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Molecular Resources for the Stored Grain *Cryptolestes* Cryptic Pest Species (Coleoptera: Laemophloeidae)

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Abstract

Recent evolutionary genetics and molecular characterisation of *Cryptolestes* (Ganglbauer) stored grain pest beetle species revealed gaps in public DNA databases that resulted in molecular diagnostic inconsistencies in publicly available sequence databases. We report the characterisation of mitochondrial DNA cytochrome oxidase I (mtCOI) genes from specimens intercepted during Australia's border biosecurity inspections, and surveys of public mtCOI gene sequences, for *Cryptolestes* species status re-assessment. We identified and characterised a new putative *Cryptolestes* species (*C.* sp. 'WTT-2016') and demonstrated the close evolutionary relationships between *C. ferrugineus* (Stephens)/*C. pusilloides* (Steel and Howe) and between *C. pusillus* (Schonherr) and the previously identified *C.* sp. 'WTT-2013' cryptic species. Confusion between *C. ferrugineus*, *C. pusiolloides*, *C. pusillus*, *C.* sp. 'WTT-2013', and *C.* sp. 'WTT-2016' highlighted a substantial and persistent taxonomic challenge within *Cryptolestes*, while low *C. spartii* (Curtis)/*C. corticinus* (Reitter) inter-specific genetic distances suggested they were the same species. Assembled and annotated mitochondrial DNA genomes (mitogenomes) of six *Cryptolestes* species identified assembly errors in published mitogenomes of *C. ferrugineus* and *C. turcicus* (Grouvelle) and misidentification of *C. pusillus*. Based on re-evaluation of genetic distances and phylogeny congruence, we proposed a *Cryptolestes* 'operational species-level genetic gap' at approximately 5% to help define *Cryptolestes* species boundaries, thereby contributing to improving agricultural biosecurity preparedness associated with this important stored grain beetle species. Our work also provides an evolutionary framework that will contribute to future understanding of ecological and environmental impact posed by this highly invasive cryptic beetle species complex.

Keywords: pest interceptions; mitochondrial DNA genomes; molecular diagnostics; flat grain beetles; species genetic gap

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1. Introduction

Accurate species identification is a major biosecurity consideration and is critical to protecting a nation's plant biosecurity in export market access, its pest-free status for global export markets, and agricultural practices. Pests of agricultural importance, such as several species of the globally widespread stored grain beetle genus, *Cryptolestes* (the flat grain beetle; FGB), have gained recognition in recent times due to potential resistance to fumigants such as phosphine [1] and difficulties of morphological species identification [2].

This has led to efforts to develop DNA-based species diagnostic methods [3,4], which also included complete mitochondrial DNA genome (mitogenome) characterisation [5–7].

To further complicate species identification via molecular diagnostics, DNA characterisation across a wide range of organisms has revealed the presence of deeply divergent genetic lineages separated by significant nucleotide distances but morphologically indistinguishable (i.e., cryptic species). Cryptic species in pest insect complexes are known [8], where significant genetic differences, life history, and behavioural traits including mating incompatibilities existed [9], knowledge of which is needed to protect both natural environments and ecosystems. While advances in genomics have significantly contributed to cryptic and putative subspecies identification [10], cryptic species have also been identified using partial mitochondrial mtCOI sequences, including in Coleoptera [11,12]. The advantage of applying genomics especially through a whole-genome sequencing (WGS) approach that contrasts from a reduced representation of a whole-genome sequencing approach (e.g., such as via RAD-Seq, GBS, and ddRAD to selectively sequence a small consistent fraction of the genome, i.e., typically CpG-rich sites via use of restriction enzymes) or via a mitochondrial genes/genomes approach is that WGS allows both nuclear and mitochondrial signatures to be analysed and compared more readily to identify introgression/hybridisation and to detect signatures of selective sweeps and incomplete lineage sorting. The WGS approach also facilitates the detection and/or characterisation of endosymbionts, especially of maternally inherited bacteria such as *Wolbachia*, *Rickettsia*, *Cardina*, and *Spiroplasma*, that could be drivers of incomplete lineage sorting and selective sweeping to complicate species confirmation via molecular diagnostics (e.g., [13–15]). The presence of *Wobachia*, *Rickettsia*, and *Spiroplasma* was reported in populations of *C. ferrugineus* from Turkey via the PCR amplification approach [16], although signatures of selective sweeps and incomplete lineage sorting in *Cryptolestes* species have not been reported.

Detection of insect pests during inspections at the border represents a unique opportunity to identify potential pest origins and arrival pathways, especially when cryptic and/or novel species are involved (e.g., [17,18]). Molecular characterisation of intercepted pest species also offers the unique opportunity to identify novel and undesirable genetic traits that could potentially be introduced should quarantine fail. Nine *Cryptolestes* species are important with regard to stored grain commodities [19] and are ranked by pest status as major (*C. ferrugineus*), intermediate (*C. turcicus* and *C. pusillus* being more important than *C. pusilloides* and *C. capensis*), or minor (*C. cornutus*, *C. divaricus*, *C. klapperichi*, *C. ugandae*). Of these, Rees [19] considered *C. ferrugineus* and *C. pusillus* as 'truly cosmopolitan', being found in North and South America, Europe and northern Asia, the Mediterranean basin, Africa, South and Southeast Asia, and Australia and Oceania (see also [20,21]). Identification is difficult owing to their small size (i.e., 1.2–2.3 mm long) and intra-specific and sexual variation. *Cryptolestes* species identification, therefore, is achieved through examination of genitalia characters [22].

Australia's grain, pulses, and oilseed industry was forecasted at approximately AUD 26 billion for the 2024/25 season [23]. Much of these commodities end up in bulk storage and so are impacted by pests such as *Cryptolestes*. Although trails using biological control agents such as *Beauveria bassiana* entomopathogenic fungus have shown promise for *C. ferrugineus* [24], control of the flat grain beetle and other stored product pests has relied heavily on phosphine, which is regarded as highly cost-effective and environmentally friendly due to its minimal impact on atmospheric ozone. Furthermore, commodities are considered generally safe from chemical residues post-fumigation [25]. Protecting the effectiveness of phosphine for the grain export industry is, therefore, a high priority as its loss due to insect resistance would, in turn, translate to significant costs for primary producers. The situation might also result in loss of export opportunities under Australia's

policy for 'nil-tolerance' of live insects in export shipments [26]. Incidences of insect resistance to phosphine are known [27], including in *C. ferrugineus* in Australia [1] and in China [28]. Potential resistance to phosphine/tolerance at a lower level has also been suspected in some *C. pusillus* and *C. pusilloides* populations from Queensland's and New South Wales's grain-producing regions.

While *C. ferrugineus*, *C. pusilloides*, and *C. pusillus* are regarded as cosmopolitan species in Australia, a fourth cryptic species (i.e., *C.* sp. 'WTT-2013') that is morphologically indistinguishable to *C. pusillus* but exhibits limited mtCOI haplotype diversity similar to recent maternal founder events has been detected in Australia's eastern regions [12]. To confirm whether *C.* sp. 'WTT-2013' likely represents a relatively recent introduced species, molecular characterisation of *Cryptolestes* species detection through biosecurity inspections at Australia's border would be a logical starting point. Detection of this cryptic species from intercepted specimens would further indicate its ongoing biosecurity risk status. Identifying the associated commodities in which the cryptic species is detected would also improve future pre-border interception efficacies.

Molecular diagnostics of *Cryptolestes* species [3,4,12] and characterisation of mitogenomes (i.e., *C. pusillus* [5], *C. ferrugineus* [6], and *C. turcicus* [7]), specifically those regularly found in stored grain products, have been reported. The outcomes of these studies clearly demonstrate the significant taxonomic confusion for the *Cryptolestes* genus and the importance of traditional taxonomy via morphological characters as the foundation to molecular diagnostics (e.g., [29,30]). Intra-specific mtCOI nucleotide diversity is typically low and varies between 0 and 2% as reported in diverse species, including *Cryptolestes* (<1%, [12]) and other coleopteran species (e.g., [31]), although diversity at 2.5–3.2% representing a potential cryptic species complex has also been reported [11]. This contrasts with studies that report infraspecific nucleotide difference of up to 7–9% for the presumed 'widely divergent' lineages of *C. ferrugineus* [32] and *C. pusillus* [3,33] but as low as 1–2% at the inter-specific level (e.g., *C. pusillus* [5] vs. *C. ferrugineus* [6]).

In this study, *Cryptolestes* species genetic diversity was detected during biosecurity inspections at Australia's border via the mtCOI DNA marker, chosen specifically for operational biosecurity diagnostic reasons, and comparison between mitogenomes of six *Cryptolestes* species is reported. Potential issues in published *Cryptolestes* mitogenomes are considered, and the possibility of significant discordance of DNA-based *Cryptolestes* species identification findings published to date are discussed to highlight the need to re-evaluate the current *Cryptolestes* species status.

2. Materials and Methods

2.1. Cryptolestes Sample Origins and Species Identification via Morphological Characters

Cryptolestes beetle specimens detected during biosecurity inspections at Australia's border were stored in >90% ethanol and were provided by the Australian Government Department of Agriculture, Fisheries and Forestry (DAFF) in individual specimen vials (Table 1). To increase confidence in species identification, including the cryptic species status of *C.* sp. 'WTT-2013', the male genitalia characters of reference culture material from *C. ferrugineus* (CSIRO), *C. pusillus* (DAFFQ), *C. pusilloides* (DAFFQ), *C. turcicus* (Julius Kuehn-Institute for Ecological Chemistry, Plant Analytics and Stored Product Protection, Berlin, Germany), and *C.* sp. 'WTT-2013' individuals (derived from field-collected material from Wilgaroi, New South Wales, and from Iandale, Queensland, Australia) were examined prior to being used to generate reference partial mtCOI sequences [12]. We note that the male genitalia character of *C.* sp. 'WTT-2013' was examined by the late Dr Michael Thomas [12]. The reproductive organs of *C. capensis* were examined by Z.H. Li (Department

of Entomology, College of Agriculture and Biotechnology, China Agricultural University, Beijing, China) as detailed [4].

Table 1. *Cryptolestes* beetle specimens intercepted at Australia's international ports by the Department of Agriculture, Fisheries and Forestry (DAFF) (previously Department of Agriculture, Water and the Environment (DAWE)/Department of Agriculture and Water Resources (DAWR)) biosecurity inspectors. Sample codes with details of the origin of the infested commodity, commodity in which insects were detected, date of detection, and putative species as identified by the Operational Science and Surveillance entomologists are provided. The confirmed species identity by DNA analysis is also provided. The number of individuals successfully sequenced (N_{SEQ}) and the number of beetles intercepted (N_T) from goods originating from the recorded countries are indicated.

	Sample Code	Country	Commodity	Date	mtCOI ID	Nseq (NT)
1	PBCRC-12	Nigeria	Sorghum	05 October 2013	*C. ferrugineus*	1 (2)
2	PBCRC-13	Thailand	Rice	10 January 2014	*C. pusilloides*	1 (1)
3	PBCRC-23 PBCRC-15 PBCRC-26	Unknown	Pot pourri	21 January 2014	*C. pusillus*	2 (7)
4	PBCRC-01 PBCRC-02 PBCRC-28	China	Dried mushrooms	23 November 2013	*C.* sp. WTT-2016	5 (46)
5	PBCRC-14	China	Dried mushrooms	21 September 2013	*C. pusilloides*	2 (4)
6	PBCRC-18 PBCRC-19	China	Reed fencing	03 October 2013	*C. turcicus*	2 (3)
7	PBCRC-17 PBCRC-22 PBCRC-06	Vietnam	Personal effects	05 December 2013	*C.* sp. WTT-2016	3 (3)
8	PBCRC-29 PBCRC-11	Solomon Is.	Palm kernel	18 October 2013	*C. ferrugineus*	2 (2)
9	PBCRC-10 PBCRC-25	USA	Peanuts	05 February 2014	*C. pusillus*	2 (4)
10	PBCRC-16 PBCRC-03	Malaysia	Personal effects	25 February 2014	*C.* sp. WTT-2016	3 (4)
11	PBCRC-07	China	Unidentified grain/seed	04 February 2014	*C.pusilloides*	2 (4)
12	PBCRC-08 PBCRC-09	China	Rhizomes	26 November 2013	*C.* sp. WTT-2013	4 (7)
13	PBCRC-05	Colombia	Green coffee beans	11 December 2013	*C.* sp. WTT-2016	1 (1)
14	PBCRC-04	Malaysia	Dried mushrooms	26 February 2014	*C.* sp. WTT-2016	1 (4)

Note: Specific items from personal effects included rice, peanuts, and dried mushrooms. 'Failed' indicates no PCR and/or sequence data were obtained. A total of 92 specimens were detected, of which Sanger sequencing were attempted on 56 randomly selected specimens (31 successful (N_{seq}); 25 failed (Table S1)). PBCRC-08 and PBCRC-09 were seized goods in mail (imported via post/parcel) to the Sydney Gateway Facility; incidents note China as the country of origin. Intercepted specimens were not morphologically characterised but instead built on DNA sequences of some morphologically confirmed species.

2.2. DNA Extraction, PCR and Sequencing

Genomic DNA (gDNA) from all *Cryptolestes* individuals intercepted was extracted individually using the Qiagen Blood and Tissue DNA extraction kit and followed the methods of Tay et al. [12] for PCR and Sanger sequencing using the described primers (CFCOI-F/-R) or the alternative PCR primer pairs Cryp_Barcode_F01 (GTTCATGAGCTG-GAATAGCAGGAAC) and Cryp_Barcode_R01 (TAARCCAATYGCTATTATWGCATAA) that amplified 779 base pairs (bp) of the mtCOI molecular diagnostics gene region. The programs Pre-Gap4 and Gap4 [34,35] were used to assemble sequence trace files. All sequences generated were assessed for premature stop codons and insertions/deletions (INDELs) to ensure no nuclear mitochondrial sequences (nuclear mitochondrial pseudogenes (Numts) had been included in analyses.

2.3. *Cryptolestes* Mitogenomes via HTS, Assembly and Annotation

The total gDNA from three *Cryptolestes* species (*C. ferrugineus*, $n = 1$; *C. pusillus*, $n = 1$; *C. pusilloides*, $n = 2$) from Australia, one *C.* sp. 'WTT-2013' (based on partial mtCOI molecular diagnostics [12]) found in goods from China, two unknown *Cryptolestes* species (temporarily named *C.* sp. 'WTT-2016') found in goods from Vietnam and Malaysia, and one species *C. turcicus* found in goods from China (Table S2) were used for constructing species-specific Illumina high throughput sequencing (HTS) genomic libraries following the protocol supplied by the manufacturer. The two randomly selected *C. pusilloides* (T6-01, N4-02) and two randomly selected *C.* sp. 'WTT-2016' species were chosen to facilitate nucleotide divergence estimates for intra-specific comparison at the mitogenome level. Assembled mitogenomes were annotated using 'MITOS' [36] and specifying the invertebrate mtDNA genetic code (5), followed by fine-scale identification of potential start/stop codons for individual protein coding genes (PCGs) within Geneious v11.1.5 (Biomatters Ltd., Auckland, New Zealand).

2.4. Comparisons of mtCOI Barcoding Gene Regions of Published *Cryptolestes* Species

To identify *Cryptolestes* species detected from biosecurity inspections at Australia's border, all partial mtCOI sequences generated were compared to the GenBank *Cryptolestes* mtCOI sequences. FaBox [37] was used to identify unique/shared mtCOI haplotypes. The corresponding mtCOI region from complete mitogenomes of *C. ferrugineus* (KT182067 [6], *C. turcicus* (KT070712 [7]), and *C. pusillus* (KT070713 [5]) was not included in the analysis due to issues within these mitogenomes (discussed below). All sequences used were trimmed to 613 bp. Gaps at the terminal regions were filled with 'N' post-trimming, prior to all molecular evolutionary analyses. Nucleotide distance estimates were used in species status delimitation with specific consideration for non-standard nucleotide divergence values across diverse organisms [38] and that large mtCOI intraspecific nucleotide divergence estimates could be associated with cryptic species [39]. For the identification of putative *C. ferrugineus*, *C. pusillus*, *C. pusilloides*, *C. turcicus*, the potential cryptic *C.* sp. 'WTT-2013', and for the evaluation of *Cryptolestes* species groups from the GenBank DNA database, the representative sequences KF241725, KF241734, KF241739, KF241723, and KF241724 were used.

2.5. Intra- and Inter-Specific Nucleotide Diversity Estimates

Available GenBank sequences (three complete mtCOI gene sequences and 133 partial mtCOI gene sequences, accessed 7 January 2021) were first identified by Blastn sequence homology search using KF241725 as input query sequence. All GenBank downloaded sequences and those from intercepted specimens were aligned using MAFFT v7.017 [40] default parameter settings (i.e., Scoring matrix: 200 PAM/k = 2; Gap open penalty: 1.53; and Offset value: 0.123) and trimmed to 613 bp (and excluding significantly shorter sequences). Intra- and inter-species nucleotide divergence estimates were calculated using MEGA7 [41] with standard error (s.e.) estimates from 500 bootstrap replications. Nucleotide substitution rates and gamma-shape parameters were also performed within MEGA7, and these parameters were used as input parameters to obtain nucleotide distance estimates using either the Kimura 2-parameter (K2P, [42]) nucleotide substitution model or the uncorrected pairwise nucleotide distances (*p*-dist) approach. We included estimates of nucleotide distances using the K2P model because this has been the most common (i.e., de facto) nucleotide substitution model used by the majority of DNA barcode and molecular diversity studies, including for *Cryptolestes* beetles (e.g., [32,33]), despite the uncorrected distance estimate model (i.e., *p*-dist) being the more appropriate approach [43,44]. The s.e. estimates representing the variables were used to obtain the respective 95% confidence intervals (C.I.; calculated

as 1.96 × s.e.) and for plotting on the *y*-axis against the estimated mean genetic distances (i.e., non-variables, *x*-axis) for visualisation of differences within and between species groups, and to allow the potential genetic gap to be identified to support species delimitation [39].

2.6. Testing of DNA Barcode Gaps

To estimate the DNA barcode gaps and to obtain statistical rigour especially for the candidate cryptic species, the automated barcode gap discovery program ABGD [45] was implemented with default settings for divergence estimates (Pmin: 0.001, Pmax: 0.1; Steps: 10; X: 1.3) and selecting the simple distance substitution model with Kimura Ts = 2.0 (default value). We also applied the Generalised Mixed Yule Coalescent (GMYC) approach [46] to estimate confidence of delimiting between putative *Cryptolestes* species using a maximum likelihood (ML) phylogeny first obtained via IQ-Tree (described below). For the GMYC approach to analyse the partial COI sequences (i.e., single locus dataset), assignment uncertainty used the Akaike information criterion (AIC)-based approach, and the datasets were analysed without outgroups to limit bias in estimates. We also selected the single-threshold (sGMYC) option to limit over-splitting of species as the recommended standard approach assuming a relatively constant evolutionary rate across the phylogeny. The GMYC analysis was carried out from the GMYC web server https://species.h-its.org/gmyc/ (accessed on 19 January 2026).

2.7. Mitogenome Protein Coding Gene (PCG) Nucleotide Divergences in Six Cryptolestes Species

To estimate nucleotide divergences between economically significant FGB species, the putative cryptic *C.* sp. 'WTT-2013' and novel *C.* sp. 'WTT-2016', all eight mitogenomes were aligned with an outgroup Cucujidae species (MK614530) using MAFFT v7.017. All 13 PCG sequences were extracted for re-alignment and end-trimming. A 71 bp region within the NAD6 gene in all individuals was removed due to the missing region in *C.* sp. 'WTT-2013' (i.e., NAD6 nt8715–8785; [47]). Aligned concatenated PCG sequences (11,024 bp) were used to calculate the *p*-dist prior to construction of a mitogenome concatenated PCG phylogeny. We also calculated the *p*-dist for the complete mtCOI gene for comparison with the concatenated PCGs *p*-dist.

2.8. Phylogenetic Analysis

2.8.1. Partial mtCOI Phylogeny

For the inference of phylogenetic relationships, a partial mtCOI maximum likelihood (ML) phylogeny on the N-terminal (i.e., 'DNA barcode') region used in the intra- and inter-specific nucleotide diversity analyses was constructed using web-based IQ-tree version 2.1 [48]. The 'automatic evolutionary rates' option via ModelFinder was selected to estimate optimal nucleotide substitution rates and evolutionary model and specifying 1000 ultra-fast bootstrap replications to estimate branch node support. Published Cucujidae beetle species' mtCOI sequences used as out groups included (i) *Cucujus clavipes* GU176341, (ii) *C. haematodes* KM439405, KM441995, (iii) *C. cinnaberinus* KM447566, (iv) *Pediacus depressus* KM441566, and (v) *Pediacus fuscus* KJ964320 and two unpublished GenBank public records, (vi) *Cucujus clavipes* KM847989 and (vii) *Pediacus fuscus* KJ203564. The phylogenetic tree was visualised via FigTree v1.4.0.

2.8.2. Concatenated Mitochondrial Protein Coding Genes Phylogeny

The ML phylogenetic relationships of the detected *C. ferrugineus*, *C. pusilloides*, *C. turcicus*, *C. pusillus*, *C.* sp. 'WTT-2013', and *C.* sp. 'WTT-2016' were inferred using 11,024 bp of trimmed and aligned concatenated sequences from the 13 mitochondrial DNA PCGs via IQ-tree as detailed and included the PCGs from the Phalacridae gen. sp. Cucujidae

beetle (MK614530 [49]) as the outgroup. The PCG partition was specified and used as an input file to enable optimal evolutionary models and substitution rates to be determined for individual PCGs within IQ-tree. The phylogeny was visualised by Dendroscope 3.

2.8.3. Analysis of Published Cryptolestes Mitogenomes

Published mitogenomes of *C. ferrugineus* (KT182067), *C. turcicus* (KT070712), and *C. pusillus* (KT070713) were compared against assembled mitogenomes of Tay et al. [47] through MAFFT alignment to identify potential contig assembly errors and to confirm species status. For potential problematic gene regions in the published mitogenomes, uncorrected *p*-dist estimates were provided against assembled mitogenomes from this study to identify likely contamination sources.

3. Results

3.1. Cryptolestes Species from Biosecurity Inspections

Between September 2013 and January 2015, a total of 92 interceptions of *Cryptolestes* beetles from contaminated plant commodities (e.g., cashews, peanuts, sorghum, green coffee beans), toys, building materials (e.g., Reed fencing), and plant parts (e.g., rhizomes) were detected during biosecurity inspections at Australia's international ports. The numbers of beetle adults and larvae intercepted in each item ranged from 1 to 46 individuals and involved diverse geographic origins (Tables 1, S1 and S2).

Of the six *Cryptolestes* species detected and identified by molecular diagnostics, four (*C. ferrugineus*, *C. pusilloides*, *C. pusillus*, *C.* sp. 'WTT-2013'; [12]) were known to be present in Australia, and two were not known to be present in Australia (i.e., *C. turcicus* and an unknown *C.* sp. 'WTT-2016'). The *C.* sp. 'WTT-2016' specimens were detected in goods that originated from China, Vietnam, Malaysia, and Colombia and were the most frequently detected species identified by molecular diagnostics. The *C.* sp. 'WTT-2016' has been identified as *C. pusilloides* [4,50] but differed significantly from the partial mtCOI sequence of a specimen morphologically identified as *C. pusilloides* [12].

3.2. Molecular Characterisation of Mitogenomes of Six Cryptolestes Species

Eight mitogenomes that belonged to six *Cryptolestes* species (1× *C. ferrugineus*; 2× *C. pusilloides*; 1× *C. pusillus*; 1× *C. turcicus*, 2× *C.* sp. 'WTT-2016'; 1× *C.* sp. 'WTT-2013') were assembled and annotated. The six *Cryptolestes* beetle species' draft mitogenomes ranged from approximately 15,186 bp to 15,341 bp, were high in A-T nucleotides (Table S2), and had 13 PCGs, 22 tRNAs, and 2 rRNA genes, similar to mitogenomes of other insects, including Coleoptera. The mitogenome gene orientations for all six *Cryptolestes* species were identical to other reported Cucujidae species.

3.3. Detection of the 'Unknown' Cryptic C. sp. WTT-2013

Four of the seven *Cryptolestes* beetles intercepted from rhizomes (PBCRC-08, PBCRC-09; Table 1 [47]) were successfully identified by molecular diagnostics, having shared high sequence homologies (99%) with the two mtCOI partial gene sequences (KF241724, KJ502178) of the putative *C.* sp. 'WTT-2013' detected in Queensland and New South Wales [12]. Furthermore, there existed 11 other partial/complete mtCOI sequences in GenBank that also shared high sequence homologies with KF241724, KJ502178 [12] and with PBCRC-08, PBCRC-09 [47]. Of the total 15 sequences, all but 2 (KF241724, KJ502178) were from China and have been assigned as *C.* sp. 'WTT-2013' (KF241724, KJ502178), *C. ferrugineus* (KT182067), *C. turcicus* (KT070712), or *C. pusillus* (KT070713, JQ708206, KC977922) (Table S2).

3.4. Detection of the Unknown Putative C. sp. 'WTT-2016'

A total of 11 intercepted *Cryptolestes* specimens on goods from Malaysia, Colombia, Vietnam, and China (Table 1) shared high sequence homologies but were sufficiently divergent from other *Cryptolestes* species and were all assigned with a putative species status (i.e., *C.* sp. 'WTT-2016'). The challenge of ascertaining the identity of *C.* sp. 'WTT-2016' is further compounded by sequences from China (KC436315, KC977916) and Germany (KM450594) being reported as *C. pusilloides* (Figure S1). A survey of literature and from this study showed that *C.* sp. 'WTT-2016' likely represents a widespread species present in South America, Asia, and Europe that has not yet been DNA-barcoded.

3.5. Cryptolestes Species Status Assessment of GenBank Sequences

Assessment of the mtCOI N-terminal (i.e., 'DNA barcode') region sequences representing the 10 *Cryptolestes* species and including all unique mtCOI haplotypes from this study resulted in 129 sequences being analysed. We identified approximately 30% (i.e., 29/129) potentially problematic and two unclassified sequences being assigned as *C. ferrugineus* (n = 15), *C. pusillus* (n = 9), *C. pusilloides* (n = 3), or *C. turcicus* (n = 2) (Figure S1). Furthermore, seven sequences were associated with either *C. corticinus* or *C. spartii*. These two species showed low nucleotide distances (average: 3.5% p-dist, range 0 to 4.57%; Table S3). Phylogenetic analyses (see below) were used to further support species status assessment.

3.6. Phylogenetic Analysis

Based on partial (613 bp) mtCOI sequences, all *Cryptolestes* species groups, including the putative *C.* sp. 'WTT-2013' and *C.* sp. 'WTT-2016', were assigned to 10 phylogenetic clades (clades A to J) representing individual species groups with high branch node confidence (80% to 100%; Figure 1). GMYC phylogenetic analysis showed high statistical support values in general for the clades identified, supporting speciation versus populations based on the single-locus (i.e., mtCOI) datasets. Samples clustered as species clusters were largely distinct due to these high node values (e.g., 98.1/100 (*C. pusilloides*); 99.9/100 (*C. corticinus*/*C. spartii*); 100/100 (*C. duplicatus*); 99.8/100 (*C. capensis*); 99.9/100 (*C. turcicus*); 99.8/100 (*C. pusillus* vs. *C.* sp. WTT-2013); 99.9/100 (*C.* sp. WTT-2016)). The node value (89/95) separating clade E and clade F (i.e., *C. ferrugineus*–A and *C. ferrugineus*–B, respectively) also supported a high confidence level with indication of potential effects from high effective population sizes or recent divergences. Individuals within these two clades could therefore represent signatures of incipient speciation events leading to high effective population size.

The major species (i.e., *C. ferrugineus*, *C. pusilloides*, *C. pusillus*, *C. capensis*, *C. turcicus*; [19]) are paraphyletic to each other. The *C.* sp. 'WTT-2016' was basal to other *Cryptolestes* species, and importantly, the cryptic *C.* sp. 'WTT-2013' retained its monophyletic position with *C. pusillus* [12]. Low bootstrap estimates for the phylogenetic placements of individual clades with respect to each other do not impact the proposed species status but rather reflect the need for more comprehensive species diversity surveys and for greater nucleotide data (i.e., from both nuclear and mitochondrial genomes) to better resolve evolutionary relationships between clades. The 10 clades identified are detailed below.

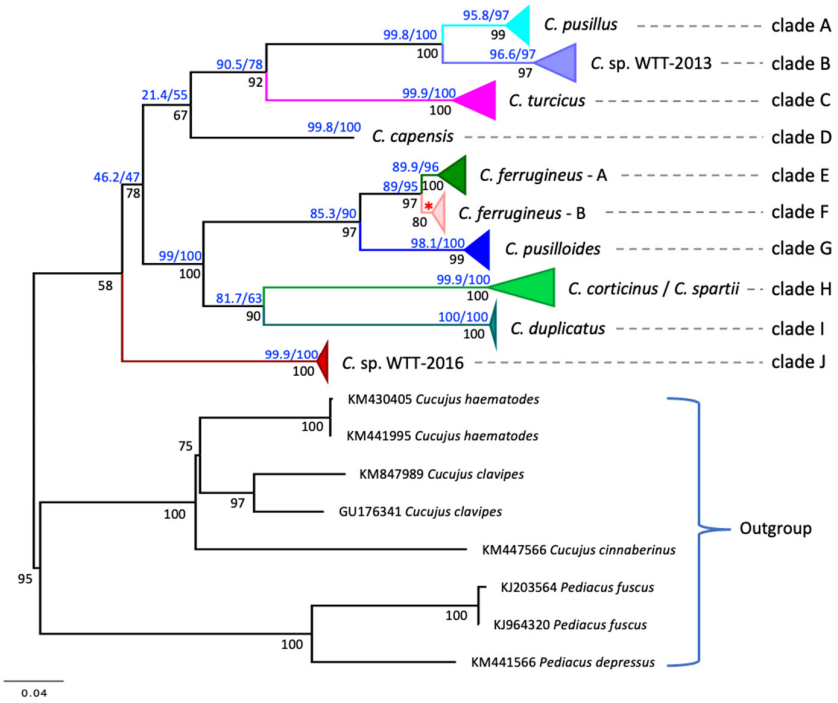

Figure 1. *Cryptolestes* phylogeny based on mtCOI partial gene sequences from GenBank and this study. Refer to Figure S1 for GenBank accession numbers and to Tay et al. [47] for sequences used. Note: Best-fit substitution model (determined using ModelFinder): TIM2 + F + I + G4; ML consensus tree log-likelihood score: −5403.4655; Bayesian information criterion score (BIC): 12,394.8391; Akaike information criterion score (AIC): 11,338.8499. Maximum likelihood bootstrap support values (in black) are provided below branch nodes, General Mixed Yule-Coalescent (GMYC) support values (in blue) are provided above branch nodes of *Cryptolestes* species. Individuals clustered into each clade are collapsed and represented as coloured triangles. **Note:** The General Mixed Yule-Coalescent phylogenetic analysis split *C. ferrugineus* individuals (JX090160, KF241728) in clade F by placing these two individuals as a basal group to clades E and F (GMYC values: 78.1/92) within the *C. ferrugineus* species. * GMYC values with reduced individuals (n = 5) for clade F branch node: 88.1/95.

3.6.1. Clade A: *Cryptolestes pusillus*

All eight *C. pusillus* haplotypes (i.e., KF241734–KF241738, KJ502175–KJ502177; [12]) and four *C. pusillus* haplotypes from GenBank (KC977917, KC977930, JQ708205, KM449111) clustered with high bootstrap support (91%). Four haplotypes (PBCRC-10, -23, -24, -25) from this study also clustered as *C. pusillus*. However, nine GenBank sequences identified as *C. pusillus* were not clustered in this clade. These included a complete mitogenome (KT070713), and eight partial mtCOI sequences (KC977918–KC977923, JQ708206, KC977931) that shared low (91–92%) sequence identity with KF241734 from Tay et al. [12].

3.6.2. Clade B: *Cryptolestes* sp. 'WTT-2013'

The *Cryptolestes* species' identity in this clade was highly discordant in the public DNA databases and has been identified as *C. ferrugineus*, *C. turcicus*, and *C. pusillus* (Figure S1). This included reports of highly divergent intra-specific nucleotide sequences (i.e., large range of *p*-dist values of 0–8.9%). Morphological (i.e., male genitalia) character assessment by Dr Michael Thomas [12] and molecular characterisation of the partial mtCOI gene (i.e., KJ502178, KF241724) have confirmed the cryptic species nature of *C.* sp. 'WTT-2013' from *C. pusillus* [12].

3.6.3. Clade C: *Cryptolestes turcicus*

Of the published (KF241723, KC977933, JQ708207, KC977913, KT070712) and unpublished (PBCRC-18, -19; China samples from within reed fencing [47]) *C. turcicus* partial mtCOI sequences analysed, only the mtCOI gene from the full mitogenome [7] did not cluster in Clade C.

3.6.4. Clade D: *Cryptolestes capensis*

Two partial mtCOI sequences of *C. capensis* [3,4] were available from GenBank. Reproductive organs were used for species identification; however, prepared slides of the genitalia characters were not kept (Z. Li, pers. comm.) and therefore could not be independently verified. The species identity of *C. capensis* is tentatively assumed to be accurate.

3.6.5. Clades E &F: *Cryptolestes ferrugineus*

Two *C. ferrugineus* sister clades (E and F) were identified and shared high sequence homologies (average *p*-dist: 2.8%; range: 1.47–3.59%; Table S3). The maximum *p*-dist between clades E and F was less than between closely related species (e.g., *C. pusillus*/*C.* sp. WTT-2013; *C. ferrugineus*/*C. pusilloides*; Figures 1 and S1). Sequences from the *C. ferrugineus* sister clades, (i.e., *C. ferrugineus*-A (17 sequences), *C. ferrugineus*-B (7 sequences)) also clustered together with a high (97%) node confidence value but excluded 16 published and unpublished '*C. ferrugineus*' sequences that shared lower (91–92%) sequence homologies with the *C. ferrugineus* Cfer-01 haplotype (KF241725).

3.6.6. Clade G: *Cryptolestes pusilloides*

In Clade G, 12 sequences reported in Tay et al. [12] and from this work (PBCRC-07, -13, -14, -27, Cpld-N4-02, Cpld-T6-01; [32]) and sequences identified as *C. ferrugineus* (JQ708204, KC977927, KC977924, KM441850, KM447067, KJ961815, KX528594, KX528597, KX528588, KX528595, and MG458967 (GenBank Unpublished)) were confidently clustered together (Figures 1 and S1). Note that two sequences (KJ961815; KM450594) used by Pentinsaari et al. [33] and Hendrich et al. [50] were voucher specimens.

3.6.7. Clade H: *Cryptolestes spartii*/*C. corticinus*

Two partial mtCOI sequences for *C. spartii* (KM450873, KM452565) and five partial mtCOI sequences for *C. corticinus* (KM447571, KJ963948, KU918377, KU912013, KJ963948) existed in GenBank. All specimens belonged to museum voucher samples [33,50]. Due to the lack of additional sequences for intra-species comparison for each of these two species, it was not possible to ascertain species status accuracy and validity. The *p*-dist estimates between these two species also approximated those observed for known inter-specific nucleotide distances (Table S3).

3.6.8. Clade I: *Cryptolestes duplicatus*

There were eight sequences from museum voucher *C. duplicatus* specimens (Germany: KM451562, KU919513, KU909614, KU908839, KU912488, KU917587; Belgium: KM440172; Sweden: KJ966304) [33,50]. All *C. duplicatus* sequences were clustered confidently as a clade.

3.6.9. Clade J: *Cryptolestes* sp. 'WTT-2016' (Putative)

The species name of this group of *Cryptolestes* beetles is not known. Of the 14 sequences used in this study, 11 were from beetles collected during biosecurity inspections and involved commodities from diverse origins. Three other partial mtCOI sequences were identified as *C. pusilloides* (from China: KC977916, KC436315; from Germany: KM450594).

Cryptolestes sp. 'WTT-2016' has therefore been recorded directly, or in goods originating, from Asia (China, Vietnam, Malaysia), Europe (Germany), and South America (Colombia).

3.7. Mitogenome Phylogeny of Cryptolestes Species

The phylogeny of the six *Cryptolestes* species based on the mitochondrial PCGs (Figure 2) was in overall agreement with the partial mtCOI gene phylogeny (i.e., *C. pusilloides*/*C. ferrugineus*, and *C. pusillus*/*C.* sp. 'WTT-2013' as closely related sister species). The *C. turcicus* species was basal to the clade that included both *C. pusillus* and *C.* sp. 'WTT-2013', while *C.* sp. 'WTT-2016' was basal to the *C. turcicus*/*C. pusillus*/*C.* sp. 'WTT-2013' clade, and branch node confidence values were also high (90–100%). A major difference between the mitogenome and the partial mtCOI phylogenies was that based on the concatenated PCG sequences, the *C. ferrugineus*/*C. pusilloides* clade was shown to be the most ancestral species clade, whereas in the partial mtCOI phylogeny which included more species, the novel *C.* sp. 'WTT-2016' had the most ancestral placement.

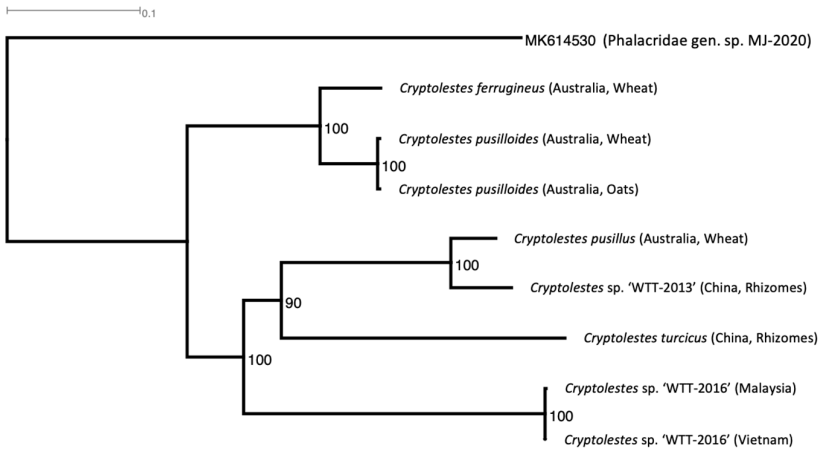

Figure 2. Phylogeny of six *Cryptolestes* species as inferred from concatenation of the 13 mitochondrial protein coding genes, with the published Cucujidae mitogenome MK614530 (Phalacridae gen. sp. MJ-2020) as an outgroup. Note: Best-fit substitution model (determined using ModelFinder): HKY + F + G4; ML consensus tree log-likelihood score: −41,276.4928 (s.e. 315.9798); BIC: 83,632.6985; AIC: 82,784.9902.

3.8. Nucleotide Distances and ABGD Analysis

Intra-specific nucleotide distances estimated for each of the nine *Cryptolestes* species in this study were typically low within each species (e.g., *C. duplicatus*: ranged from 0.0 to 0.0049: *C. corticinus*/*C. spartii*: 0.0 to 0.0457; Table S3). Plotting the average between species nucleotide distance estimates based on either the *p*-dist or the K2P evolutionary models showed the K2P estimates to be typically higher than the *p*-dist estimates, although at the intra-specific level, both estimates were similar (Figure 3). A clear nucleotide distance 'gap' was consistently evident across analyses of intra- and inter-specific comparisons at approximately 5–6%. The two *C. ferrugineus* sister clades (i.e., clades E and F; Figure 1) were assigned as a single species based on partial mtCOI nucleotide distance estimates. Inter-specific nucleotide distances of *C. ferrugineus*/*C. pusilloides* and *C. pusillus*/*C.* sp. 'WTT-2013' were approximately 7.67% based on the *p*-dist approach (10.8–12.4% by the K2P model) and further supported the close relation of these species (Figure 3a; Table S3). Inter-specific estimates of nucleotide distance between the other *Cryptolestes* species were otherwise large (*p*-dist: 13.78–17.94%; K2P: 15.2–19.4%) (Table S3).

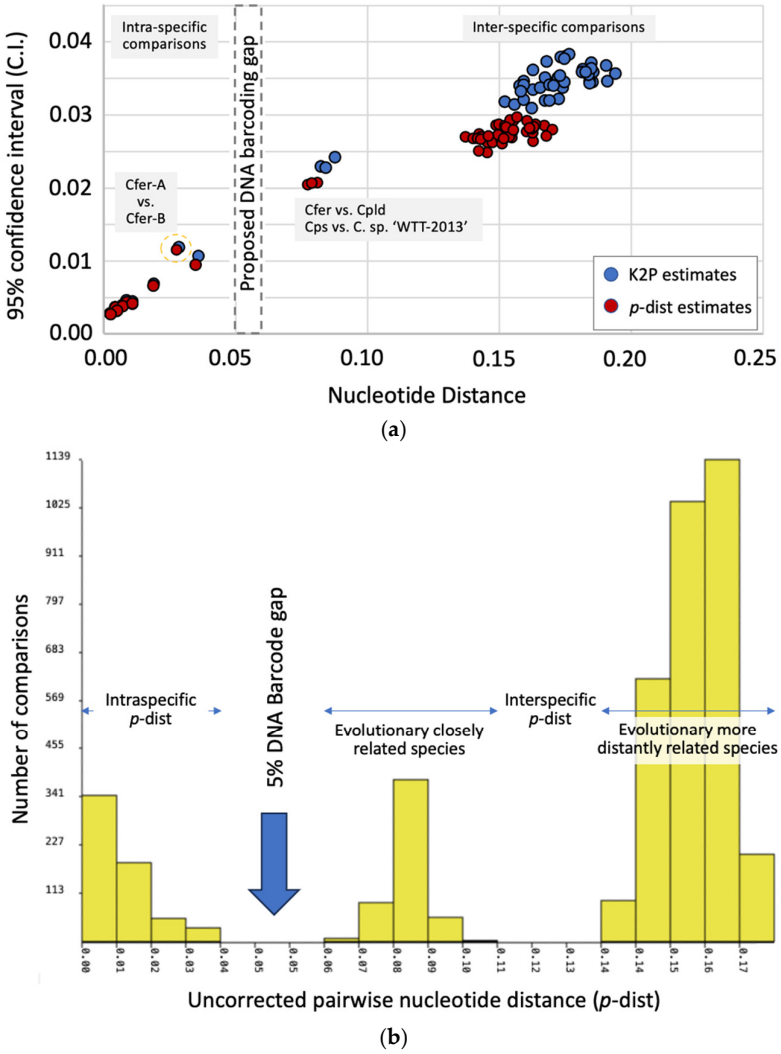

Figure 3. Intra- and inter-specific barcode gaps estimates of *Cryptolestes* species. (**a**) Scatter plots showing both expected low and high nucleotide distances for within and between species, respectively. A proposed 'DNA barcoding gap' (dashed line box at 5–6%) is shown. The close genetic relationships between *C. ferrugineus*/*C. pusilloides* (Cfer vs. Cpld) and *C. pusillus*/*C.* sp. 'WTT-2013' (Cpls vs. *C.* sp. 'WTT-2013') are also shown. *C. ferrugineus* sister clades E and F (Figure 1) are represented by Cfer-A and Cfer-B, respectively (dashed-line circle). *Y*-axis is the 95% confidence interval (C.I). Average intra-specific nucleotide estimates also include those between *C. spartii* and *C. corticinus*. Estimates provided were for K2P and *p*-dist models. (**b**) DNA barcode gaps identified by ABGD for the *Cryptolestes* species analysed in this study. The blue arrow shows the 5% barcode gap that separated intra-specific individuals and inter-specific individuals based on nucleotide distances. A DNA barcode gap at approximately 12–13% that separates evolutionarily closely related and more distantly related *Cryptolestes* species is also evident.

The ABGD analysis placed the barcode gap at 5% to differentiate between *Cryptolestes* species (Figure 3b) and delimited the same nine operational taxonomic units (OTUs) [i.e., Clades A to D, E + F, G to J; Figure 1] as inferred from the maximum likelihood (ML) phylogenetic approach that included placing *C. cortinicus* and *C. spartii* as a single OTU. A main difference between the ABGD analysis and the ML phylogeny approach was that the ABGD analysis did not differentiate *C. ferrugineus* into separate but closely related sister clades.

3.9. Cryptolestes Mitogenome PCG Nucleotide Distances

Intra-specific nucleotide distances based on concatenated mitogenome PCG sequences (11,024 bp) similarly led to the detection of low estimates (*p*-dist < 1%) for the two *C.* sp. 'WTT-2016' and the two *C. pusilloides*. Inter-specific *p*-dist estimates between closely related species were approximately 6.3–6.8%, while between distantly related *Cryptolestes* species, *p*-dist estimates were generally higher (15–18%, Table S4).

3.10. Analyses of Published Cryptolestes Mitogenomes

Sequence alignment of *C. ferrugineus* (KT182067), *C. turcicus* (KT070712), and *C. pusillus* (KT070713) mitogenomes showed that KT182067 and KT070713 were overall similar (*p*-dist: 2.15%; expected at intra-specific level, Table S4) with the difference largely caused by an approximately 2300 bp nucleotide dissimilarity that spanned the COIII-trnG-ND3-trnA-trnR-trnN-trnS1-trnE-trnF-ND5 gene region. Multiple sequence alignment of KT182067 (*C. ferrugineus*) with assembled mitogenomes of the six species from this study suggested contig assembly errors for this KT182067 mitogenome (Figure S2a,b).

Similarly, comparison of the *C. turcicus* mitogenome (KT070712) with mitogenomes from this study indicated KT070712 to be predominantly *C. turcicus*, with the exception of an approximately 700 bp region at the mtCOI barcoding gene region, where it matched with high sequence homology to the *C.* sp. 'WTT-2013' mtCOI gene. This provided evidence that the published *C. turcicus* mitogenome (KT070712) was a chimera likely caused by contamination/contig assembly errors (Figure S3a,b).

Excluding two missing mitogenome regions (i.e., a 93 bp region from the *C.* sp. 'WTT-2013' ND6 gene and an approximately 407 bp mitogenome region from the AT-rich region; [47]), the published *C. pusillus* mitogenome (KT070713) and the *C.* sp. 'WTT-2013' mitogenome [47] shared 99.23% sequence identity, suggesting that they were the same species. The species identity of KT070713 and *C.* sp. 'WTT-2013' will require confirmation.

4. Discussion

In this study, molecular diagnoses of *Cryptolestes* beetles detected during biosecurity inspections at Australia's border, and re-analyses of publicly available partial mtCOI sequences and mitogenomes, demonstrated the pervasiveness of species misidentification in this agriculturally important beetle genus. The previously detected cryptic *C.* sp. 'WTT-2013' in eastern Australia [12] is also likely an introduced exotic species with a probable Asian origin. Furthermore, while *C. ferrugineus* and *C. pusillus* have been regarded as the most widespread and agriculturally important species [19], the novel *C.* sp. 'WTT-2016' represented the most frequently intercepted species in Australia from diverse agricultural commodities, building material, and personal effects and potentially has a wide geographic presence in Asia, Europe, and South America and an unknown presence in North America and Africa as no *Cryptolestes*-contaminated goods were intercepted from these two continents during the sampling period. With the species identified from continents with similar latitudes to the North American and African continents and international movements of contaminated goods to or from globally diverse regions, *C.* sp. 'WTT-2016' therefore represents a species that has the potential to successfully establish in Australia due to regular interceptions and its presence in diverse eco-climatic zones. Accidental introductions of this species are likely, as exemplified by the recent spread of various highly invasive insect pest species that have been shown to be associated with anthropogenic-related activities (e.g., [11,12,51–55]).

Challenges posed by cryptic species to traditional morphological taxonomic characterisation via internal and external morphological characters are well-known in arthropods, including in ecologically, horticulturally, and agriculturally important species

(e.g., [11,56–58]; see also review [8]). While incorrect molecular diagnostics relating to cryptic species can be compounded by pseudogenes (Numts; best exemplified in the whiteflies *Bemisia tabaci* cryptic species complex, e.g., between *B. tabaci* 'MEAM2' [59,60] vs. [61]); *B. tabaci* 'SSA4' ([62,63] vs. [64] that identified incipient speciation), incorporating full mitochondrial genomes, multi-gene markers, and genome-wide SNP analyses could clarify species status and identify potential selective sweeps and incomplete lineage sorting with maternally inherited endosymbionts as drivers of evolution (e.g., [13,65]). Similarly, confusion in *Cryptolestes* species identification (e.g., [3,4,32,50]) demonstrate the significant taxonomic challenge in this beetle genus, including in the identification between *C. pusillus* and the cryptic *C.* sp. 'WTT-2013' based on genitalia morphological characterisation [12], and, as evidenced in this study, also extends to museum voucher specimens.

Possible factors underpinning poor species identification may include (i) the unanticipated presence of cryptic species, (ii) the expectation that voucher *Cryptolestes* species from different museums agreed, and (iii) possible laboratory/analytical-related mistakes, including potential PCR contaminations or contig assembly errors. Contig assembly errors and failure to cross-check assembled genomes/DNA contigs can exacerbate confusion relating to molecular diagnostic efforts, with potential significant biosecurity and pest management implications if uncorrected. These are especially important in cryptic and highly invasive insect pest species and could be factors that underpin the persistent molecular diagnostics-related misidentification of *Cryptolestes* (Figure S1).

In the reanalysis of published mitochondrial genomes for *C. ferrugineus* and *C. turcicus*, potential contig assembly issues associated with DNA contamination were identified (Figures S2 and S3). The unexpected presence of cryptic species (e.g., *C.* sp. 'WTT-2013', *C.* sp. 'WTT-2016') and potentially inconsistent species morphological characterisation of voucher specimens also contributed to species status confusion in the *Cryptolestes* genus that could result in inconsistent intra- and inter-specific nucleotide sequence divergence patterns. For example, a study of *Cryptolestes* species diversity from China, USA, and the Czech Republic [4] identified highly divergent sequences within *C. pusillus*. The authors concluded that intra-specific nucleotide diversity in *C. ferrugineus* and *C. pusillus* varied between 0 and as high as 8.9% but was, however, low in *C. pusilloides* and *C. turcicus* (max. < 1%). Similarly, evidence of species misidentification involving voucher samples could be seen from Pentinsaari et al. [33], where sequences of voucher *C. ferrugineus* samples (KJ964655, KJ961815) clustered confidently either within the *C. ferrugineus* clade (i.e., KJ964655) or with Clade G (*C. pusilloides*; KJ961815). Similar confusion surrounded other voucher *C. ferrugineus* specimens' partial mtCOI sequences [50], and it was also evident that these individuals clustered confidently in Clade G (i.e., KM441850, KM447067; Figure S1).

Various partial mtCOI gene studies showed that in Coleoptera, interspecific nucleotide distances ranged from as low as 2.5–3.2% [11] to approximately 4.4–12.8% [66,67]. They also highlighted the need to carefully assess the appropriate DNA barcoding gap value to adopt for the target species. Similar *p*-dist estimates with other gene regions [68] and evidence presented here therefore support a need for *Cryptolestes* species taxonomic revision. This study to characterise *Cryptolestes* intra- and inter-specific nucleotide distances showed that at the within-species level, partial mtCOI gene *p*-dist estimates typically ranged between 0 and 4.6% at the intra-specific level and from approximately 7.5 to 17.9% for between evolutionarily more divergent *Cryptolestes* species (Figure 3a,b; Table S3). Based on our findings, a tentative *Cryptolestes* 'barcoding gap' is proposed at between approximately 5 and 6% mtCOI nucleotide distance (Figure 3a,b), which lends support to the notion that *C.* sp. 'WTT-2013' is likely to be a cryptic species of *C. pusillus* [12] (Figure S1, Table S3).

Phylogenetic analysis based on concatenated mitogenome PCGs also supported the species status of *C.* sp. 'WTT-2013' and contrasted with the significant branch length difference observed for intraspecies (i.e., *C. pusilloides*; *C.* sp. 'WTT-2016'; Figure 2). Analysis of the assembled mitogenomes showed subtle nucleotide differences between *C. pusillus* and *C. pusilloides*, such as the coding sequence length for the NAD2 gene, where *C. pusillus* exhibited two amino acid residues longer than *C.* sp. WTT-2013 just prior to the predicted stop codon [47]. Mating compatibility studies have been applied to demonstrate cryptic species status [9,69] and could be considered for *Cryptolestes*.

Using limited microsatellite DNA markers, Toon et al. [32] reported limited introgression between two highly diverged and geographically separated *C. ferrugineus* partial mtCOI gene lineages, which the authors attributed to historical population structure associated with early Pleistocene climate changes. While introgression between closely related insect species has been shown to be more widespread than previously reported, especially when analysed using genome-wide single-nucleotide polymorphic (SNP) markers or via a whole-genome resequencing approach (e.g., [10,64,70]), findings from this study suggested that the two very divergent *C. ferrugineus* lineages of Toon et al. [32] likely represented C. *ferrugineus* and *C. pusilloides*. The likelihood of introgression between closely related *Cryptolestes* species (e.g., *C. pusillus* and *C.* sp. 'WTT-2013') and the possibility of selective sweeps and incomplete lineage sorting (e.g., due to infections by maternally inherited bacteria [16]) therefore represent priority areas to be investigated via a whole-genome sequencing approach and population genomic studies.

The high biosecurity interception frequencies of *C.* sp. 'WTT-2013' and *C.* sp. 'WTT-2016' and the prevalence of species status confusion associated with molecular diagnostics via partial mtCOI genes deposited in public DNA databases highlighted the importance and need for accurate species identification in the *Cryptolestes* flat grain beetle species complex. High haplotypes diversity of *C.* sp. 'WTT-2013' from Chinese populations and with individuals detected in goods originating from China suggested that this species likely has an Asian origin. The phosphine resistance status of *C.* sp. 'WTT-2013' and of the globally widespread *C.* sp. 'WTT-2016' is currently unknown but given the high interception frequencies of *C.* sp. 'WTT-2016' that originated from globally diverse locations enables us to conclude that this species represents the greatest ongoing interception risk and therefore a strong candidate for future establishment in countries where it has not yet been established (e.g., in Australia).

While phosphine resistance in populations of *C. ferrugineus* from Australia's eastern states are known (e.g., [71]), phosphine resistance status in other grain-producing states of Australia should be re-assessed given the propensity for species misidentification (e.g., [28]). Similarly, varying levels of phosphine resistance in non-Australian *C. ferrugineus* populations has been reported, including in Bangladesh [72,73], USA [27], Africa (Ivory Coast, Cameroon), Philippines [74], Indonesia [75], Turkey [76], and Greece [77]. In many cases, the precise species involved will require re-evaluation since our molecular diagnostic and mitogenome characterisation work has found evidence of species status confusion not only for *C. ferrugineus* but also for other *Cryptolestes* species that are regarded as of global importance [19]. Similarly, a wide range of phosphine resistance levels in Chinese populations of *C. ferrugineus* have also been reported and may potentially include mixed species [28,78,79]. Moreover, accurate species identification is especially important to limit the spread of resistance genes between individuals of cosmopolitan species and through introgression/hybridisation between evolutionarily more closely related species.

While various *Cryptolestes* species are significant pests of stored products, species within the genus are also highly invasive [2,12,80], likely as a result of global movements of grain and agricultural commodities and, as this study also showed, through movements

of personal effects. Environmental monitoring [80] confirmed new distribution records for *C. cornutus* in Florida, USA, in 2005, and in Hawaii since 2003. Similarly, *C. klapperichi* was also confirmed in Florida in 2005 after earlier confirmations in the US Virgin Islands, St. Lucia, and the Galapagos Islands [80,81]. Despite reports of exotic *Cryptolestes* species being established in non-native ranges (e.g., [12,80,81]), the biology of many species and the ecological impact on their newly established environments largely remained unknown [81]. Laboratory studies of species considered as of economic importance especially to the grain industries have found species such as *C. ferrugineus* and *C. turcicus* to be behaviourally dominant or highly successful competitors [21,82,83] and under appropriate environmental conditions can reproduce quickly to build up significant population size, potentially to outcompete and to predate on native arthropods [81,84]. Poor taxonomy as identified in this study therefore highlighted the challenges facing taxonomists, ecologists, and biologists, especially relating to understanding the ecological impacts of native and introduced *Cryptolestes* species that are not considered as economically important [81].

This work highlights the importance of integrating classical taxonomy via examination of internal reproductive organs of *Cryptolestes* [2,12,80–83,85–87] with molecular genomic advances and of extensively surveying intra-specific genetic diversity to better gauge nucleotide boundaries for species delimitation. There is a need to revise the taxonomy of *Cryptolestes* and to update the existing list of known *Cryptolestes* species to include putative cryptic and novel species (e.g., such as for the potentially misidentified '*C. pusillus*' species (GenBank submitted but unpublished partial mtCOI sequences ON753799.1–ON753803.1, and ON753806.1–ON753808.1 (accessed date 20 December 2025) reported in Thailand but which showed the closest match (97.41% sequence identity) to CspWTT2013-Chn10 [38]) identified to date. Future adoption of whole-genome HTS methods should be priority research areas to facilitate undertaking of metagenomic (i.e., needed for endosymbiont characterisation) and population genomic analyses (i.e., to identify signatures of introgression/hybridisation; selective sweeps, track movements of resistance genes, and estimate resistance allele frequencies; and better estimates of intraspecific *p*-dist range) for *Cryptolestes* species that incorporate also ecological data to contribute to food security, agricultural (e.g., for the stored grain primary industry sector), and environmental biosecurity preparedness.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d18020096/s1, Figure S1: Re-assignment of 158 par3al *Cryptolestes* mtCOI sequences (570–613 bp) from GenBank (n = 129) and generated from this study (n = 29); Figure S2: Schematic representation of the published *C. ferrugineus* (KT182067; [6]) mitogenome aligned to the mitogenomes of [a] *C.* sp. 'WTT-2013' [38], and [b] *C. pusilloides* [38]; Figure S3: Schematic representation of the published *C. turcicus* (KT070712; [7]) mitogenome aligned to the mitogenomes of: [a] *C. turcicus* (this study; see [38]), and [b] *C.* sp. WTT-2013 (this study; see [38]); Table S1: A list of Cucujidae beetles (n = 25) detected during biosecurity inspections at Australia's border that failed to PCR amplify for Sanger sequencing. Sample codes not provided are indicated by 'N/A'. Number of intercepted beetles are indicated by 'n'; Table S2: Draft mitochondrial DNA genomes of six *Cryptolestes* species from Australia (*C. ferrugineus*, *C. pusilloides*, *C. pusillus*), China (*C. turcicus*, *C.* sp. 'WTT-2013'), and Malaysia and Vietnam (*C.* sp. 'WTT-2016'); Table S3: Estimates of pairwise nucleotide distances between *Cryptolestes* species as assigned through the phylogenetic analysis; Table S4: Intra- and inter-specific uncorrected pair-wise nucleotide distances (*p*-dist) between six *Cryptolestes* species based on 13 concatenated mitochondrial protein coding genes (11,024 bp).

Author Contributions: Conceptualization, W.T.T. and P.D.B.; methodology, W.T.T. and M.P.; formal analysis, W.T.T.; investigation, P.D.B. and W.T.T.; resources, W.T.T.; data curation, W.T.T. and M.P.; writing—original draft preparation, W.T.T., D.K., M.P. and S.B.; writing—review and editing, W.T.T., D.K. and S.B.; visualization, W.T.T.; supervision, W.T.T.; project administration, W.T.T.; funding acquisition, P.D.B. and W.T.T. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data supporting reported results can be found at <https://data.csiro.au/collection/csiro:62249> (accessed on 29 December 2025) as detailed in reference [47].

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Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Collins, P.J. Resistance to grain protectants and fumigants in insect pests of stored products in Australia. In Proceedings of the 1st Australian Postharvest Technical Conference, Canberra, Australia, 26–29 May 1998.
2. Halstead, D.G.H. Keys for the Identification of Beetles Associated with Stored Products. 2. Laemophloeidae, Passandridae and Silvanidae. *J. Stored Prod. Res.* **1993**, *29*, 99–197. [CrossRef]
3. Varadinova, Z.; Wang, Y.J.; Kucerova, Z.; Stejskal, V.; Opit, G.; Cao, Y.; Li, F.J.; Li, Z.H. COI barcode based species-specific primers for identification of five species of stored-product pests from genus *Cryptolestes* (Coleoptera: Laemophloeidae). *Bull. Entomol. Res.* **2015**, *105*, 202–209. [CrossRef] [PubMed]
4. Wang, Y.J.; Li, Z.H.; Zhang, S.F.; Varadinova, Z.; Jiang, F.; Kucerova, Z.; Stejskal, V.; Opit, G.; Cao, Y.; Li, F.J. DNA barcoding of five common stored-product pest species of genus *Cryptolestes* (Coleoptera: Laemophloeidae). *Bull. Entomol. Res.* **2014**, *104*, 671–678. [CrossRef] [PubMed]
5. Li, L.; Liu, G.; Sun, T.; Xin, T.; Li, M.; Zou, Z.; Xia, B. Complete mitochondrial genome of *Cryptolestes pusillus* (Coleoptera: Laemophloeidae). *Mitochondrial DNA A DNA Mapp. Seq. Anal.* **2016**, *27*, 3703–3704. [CrossRef]
6. Sun, T.Y.; Li, L.; Xin, T.; Wang, Y.; Xia, B. The complete mitochondrial genome of *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae). *Mitochondrial DNA A DNA Mapp. Seq. Anal.* **2016**, *27*, 3676–3677. [CrossRef]
7. Sun, T.Y.; Liu, G.; Li, L.; Xin, T.; Lei, K.; Xia, B. The complete mitochondrial genome of *Cryptolestes turcicus* (Grouvelle) (Coleoptera: Laemophloeidae). *Mitochondrial DNA A DNA Mapp. Seq. Anal.* **2016**, *27*, 3701–3702.
8. De Barro, P.J.; Liu, S.S.; Boykin, L.M.; Dinsdale, A.B. *Bemisia tabaci*: A Statement of Species Status. *Annu. Rev. Entomol.* **2011**, *56*, 1–19. [CrossRef]
9. Liu, S.S.; Colvin, J.; De Barro, P.J. Species Concepts as Applied to the Whitefly *Bemisia tabaci* Systematics: How Many Species Are There? *J. Intgr. Agr.* **2012**, *11*, 176–186. [CrossRef]
10. Anderson, C.J.; Tay, W.T.; McGaughran, A.; Gordon, K.; Walsh, T.K. Population structure and gene flow in the global pest, *Helicoverpa armigera*. *Mol. Ecol.* **2016**, *25*, 5296–5311. [CrossRef]
11. Rugman-Jones, P.F.; Hoddle, C.D.; Hoddle, M.S.; Stouthamer, R. The Lesser of Two Weevils: Molecular-Genetics of Pest Palm Weevil Populations Confirm *Rhynchophorus vulneratus* (Panzer 1798) as a Valid Species Distinct from *R. ferrugineus* (Olivier 1790), and Reveal the Global Extent of Both. *PLoS ONE* **2013**, *8*, e78379. [CrossRef]
12. Tay, W.T.; Beckett, S.J.; De Barro, P.J. Phosphine resistance in Australian *Cryptolestes* species (Coleoptera: Laemophloeidae): Perspectives from mitochondrial DNA cytochrome oxidase I analysis. *Pest Manag. Sci.* **2016**, *72*, 1250–1259. [CrossRef] [PubMed]
13. Jiggins, F.M. Male-killing Wolbachia and mitochondrial DNA: Selective sweeps, hybrid introgression and parasite population dynamics. *Genetics* **2003**, *164*, 5–12. [CrossRef] [PubMed]
14. Cariou, M.; Duret, L.; Charlat, S. The global impact of *Wolbachia* on mitochondrial diversity and evolution. *J. Evol. Biol.* **2017**, *30*, 2204–2210. [CrossRef] [PubMed]
15. Graham, R.I.; Wilson, K. Male-killing *Wolbachia* and mitochondrial selective sweep in a migratory African insect. *BMC Evol. Biol.* **2012**, *12*, 204. [CrossRef]
16. Koçak, E.; Ünal, H. Endosymbiont Microorganisms in Rusty Grain Beetle *Cryptolestes ferrugineus* (L.) Populations. *Turk. J. Agric. Food Sci. Technol.* **2019**, *7*, 93–96.

17. Tay, W.T.; Rane, R.V.; Padovan, A.; Walsh, T.K.; Elfekih, S.; Downes, S.; Nam, K.; d'Alençon, E.; Zhang, J.; Wu, Y.; et al. Global population genomic signature of *Spodoptera frugiperda* (fall armyworm) supports complex introduction events across the Old World. *Commun. Biol.* **2022**, *5*, 297. [CrossRef]
18. Handayani, A.; Wagiman, F.X.; Indarti, S.; Suputa, S. Insect Quarantine Status in Association with Imported Commodities from Timor Leste Passed through Agricultural Quarantine Ware of Mota'ain-District of Belu. *J. Perlindungan Tanam. Indones.* **2019**, *23*, 75–84. [CrossRef]
19. Rees, D. *Insects of Stored Products*; CSIRO Publishing: Melbourne, Australia, 2004.
20. Berhe, M.; Subramanyam, B.; Chichaybelu, M.; Demissie, G.; Abay, F.; Harvey, J. Post-Harvest Insect Pests and Their Management Practices for Major Food and Export Crops in East Africa: An Ethiopian Case Study. *Insects* **2022**, *13*, 1068. [CrossRef]
21. Bharathi, V.S.K.; Jian, F.; Jayas, D.S. Biology, ecology, and behavior of rusty grain beetle (*Cryptolestes ferrugineus* (Stephens)). *Insects* **2023**, *14*, 590. [CrossRef]
22. Holloway, J.C.; Mayer, D.G.; Daglish, G.J. Flight activity of *Cryptolestes ferrugineus* in southern New South Wales, Australia. *J. Pest Sci.* **2018**, *91*, 1353–1362. [CrossRef]
23. Grains Australia. Grains. Available online: https://grainsaustralia.com.au/grains (accessed on 19 December 2025).
24. Tekiner Aydin, N.; Tozlu, E.; Tozlu, G. Potential for the use of the entomopathogenic fungus *Beauveria bassiana* in the biological control of *Cryptolestes ferrugineus* and *Acanthoscelides obtectus*. *Turk. J. Biod.* **2023**, *6*, 88–96. [CrossRef]
25. Chaudhry, M.Q. Phosphine resistance. *Pestic. Outlook* **2000**, *11*, 88–91. [CrossRef]
26. Australian Government. Export process instruction. In *Inspection of Prescribed Grain and Plant Products for Export*; Version 8; Document ID: IMLS-12-3853; DAFF: Canberra, Australia, 2024.
27. Konemann, C.E.; Hubhachen, Z.; Opit, G.P.; Gautam, S.; Bajracharya, N.S. Phosphine Resistance in *Cryptolestes ferrugineus* (Coleoptera: Laemophloeidae) Collected from Grain Storage Facilities in Oklahoma, USA. *J. Econ. Entomol.* **2017**, *110*, 1377–1383. [CrossRef] [PubMed]
28. Chen, E.-H.; Shen, D.-R.; Du, W.-W.; Meng, H.-J.; Tang, P.-A. Cuticle protein genes are involved in phosphine resistance of *Cryptolestes ferrugineus*. *Sci. Agric. Sin.* **2023**, *56*, 1696–1707.
29. Polaszek, A.; Otim, M.H.; Briscoe, A.; Court, L.; Macfadyen, S.; Schmidt, S.; Geng, H.; Tay, W.T. Morphological and molecular description, and draft mitogenome, of a new *Encarsia* species (Hymenoptera: Aphelinidae), a parasitoid of the *Bemisia tabaci* species complex (Hemiptera: Aleyrodidae) on cassava in Uganda. *J. Nat. Hist.* **2025**, *59*, 2441–2461. [CrossRef]
30. Conde, S.; Monteiro, F.; Catarino, S.; Ferreira, M.R.; Ferreira, S. Uninvited guests: New stored mangrove rice insect pests in Guinea-Bissau. *J. Stored Prod. Res.* **2025**, *111*, 102567. [CrossRef]
31. Tay, W.T.; Marshall, S.D.G.; Popa-Baez, A.D.; Dulla, G.F.J.; Blas, A.L.; Sambiran, J.W.; Hosang, M.; Millado, J.B.H.; Melzer, M.; Rane, R.V.; et al. Alternative DNA Markers to Detect Guam-Specific CRB-G (Clade I) *Oryctes rhinoceros* (Coleoptera: Scarabaeidae) Indicate That the Beetle Did Not Disperse from Guam to the Solomon Islands or Palau. *Diversity* **2025**, *16*, 634. [CrossRef]
32. Toon, A.; Daglish, G.J.; Ridley, A.W.; Emery, R.N.; Holloway, J.C.; Walter, G.H. Random Mating Between Two Widely Divergent Mitochondrial Lineages of *Cryptolestes ferrugineus* (Coleoptera: Laemophloeidae): A Test of Species Limits in a Phosphine-Resistant Stored Product Pest. *J. Econ. Entomol.* **2016**, *109*, 2221–2228. [CrossRef]
33. Pentinsaari, M.; Hebert, P.D.N.; Mutanen, M. Barcoding Beetles: A Regional Survey of 1872 Species Reveals High Identification Success and Unusually Deep Interspecific Divergences. *PLoS ONE* **2014**, *9*, e108651. [CrossRef]
34. Staden, R.; Beal, K.F.; Bonfield, J.K. The Staden package, 1998. *Methods Mol. Biol.* **2000**, *132*, 115–130.
35. Staden, R. The Staden sequence analysis package. *Mol. Biotechnol.* **1996**, *5*, 233–241. [CrossRef]
36. Bernt, M.; Donath, A.; Juhling, F.; Externbrink, F.; Florentz, C.; Fritzsch, G.; Pütz, J.; Middendorf, M.; Stadler, P.F. MITOS: Improved de novo metazoan mitochondrial genome annotation. *Mol. Phylogenet. Evol.* **2013**, *69*, 313–319. [CrossRef]
37. Villesen, P. FaBox: An online toolbox for FASTA sequences. *Mol. Ecol. Notes* **2007**, *7*, 965–968. [CrossRef]
38. Cognato, A.I. Standard percent DNA sequence difference for insects does not predict species boundaries. *J. Econ. Entomol.* **2006**, *99*, 1037–1045. [CrossRef] [PubMed]
39. Dinsdale, A.; Cook, L.; Riginos, C.; Buckley, Y.M.; De Barro, P. Refined Global Analysis of *Bemisia tabaci* (Hemiptera: Sternorrhyncha: Aleyrodoidea: Aleyrodidae) Mitochondrial Cytochrome Oxidase 1 to Identify Species Level Genetic Boundaries. *Ann. Entomol. Soc. Am.* **2010**, *103*, 196–208. [CrossRef]
40. Katoh, K.; Misawa, K.; Kuma, K.; Miyata, T. MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* **2002**, *30*, 3059–3066. [CrossRef]
41. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef]
42. Kimura, M. A Simple Method for Estimating Evolutionary Rates of Base Substitutions through Comparative Studies of Nucleotide-Sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef]
43. Srivathsan, A.; Meier, R. On the inappropriate use of Kimura-2-parameter (K2P) divergences in the DNA-barcoding literature. *Cladistics* **2011**, *28*, 190–194. [CrossRef]

44. Collins, R.A.; Boykin, L.M.; Cruickshank, R.H.; Armstrong, K.F. Barcoding's next top model: An evaluation of nucleotide substitution models for specimen identification. *Methods Ecol. Evol.* **2012**, *3*, 457–465. [CrossRef]
45. Puillandre, N.; Lambert, A.; Brouillet, S.; Achaz, G. ABGD, Automatic Barcode Gap Discovery for primary species Delimitation. *Mol. Ecol.* **2012**, *21*, 1864–1877. [CrossRef] [PubMed]
46. Fujisawa, T.; Barraclough, T.G. Delimiting Species Using Single-Locus Data and the Generalized Mixed Yule Coalescent Approach: A Revised Method and Evaluation on Simulated Data Sets. *Syst. Biol.* **2013**, *62*, 707–724. [CrossRef] [PubMed]
47. Tay, T.; Piper, M.C.; Beckett, S.J.; Kunz, D.; De Barro, P.J. Molecular Resources for the Stored Grain *Cryptolestes* Cryptic Pest Species (Coleoptera: Laemophloeidae): Mitochondrial Genomes and Partial mtDNA COI Sequences. v1. CSIRO. Data Collection. Available online: https://data.csiro.au/collection/csiro%3A62249v1 (accessed on 10 April 2024).
48. Minh, B.Q.; Schmidt, H.A.; Chernomor, O.; Schrempf, D.; Woodhams, M.D.; von Haeseler, A.; Lanfear, R. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Mol. Biol. Evol.* **2020**, *37*, 1530–1534. [CrossRef] [PubMed]
49. Jin, M.J.; Zwick, A.; Slipinski, A.; Marris, J.W.M.; Thomas, M.C.; Pang, H. A comprehensive phylogeny of flat bark beetles (Coleoptera: Cucujidae) with a revised classification and a new South American genus. *Syst. Entomol.* **2020**, *45*, 248–268. [CrossRef]
50. Hendrich, L.; Moriniere, J.; Haszprunar, G.; Hebert, P.D.N.; Hausmann, A.; Kohler, F.; Balke, M. A comprehensive DNA barcode database for Central European beetles with a focus on Germany: Adding more than 3500 identified species to BOLD. *Mol. Ecol. Resour.* **2015**, *15*, 795–818. [CrossRef]
51. Elfekih, S.; Etter, P.; Tay, W.T.; Fumagalli, M.; Gordon, K.; Johnson, E.; De Barro, P. Genome-wide analyses of the *Bemisia tabaci* species complex reveal contrasting patterns of admixture and complex demographic histories. *PLoS ONE* **2018**, *13*, e0190555. [CrossRef]
52. Rane, R.; Walsh, T.K.; Lenancker, P.; Gock, A.; Dao, T.H.; Nguyen, V.L.; Khin, T.N.; Amalin, D.; Chittarath, K.; Faheem, M.; et al. Complex multiple introductions drive fall armyworm invasions into Asia and Australia. *Sci. Rep.* **2023**, *13*, 660. [CrossRef]
53. Hoffmann, B.D.; Tay, W.T.; Blas, A.L. Biosecurity interceptions of coconut rhinoceros beetle *Oryctes rhinoceros*. *Manag. Biol. Invasions* **2024**, *15*, 437–443. [CrossRef]
54. Magalhaes, V.S.; Czepak, C.; van Niekerk, M.; Du Plessis, H.; Court, L.; Tay, W.T. *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae) draft mitogenomes and insecticide resistance gene characterisation support multiple maternal lineages in invasive African, Asian, and European populations. *Bull. Entomol. Res.* **2025**, *115*, 437–451. [CrossRef]
55. Lopes-da-Silva, M.; Sanches, M.M.; Stancioli, A.R.; Alves, G.; Sugayama, R. The Role of Natural and Human-Mediated Pathways for Invasive Agricultural Pests: A Historical Analysis of Cases from Brazil. *Agric. Sci.* **2014**, *5*, 634–646. [CrossRef]
56. Rugman-Jones, P.F.; Au, M.; Ebrahimi, V.; Eskalen, A.; Gillett, C.P.D.T.; Honsberger, D.; Husein, D.; Wright, M.G.; Yousuf, F.; Stouthamer, R. One becomes two: Second species of the *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae) species complex is established on two Hawaiian Islands. *PeerJ* **2020**, *8*, e9987. [CrossRef] [PubMed]
57. Smith, S.M.; Gomez, D.F.; Beaver, R.A.; Hulcr, J.; Cognato, A.I. Reassessment of the species in the *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae) complex after the rediscovery of the "Lost" Type specimen. *Insects* **2019**, *10*, 261. [CrossRef] [PubMed]
58. Stouthamer, R.; Rugman-Jones, P.; Thu, P.Q.; Eskalen, A.; Thibault, T.; Hulcr, J.; Wang, L.-J.; Jordal, B.H.; Chen, C.-Y.; Cooperband, M.; et al. Tracing the origin of a cryptic invader: Phylogeography of the *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae) species complex. *Agric. Forest Entomol.* **2017**, *19*, 366–375. [CrossRef]
59. Delatte, H.; Reynaud, B.; Granier, M.; Thornary, L.; Lett, J.M.; Goldbach, R.; Peterschmitt, M. A new silverleaf-inducing biotype Ms of *Bemisia tabaci* (Hemiptera: Aleyrodidae) indigenous to the islands of the south-west Indian Ocean. *Bull. Entomol. Res.* **2005**, *95*, 29–35. [CrossRef]
60. Karut, K.; Kaydan, M.B.; Tok, B.; Döker, İ.; Kazak, C. A new record for *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) species complex of Turkey. *J. Appl. Entomol.* **2015**, *139*, 158–160. [CrossRef]
61. Tay, W.T.; Elfekih, S.; Court, L.N.; Gordon, K.H.J.; Delatte, H.; De Barro, P.J. The Trouble with MEAM2: Implications of Pseudogenes on Species Delimitation in the Globally Invasive *Bemisia tabaci* (Hemiptera: Aleyrodidae) Cryptic Species Complex. *Genome Biol. Evol.* **2017**, *9*, 2732–2738. [CrossRef]
62. Berry, S.D.; Fondong, V.N.; Rey, C.; Rogan, D.; Fauquet, C.M.; Brown, J.K. Molecular evidence for five distinct *Bemisia tabaci* (Homoptera: Aleyrodidae) geographic haplotypes associated with cassava plants in sub-Saharan Africa. *Ann. Entomol. Soc. Am.* **2004**, *97*, 852–859. [CrossRef]
63. Wosula, E.N.; Chen, W.; Fei, Z.; Legg, J.P. Unravelling the Genetic Diversity among Cassava *Bemisia tabaci* Whiteflies Using NextRAD Sequencing. *Genome Biol. Evol.* **2017**, *9*, 2958–2973. [CrossRef]
64. Elfekih, S.; Tay, W.T.; Polaszek, A.; Gordon, K.H.J.; Kunz, D.; Macfadyen, S.; Walsh, T.K.; Vyskočilová, S.; Colvin, J.; De Barro, P.J. On species delimitation, hybridization and population structure of cassava whitefly in Africa. *Sci. Rep.* **2021**, *11*, 7923. [CrossRef]
65. Klopfstein, S.; Christian, K.; Baur, H. *Wolbachia* endosymbionts distort DNA barcoding in the parasitoid wasp genus *Diplazon* (Hymenoptera: Ichneumonidae). *Zool. J. Linn. Soc.* **2016**, *177*, 541–557. [CrossRef]

66. Aykut, M.; Yildirim, Y.H.; Tusun, S.; Fery, H. *Deronectes kabilcevz* sp. n. and *D. propedoriae* sp. n. from south-eastern Anatolia (Turkey) (Coleoptera, Dytiscidae, Hydroporinae). *Zootaxa* **2019**, *4691*, 589–600. [CrossRef] [PubMed]
67. Monaghan, M.T.; Balke, M.; Gregory, T.R.; Vogler, A.P. DNA-based species delineation in tropical beetles using mitochondrial and nuclear markers. *Phil. Trans. R. Soc. B* **2005**, *360*, 1925–1933. [CrossRef] [PubMed]
68. Machado, A.; Rodriguez-Exposito, E.; Lopez, M.; Hernandez, M. Phylogenetic analysis of the genus *Laparocerus*, with comments on colonisation and diversification in Macaronesia (Coleoptera, Curculionidae, Entiminae). *Zookeys* **2017**, *651*, 1–77. [CrossRef] [PubMed]
69. Vyskocilova, S.; Tay, W.T.; van Brunschot, S.; Seal, S.; Colvin, J. An integrative approach to discovering cryptic species within the *Bemisia tabaci* whitefly species complex. *Sci. Rep.* **2018**, *8*, 10886. [CrossRef]
70. Anderson, C.J.; Oakeshott, J.G.; Tay, W.T.; Gordon, K.H.J.; Zwick, A.; Walsh, T.K. Hybridization and gene flow in the mega-pest lineage of moth, *Helicoverpa*. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 5034–5039. [CrossRef]
71. Kaur, R.; Nayak, M.K. Developing effective fumigation protocols to manage strongly phosphine-resistant *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae). *Pest Manag. Sci.* **2014**, *71*, 1297–1302. [CrossRef]
72. Nayak, M.; Holloway, J.; Pavic, H.; Head, M.; Reid, R.; Patrick, C. Developing strategies to manage highly phosphine resistant populations of flat grain beetles in large bulk storages in Australia. In *Proceedings of the 10th International Working Conference on Stored Product Protection, Estoril, Portugal, 27 June–2 July 2010*; Julius Kühn-Institut: Quedlinburg, Germany, 2010; Volume 425, pp. 396–401.
73. Sakka, M.K.; Götze, M.-C.; Athanassiou, C.G. Phosphine Susceptibility Screening of Three Different Stored Product Beetle Species by Using Three Diagnostic Techniques. *Agriculture* **2025**, *15*, 1904. [CrossRef]
74. Acda, M.A.; Mangoba, M.A.; Mesa, V.G.; Dela Cruz, M.V. Phosphine Resistance in the Philippines. *Philipp. Ent.* **2018**, *32*, 133–146. [CrossRef]
75. Parasian, F.; Trisyono, Y.A.; Martono, E. Resistance of *Ahasverus advena* and *Cryptolestes ferrugineus* to phosphine on imported cocoa beans from Cameroon, Ivory Coast, and Dominican Republic. *JPTI* **2018**, *22*, 173. [CrossRef]
76. Koçak, E.; Yilmaz, A.; Alpkent, Y.; Bilginturan, S. Phosphine resistance of rusty grain beetle *Cryptolestes ferrugineus* (Coleoptera: Laemophloeidae) populations in Turkey. *Sci. Papers. Ser. A Agron.* **2018**, *LXI*, 286–290.
77. Agrafioti, P.; Kaloudis, E.; Kateris, D.; Athanassiou, C.G. Assessment of phosphine resistance in major stored-product insects in Greece using two diagnostic protocols. *Insects* **2024**, *15*, 802. [CrossRef]
78. Daglish, G. Phosphine Resistance in Insect Pests of Stored Grain. 1999. Available online: http://aciar.gov.au/project/pht/1994/015 (accessed on 23 December 2025).
79. Ling, Z. Development and countermeasures of resistance in stored grain insects in Guangdong of China. In *Proceedings of the 7th Proceedings IWCSPP, Beijing, China, 14–19 October 1998*; Jin, Z., Liang, Q., Liang, Y., Tan, X., Guan, L., Eds.; CAB Int.: Wallingford, UK, 1999; pp. 642–647.
80. Thomas, M.C. New distribution records for two species for *Cryptolestes* Ganglauer (Coleoptera: Laemophloeidae). *Insecta Mundi* **2005**, *19*, 88.
81. Thomas, M.C. A revision of the New World species of *Cryptolestes* Ganglbauer (Coleoptera: Cucujidae: Laemophloeinae). *Insecta Mundi* **1988**, *2*, 43–65.
82. Rilett, R.O. The biology of *Laemophloeus ferrugineus* (Steph.). *Can. J. Res.* **1949**, *27*, 112–148. [CrossRef] [PubMed]
83. Lefkovitch, L.P. The biology of *Cryptolestes turcicus* (Grouvelle) (Coleoptera: Cucujidae), a pest of stored and process cereals. *J. Zool.* **2009**, *138*, 23–25. [CrossRef]
84. Lefkovitch, L.P. Difference between six species of *Cryptolestes* (Coleoptera, Cucujidae) in susceptibility to Methyl Bromide vapour. *Bull. Entomol. Res.* **1965**, *56*, 197–200. [CrossRef]
85. Banks, H.J. Identification of stored product *Cryptolestes* spp. (Coleoptera: Cucujidae): A rapid technique for preparation of suitable mounts. *J. Aust. Entomol. Soc.* **1979**, *18*, 217–222. [CrossRef]
86. Biege, C.R.; Partida, G.J. Taxonomic characters to identify three species of *Cryptolestes* (Coleoptera: Cucujidae). *J. Kan. Entomol. Soc.* **1976**, *49*, 161–164.
87. Green, M. *Cryptolestes klapperichi* Lefkovitch in stored products and its identification (Coleoptera: Cucujidae). *J. Stored Prod. Res.* **1979**, *15*, 71–72. [CrossRef]

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