotic *Trogoderma* and the Australian native *Trogoderma*; fig. 3b) are minimal. One of the main external differences between the exotic and Australian native *Trogoderma* are the lighter-coloured patterns in the integument of the elytra of the exotic *Trogoderma* and setae colour. Interestingly, morphological comparisons between the Australian native *Trogoderma* and *Trogoderma* sample 96 were negligible, yet the phylogenetic trees grouped these individuals at opposite ends of the tree and a ML distance >0.29. The conservation of morphological characters is not uncommon and has been observed in frogs (Bossuyt & Milinkovitch, 2000; Chiari *et al.*, 2004), bacteria (Degnan *et al.*, 2005) and other insects (Buckley *et al.*, 2009). This suggests parallelism of the *Trogoderma* characteristics and that, even with major morphological taxonomic revision, the paraphyly in *Trogoderma* may not be easily resolved.

A high degree of branch swapping was observed when the Parsimony and Bayesian approaches were compared, despite Bayesian generating trees with high levels of posterior probability support on all nodes. This lack of agreement

Table 5. Time to most recent common ancestor using BEAST.

Genus	Mean (MA)	95% HPD lower	95% HPD upper
Root	237	224	247
<i>Dermestes</i>	209	198	217
<i>Attagenus</i>	194	180	205
<i>Anthrenus</i>	181	168	192
<i>Orphinus</i>	157	146	165
<i>Trogoderma</i> / <i>Anthrenocerus</i>	141	137	159
Australian <i>Trogoderma</i> / <i>Anthrenocerus</i>	128	122	143
Exotic <i>Trogoderma</i>	102	96	119

between Bayesian and Parsimony trees is not uncommon and is a phenomena previously observed (Shoup & Lewis, 2003; Khan *et al.*, 2008). To explain this lack of congruence between the two phylogenetic approaches, three commonly attributed causes were investigated: homoplasy, gene divergence rates and lineage sorting (O’Huigin *et al.*, 2002; Cantarel *et al.*, 2006; Rasmussen & Kellis, 2007; McCormack *et al.*, 2009).

The saturation plots illustrated that transitions and transversion saturation had been reached at the third position, suggesting that homoplasy is present within our dataset. The presence of homoplasy at the third position may make it difficult for Parsimony to generate accurate trees as no nucleotide substitution models are employed and the tree with least number of steps is preferred (Page & Holmes, 1998). Furthermore, at the second position, only three polymorphisms between *T. granarium* (T124), T107 and T134 were observed, making these species look more closely related than they are at other base sites. In this study, both the presence of homoplasy and invariable sites may have contributed to inaccuracies between trees.

The ML distances for *COI* and *CYTb* were considerably high, with the *Trogoderma* distance only 0.21 (between T170 and T107; table 4). Cytochrome b, which has previously been reported as a slower evolving gene (Castresana, 2001), in our study, mutated at a greater rate than *COI*. In comparison, minimal divergence was observed within the 18S. Previous studies examining the effect that divergence has on tree accuracy concluded that accuracy and divergence are proportional (Cantarel *et al.*, 2006; Rasmussen & Kellis, 2007). Rapidly evolving genes can write over the phylogenetic signals, whereas slowly evolving genes may have insufficient informative sites. In our case, either increasing the number of closely related *Trogoderma* species or a gene evolving slower than *COI* and *CYTb* would likely help increase support within the discordant zone.

Alternatively, the polytomy observed in the Parsimony trees and the short internal branch lengths within the Bayesian trees may represent hard polytomy or lineage sorting. Lineage sorting is a process whereby three or more species radiate rapidly such that an ancient polymorphism is retained in all descendants and by chance each species may share a set of alleles at different loci (McCracken & Sorenson, 2005). Therefore, the Parsimony polytomy and short internal nodes from the Bayesian analysis may signify rapid radiation (hard polytomy) and lineage sorting, not in inaccuracies in the phylogenetic models or selected genes.

The interpretation of the biogeographical distributions and TMRCA (BEAST) for *Anthrenus*, *Anthrenocerus*, *Attagenus*, *Dermestes*, *Orphinus* and *Trogoderma* allows postulations about the origins of the Dermestidae family. Current biogeographical distribution of *Attagenus* species includes: the southern Palaearctic (45%), Ethiopian (35%), Oriental (7%) and Nearctic (5%) regions (Mroczkowski, 1968), which were all connected as part of the supercontinent Pangaea (Li & Powell, 2001). For *Dermestes*, the majority of the 73 species are found in the Palaearctic (50%) and Nearctic (26%) (Mroczkowski, 1968). Approximately 175 MA, Pangaea began to break-up and a rift formed between North America and Africa, resulting in the continents Laurasia and Gondwana (Li & Powell, 2001). With a high proportion of *Attagenus* and *Dermestes* found on both Laurasia and Gondwana, the origins must be earlier than this vicariant event. Estimation of date of origin by BEAST placed the *Attagenus* and *Dermestes* samples near the base of the tree (fig. 4), a similar position to that in the morphological study by Kiselyova & McHugh (2006), with a value of 209 MA (± 33 MA). Furthermore, molecular data suggests angiosperms evolved at, or after, 190 MA (Sanderson & Doyle, 2001), which together suggests that the origin of the Dermestidae family is the mid-early Jurassic (>175 MA) on the Pangean supercontinent.

Orphinus appear to have a Gondwanan distribution, with the majority of species found in the Australian, Oriental and Ethiopian regions (Mroczkowski, 1968). Based on biogeographical data, it seems reasonable to suggest that the origins of *Orphinus* are Ethiopian and prior to the west Gondwana (Africa, Arabia and South America) and east Gondwana (Australia, Antarctica, Madagascar and India) split, approximately 160 MA (Li & Powell, 2001). This placement of origin was supported by BEAST, which estimated the common ancestor of *Orphinus* at 157 MA. Future studies will need to include *Orphinus* from multiple biogeographical locations to determine if this group is monophyletic.

The origins of the *Trogoderma* and *Anthrenocerus* clade, excluding sample T96, were estimated at 141 MA. Using this date, the scenario, proposed by Kiselyova & McHugh (2006), that *Trogoderma* followed the dispersal of marsupial mammals (carcasses of which are a likely food source for larvae) from North America to South America and in to Australia around 30 to 65 MA is doubtful. Rather, it is plausible to suggest that *Trogoderma*'s origins are East Gondwanan, whereby they inhabited both the Indian and Australian continents. The exotic *Trogoderma*, inhabited the Indian continent which collided with Asia approximately 55 MA (Klootwijk *et al.*, 1992), allowing this genus to spread east into the Palaearctic. Rather than *Trogoderma* migrating from North America to South America and then to Australia, it is more likely that migration was in the opposite direction. We base this scenario on a number of factors. First, the number of *Trogoderma* species decreases as we move away from Australia. Second, the lack of species present within the Ethiopian region and the date estimated by BEAST suggest that *Trogoderma* were not present prior to the Gondwana split. Third, several nodes within the *Trogoderma* clade are greater than 100 MA. Finally, this scenario provides a link between the Australian and Exotic *Trogoderma*. To further confirm this scenario, North and South American samples are required, and this could be a potential avenue of future work.

The phylogenetic analysis provided an interesting insight into the evolution of the *Trogoderma* genus, as well as several Dermestidae genera. While this study was unable to resolve the polyphyly reported using the current taxonomic nomenclature, this study did highlight three interesting *Trogoderma* groups. The species within these groups need to be further scrutinised to determine if they have unique characteristics which have been overlooked, thus resulting in the clumping of the species. However, it seems possible that *Trogoderma* is a common ancestor to a proportion of the Dermestidae genera, especially given the limited morphological characteristics that differ between *Anthrenocerus*, *Orphinus* and *Trogoderma* and the minimal dietary differences between *Trogoderma* and all other dermestids. This study also showed the importance of a well-sampled dataset, especially if polytomy is caused by a lack of closely related samples, as the addition of new species may alter the topology of the phylogenetic trees and lead to inaccurate assumptions.

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