Molecular phylogenetics of a South Pacific sap beetle species complex (Carpophilus spp., Coleoptera: Nitidulidae)

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ABSTRACT

Several species of sap beetles in the genus Carpophilus are minor pests of fresh produce and stored products, and are frequently intercepted in biosecurity operations. In the South Pacific region, the superficially similar species C. maculatus and C. oculatus are frequently encountered in these situations. Three subspecies of C. oculatus have been described, and the complex of these four taxa has led to inaccurate identification and questions regarding the validity of these taxa. A molecular phylogenetic study using the mitochondrial gene cytochrome c oxidase I (COI) and two nuclear markers comprising the rDNA internal transcribed spacer 2 (ITS2) and the D1–D2 region of the large (28S) ribosomal RNA subunit showed that C. maculatus, and C. o. cheesmani were easily differentiated from the two other subspecies of C. oculatus. COI also showed differentiation between C. o. gilloglyi and C. o. oculatus, but this was not shown when third codon positions were removed and when RY-coding analyses were conducted. Generalised mixed Yule-Coalescent (GMYC) models were fitted to trees estimated from the COI data and were analysed using a multimodel approach to consider the evidence for three taxonomic groupings of the C. oculatus group. While the arrangement with the highest cumulative weight was not the arrangement ultimately accepted, the accepted taxonomy also had an acceptable level of support. ITS2 showed structure within C. oculatus, however C. o. oculatus was resolved as paraphyletic with respect to C. o. gilloglyi. COI showed evidence of sequence saturation and did not adequately resolve higher relationships between species represented in the dataset. 28S resolved higher relationships, but did not perform well at the species level. This study supports the validity of C. maculatus as a separate species, and provides sufficient evidence to raise C. o. cheesmani to the level of species. This study also shows significant structure within and between C. o. gilloglyi and C. o. oculatus, giving an indication of recent speciation events occurring.

To highlight the interesting biology between these two taxa, C. o. cheesmani and C. o. oculatus, these results give clarity regarding the taxonomic status of C. maculatus and the subspecies of C. oculatus and provide a platform for future systematic research on Carpophilus.

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1. Introduction

The sap beetle genus Carpophilus (Coleoptera: Nitidulidae) contains over 200 species found throughout the world. Both adults and larvae live and develop in organic matter, including rotting fruit and stored products, which has made some species pests in orchards and warehouses. Carpophilus davidsoni Dobson, C. hemipterus (Linnaeus) and C. mutilatus Erichson have emerged as serious pests of stone fruit in Australia with the decrease in use of broad-spectrum insecticides (Hossain and Williams, 2003; James et al., 1997; James and Voge, 2000), while C. lugubris Murray is an important pest of corn in the United States (Dowd, 2000) and C. sayi Parsons has been implicated in the transmission of oak wilt disease (Cease and Juzwik, 2001). The species most commonly found in stored products are C. hemipterus, C. dimidiatus (Fabricius), C. ligneus Murray and C. obsoletus Erichson (Dobson, 1955). A number of species are also associated with flowers and are important pollinators, particularly of the Annonaceae (Nagel et al., 1989). In the tropical South Pacific region, C. maculatus Murray and C. oculatus Murray are widespread species that are commonly found in agricultural situations, and have been intercepted in exported fresh produce (Archibald and Chalmers, 1983; Leschen and Marris, 2005).

There has been much published on the ecology and control of the group, as it is considered to be the most economically important genus within the Nitidulidae (Connell, 1981). However, the taxonomy of the genus remains problematic, with the last global revision of the group dating to the 19th Century (Murray, 1864). Since Murray’s seminal work, several new species have been
described, most notably by Dobson and Kirejtshuk (e.g. Dobson, 1952, 1993a; Kirejtshuk, 2001). Not withstanding this, the genus remains in need of a thorough taxonomic revision, as most species are poorly defined and morphologically variable, with subtle characters defining the species. This taxonomic uncertainty impacts on the ability to understand their relative invasiveness and risk.

Within the Nitidulidae, Carpophilus is placed in the subfamily Carpophilinae (Kirejtshuk, 2008). While no formal systematic studies have yet been published on the relationships of higher taxa within the Nitidulidae, it is believed that the Carpophilinae form a single lineage with the Epuraeinae and Amphicrossinae (Kirejtshuk, 2008), an arrangement that is consistent with preliminary molecular systematic results from the family (A. Cline pers. comm.). The genus is easily identified by having two exposed abdominal tergites and a button-like male 8th sternite (Gillogly, 1962; Leschen and Marris, 2005). It is morphologically conservative between species within the genus and there are a number of groups containing several species with very similar appearance and habits. Carpophilus is classified into nine subgenera: Carpophilus Stephens sensu stricto, Megacarpolus Reitter, Semocarpolus Kirejtshuk, Caplocarpolus Kirejtshuk, Askocarpolus Kirejtshuk, Plagennipolus Kirejtshuk, Ecomorphus Motschulsky, Caplothorax Kirejtshuk and Myothorax Murray (Kirejtshuk, 2008). However, the exact limits of these subgenera have not been expanded on in the context of an overall review of Carpophilus systematics. The majority of these subgenera are fairly small, consisting of only a few species, most of which are confined to South-East Asia. The exceptions are Carpophilus s. str., Ecomorphus and Myothorax, which are large and cosmopolitan.

Carpophilus maculatus and C. oculatus are widely distributed through the South Pacific, with C. maculatus also being found in Southeast Asia, South America and the West Indies. The two species are similar, with the key character that differentiates between them being the extent of red colouration on the elytra. However, this patterning is very variable and there is significant overlap in the patterns possessed by both species. Dobson (1993b) reviewed C. oculatus and described three subspecies based on differences in pronotal punctuation and male genitalia. The nominate subspecies, C. o. oculatus is found from Tahiti through the central Pacific to New Caledonia. There is significant overlap in geographic range between this subspecies and C. o. gigilogyi Dobson which is found from Easter Island to Fiji and Micronesia. The last subspecies, C. o. cheesemani Dobson is confined to Vanuatu. Morphological evidence for monophyly of the C. oculatus subspecies include the distinctive ring-shaped elytral colour pattern and the pronotal length/width ratio, however there are no clear and unambiguous synapomorphies that define C. oculatus. A sister taxon relationship is suggested between C. oculatus and C. maculatus by the sculpturing of the prosternum and by the shape of the male genitalia. The genitalic similarity is particularly striking between C. maculatus and C. o. gigilogyi, with the shape of the parameres of these two species being nearly identical. These similarities, and the variation in colour pattern has led some to surmise that these two species form a single, hyper-variable species (Leschen and Marris, 2005).

In this study, the monophyly of C. oculatus and its relationship with C. maculatus was tested using molecular systematic techniques. The opportunity was also taken to provide a preliminary glimpse into the systematics of Carpophilus, by sampling a range of species within the genus and with an emphasis on species within the subgenus Myothorax. A molecular phylogeny of Carpophilus was constructed based on sequences of mitochondrial and nuclear gene regions from as many species as it was possible to obtain. This was used primarily to test the monophyly of C. oculatus and its subspecies, and to test for a sister-taxon relationship between C. oculatus and C. maculatus. It also provides a basis for the systematics of Carpophilus in general and will be useful for guiding future taxonomic work on the genus.

2. Methods

2.1. Taxon coverage and specimens

To test the monophyly of C. oculatus, the three subspecies of this species were collected preferentially. The relationship of C. oculatus with C. maculatus was also targeted, given the morphological similarity between them. Beyond this, other Carpophilus species were collected opportunistically by the senior author and colleagues, primarily from the Pacific.

Specimens were collected by hand from rotting fruit and vegetables and preserved in propylene glycol in the field for transporting back to New Zealand. In the laboratory, specimens were sorted and temporarily stored in 100% ethanol. A single leg was removed from each specimen to be used for molecular analysis and the remainder card-mounted as a voucher specimen and deposited in the Lincoln University Entomology Museum (LUNZ).

2.2. Molecular methods

DNA was extracted using the prepGEM DNA extraction kit (ZyGem Ltd., Hamilton, New Zealand). Incubation consisted of 30 min at 75 °C and 5 min at 95 °C. This longer incubation period ensured that dehydrated tissues had sufficient time to rehydrate and lyse. DNA was amplified using a 10 μl PCR reaction containing 0.25 U Expand High-Fidelity Taq (Roche Applied Science, Indianapolis, IN, USA), 0.2 mM dNTPs, 2 mM MgCl2 and 0.3 mM of both forward and reverse primers. To encourage DNA amplification in difficult species, 2 μl GC Rich mixture (Roche) was added to the reaction when necessary. The 5’ end of the cytochrome c oxidase subunit I (COI) mitochondrial gene, the D1–D2 region of the 28S ribosomal RNA gene and the internal transcribed spacer 2 (ITS2) region in the nuclear ribosomal encoding cistrons were amplified using the primers listed in Table 1. For COI the combination LCO1490/HC02198 was used preferentially, with TY-J-1460/C1-N-2191 used when these amplifications were unsuccessful. ITS2 amplifications required a primer concentration of 0.5 mM. Reactions were run on a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturation of 94 °C for 2 min, followed by 40 cycles of 94 °C (15 s), 45 °C (30 s) and 72 °C (75 s), and with a final extension at 72 °C for 7 min. An annealing temperature of 54 °C was used for 28S reactions. Successful amplification was confirmed by running PCR products in 1% agarose gels made using a NaOH/borate buffer with a pH of 8.0; these were run at 170 V and 50 mA for 15 min. Gels were stained during casting with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) and viewed with a GeneWizard Gel imaging system (SynGene, Cambridge, England). PCR products were sequenced in 10 μl reactions containing 0.3 μl of PCR product, 0.5 μl BigDye® Terminator (v3.1) (Applied Biosystems), 2 μl BigDye® Sequencing buffer (v1.1/3.1) and 0.8 μM of the primers used for amplification. Products were sequenced in both directions. PCR cleanup was done with CleanSEQ® Dye Terminator Removal kit (Agencourt Bioscience Corporation, Beverly, MA, USA). Sequences were read using a Long Read Sequencing Protocol on an ABI Prism® 3100-Avant Genetic Analyzer (Applied Biosystems).

2.3. Analysis

Sequences were aligned by eye using the manual alignment software BoEdn (Hall, 1999). COI data were analysed using four methods; (1) Original nucleotide (NT) coded data, (2) Third codon positions excluded, (3) Third codon positions RY-coded, and (4) All data RY-coded (Phillips et al., 2004), while the other two loci were unaltered. Loci were analysed independently, due to the
**Table 1**
Markers and PCR primer combinations used in this research.

<table>
<thead>
<tr>
<th>Marker</th>
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<th>Primer sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COI</td>
<td>LCO1490</td>
<td>5’-GCT CAA AAT AAT ATA TTT G-3’</td>
<td>Folmer et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>HC02198</td>
<td>5’-TAA ACT TCA GGG GTA CAA AAA AAT CA-3’</td>
<td>Folmer et al. (1994)</td>
</tr>
<tr>
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<td>TY-J-1460</td>
<td>5’-TAC ATT TTA TCC AAA CTT CCC CC-3’</td>
<td>Simon et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>C1-N-2191</td>
<td>5’-CCC GGT AAA ATT AAA ATA TAA ACT TC-3’</td>
<td>Simon et al. (1994)</td>
</tr>
<tr>
<td>28S</td>
<td>LSUf2</td>
<td>5’-ACA CTT ACC DTR AGG CAA AAT TG-3’</td>
<td>Sonnenberg et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>LSUrev1</td>
<td>5’-TAC TAG AAG GGA CTA GTA GTC-3’</td>
<td>Sonnenberg et al. (2007)</td>
</tr>
<tr>
<td>ITS2</td>
<td>CAS5p8Bsc</td>
<td>5’-TAGA CAT CGA CAT TTY GAA CGC ACA T-3’</td>
<td>Ji et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>CAS28sB1d</td>
<td>5’-TTC TTT TCC TTC C1-N-2191-3’</td>
<td>Ji et al. (2003)</td>
</tr>
</tbody>
</table>

Inconsistencies in phylogenetic inference when genes with differing histories are concatenated (Kubatko and Degnan, 2007), and the lack of multiple samples for many species precluded the use of coalescent-based approaches (Heled and Drummond, 2010).

Maximum likelihood (ML) analyses for all loci were run using PhyML 3.0 (Anisimova and Gascuel, 2006; Guindon and Gascuel, 2003), and support for the topology calculated using parametric bootstrap procedures with 1000 replicates. jModelTest (Posada, 2008) was used to determine the appropriate models of molecular evolution, based on the Akaike information criterion (AIC). Saturation plots of the p-distance against ML distances calculated from trees, were constructed in R using functions from the ape package (Paradis et al., 2004; R Development Core Team, 2012). Pairwise distances are reported as both raw p-distances and as model-corrected distances to illustrate the uncertainty in the derivation of genetic distance estimates (Collins et al., in press).

Ultrametric COI trees for generalised mixed Yule-Coalescent (GMYC) analysis were estimated from the NT-coded COI data using BEAST 1.6.2 (Drummond and Rambaut, 2007; Drummond et al., 2006) using an HKY + I + G model of evolution, a relaxed lognormal molecular clock and default priors. A lognormal molecular clock model was justified with the ucldev.stddev value of 1.384 indicating considerable rate heterogeneity. The MCMC chain was run for a total of 50.7 million generations, and trees were sampled every 1000 generations. A maximum clade credibility (MCC) tree was made using Treeannotator from the sampled trees after removing a standard burn-in of the first 10% trees of each run. GMYC (Pons et al., 2008) was used to determine the appropriate models of molecular evolution, based on the Akaike information criterion (AIC). Saturation plots of the p-distance against ML distances calculated from trees, were constructed in R using functions from the ape package (Barash et al., 2006; R Development Core Team, 2012). Pairwise distances are reported as both raw p-distances and as model-corrected distances to illustrate the uncertainty in the derivation of genetic distance estimates (Collins et al., in press).

COI and 28S was sequenced from all species, with the exception of C. obsoletus for which 28S was unable to be amplified. ITS2 was only sequenced from selected species within the Myothorax subgenus. Outgroups were selected from available specimens and previously published sequences of other species within the Nitidulidae. These represent two subfamilies outside of the Carpophilinae and included Epuraea signata Broun and E. ocularis Fairmaire (Epuraeinae), unidentified Conotelus species (Cillaeinae), and Aethina concolor (Macleay), Omosia discoides (Fabricius), three Meligethes species and unidentified specimens of Phenolia and Steldota (Nitidulinae).

### 3. Results

#### 3.1. Taxon sampling

Specimens of 17 species of Carpophilus were sequenced. These represented four subgenera, as proposed by Kirejtshuk (2008). Semocarpus was represented by a single species, C. marginellus; Carpophilus by three species (C. lugubris, C. obsoletus and C. hemipterus); Ecomorphus by four (C. antiquus Melsheimer, C. discoides Leconte, C. bakewelli Murray and C. corticinus Erichson); and Myothorax by eight species (C. dimidiatus, C. mutilatus, C. davidsoni, C. gavenni Dobson, C. nepos Murray, C. maculatus, C. ocularis, C. robustus Murray and C. schidtelti Murray). A single species of Urophorus (U. humeralis [Fabricius]) was also included in the dataset. COI and 28S were sequenced from all species, with the exception of C. bakewelli and C. obsoletus for which 28S was unable to be amplified. ITS2 was only sequenced from selected species within the Myothorax subgenus. Outgroups were selected from available specimens and previously published sequences of other species within the Nitidulidae. These represent two subfamilies outside of the Carpophilinae and included Epuraea signata Broun and E. ocularis Fairmaire (Epuraeinae), unidentified Conotelus species (Cillaeinae), and Aethina concolor (Macleay), Omosia discoides (Fabricius), three Meligethes species and unidentified specimens of Phenolia and Steldota (Nitidulinae).

#### 3.2. Alignments and variation

A total of 200 sequences over the three markers were obtained. Sequences have been submitted to Genbank with accession numbers GU217433–GU217530 for COI, GU217391–GU217432 for 28S and GU217338–GU217390 for ITS2. Specimen details are available as Supplementary Material (Table 6). Sequence polymorphism data for each gene are presented in Table 2.

28S and ITS2 alignments required the addition of alignment gaps because of the presence of indels. While there are no indels in the COI dataset, 11 specimens had missing values from short sequencing runs. The maximum number of missing bases in any one specimen was 141, with the median number being 9. Base frequencies of the three markers are typical for insects (Lin and Danforth, 2004).

Although COI and 28S had roughly equivalent levels of variable sites (Table 2), COI had more parsimony-informative sites than 28S, and was better at distinguishing between species and subspecies of Carpophilus (Fig. 2 c.f. Fig. 3). Distribution of parsimony-informative sites in COI across first, second and third codon positions respectively was 37, 5, and 159, a result consistent with most protein-coding genes (Xia, 1998). The saturation plot (Fig. 1) for COI showed deviation from linearity, suggesting that the marker is unsuitable for inferring higher relationships. Both nuclear markers are

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<td>Simon et al. (1994)</td>
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<td>28S</td>
<td>LSUf2</td>
<td>5’-ACA CTT ACC DTR AGG CAA AAT TG-3’</td>
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<td>5’-TTC TTT TCC TTC C1-N-2191-3’</td>
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<td>COI</td>
<td>28S</td>
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<tr>
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<td>C</td>
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<td>16.9</td>
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<td>T</td>
<td>35.5</td>
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</table>
essentially linear, showing no evidence of saturation at the level investigated here.

3.3. Phylogenetic analyses

3.3.1. COI
3.3.1.1. Maximum likelihood analyses. In all four COI ML analyses, C. o. gilloglyi + C. o. oculatus are shown to form a monophyletic clade. This clade is supported by >50% bootstrap values in all analyses, with the exception of the dataset where third codon positions were excluded. None of the analyses resolved the sister-group relationship of C. o. cheesmani with statistical support or placed them with the other two C. oculatus subspecies. The full NT-coded dataset resolves C. o. gilloglyi and C. o. oculatus as being reciprocally monophyletic, while all other datasets resolve C. o. gilloglyi as paraphyletic with respect to C. o. oculatus.

Maximum likelihood of the nucleotide-coded COI data inferred a single tree of -ln likelihood 8179.84071 from a TPM3uf + I + G model with nucleotide frequencies and rate parameters as shown in Tables 2 and 3. The tree (Fig. 2) grouped conspecific taxa with each other, and were supported with high bootstrap values. In particular, the monophyly of C. o. oculatus and C. o. gilloglyi is supported by a bootstrap of 72%. However, higher relationships between species and between genera are not resolved with any degree of certainty. Support for deeper nodes were not increased by the removal of third codon positions or RY coding the data (see Supplementary Material).

The distances between the subspecies of C. oculatus are given in Table 4. In addition, C. o. gilloglyi was shown to have a very deep split (16.5% mean ML pairwise distance; 7.41% mean p-distance) between western specimens collected in Fiji (including Rotuma) and eastern populations from the Kermadec Islands to French Polynesia, including Tonga. The distinction between these two groups is retained throughout the modifications to the dataset, though the exact relationships vary between the modifications (see Supplementary Material). When third codons are removed, the Fijian clade of C. o. gilloglyi is nested within C. o. oculatus, while the eastern clade is paraphyletic to the clade formed by the former groups. These relationships, however, do not have bootstrap support, while the monophyly of C. o. cheesmani is supported with a bootstrap of 74.3%. In both RY-coded datasets, the Fijian clade is sister to the Eastern clade + C. o. oculatus (see Supplementary Material).

A deep split is also seen between Australian and Pacific populations of C. maculatus, with a mean ML pairwise distance between the clades of 14.52% (6.01% mean p-distance). There was also significant structuring within the Pacific clade, with a mean ML pairwise distance of 4.67% (1.9% mean p-distance). Within a single, small island (Wallis), a specimen was 3.87% ML distance (2.62% p-distance) different from its conspecifics from the same island.

Within other species with a sample size that allows the calculation of intra-specific distances, C. nepos had a mean ML pairwise distance of 4.18% (1.59% mean p-distance) from three specimens collected in New Caledonia, Tahiti and a laboratory culture from the USA. In ML analyses, the specimen from New Caledonia was resolved as basal to the other two specimens, with support of 59.4%, while in the Bayesian tree, the specimen from the USA was basal, making the Pacific specimens monophyletic (posterior probability (PP) = 0.57). The ML distance between the Pacific specimens was 3.62% (0.71% p-distance). Carpophilus mutilatus was represented by three specimens from Hawaii, Tahiti and Nauru, and had a mean ML pairwise distance of 0.42% (0.16% mean p-distance). In both ML and Bayesian analyses, the Nauru specimen was basal to the other two specimens (bootstrap: 57.9%; PP = 0.76). The relationships of these two species were not resolved in any of the analyses with any degree of support, with the exception of a C. nepos – C. davidsoni
Fig. 2. ML tree of nucleotide-coded COI sequences. Bootstrap values are shown above nodes with greater than 50% support. Three letter codes signify general locality of specimens: AUS–Australia, BIS–Bismarck Islands, FIJ–Fiji, HAW–Hawaii, KER–Kermadec Islands, NAU–Nauru, NCL–New Caledonia, NZL–New Zealand, SAM–Samoa, SOC–Society Islands (French Polynesia), TON–Tonga, TUB–Austral Islands (French Polynesia), TUV–Rotuma Island (close to Tuvalu), USA–United States of America, VAN–Vanuatu.
sister group relationship being resolved in the ITS2 analysis with a bootstrap value of 66%. Unfortunately, *C. mutilatus* is not represented in this dataset.

### 3.3.1.2. Bayesian analyses
Consistent with the ML analyses, *C. oculatus* was not resolved as being monophyletic on the Bayesian MCC tree (Fig. 6). The combination of *C. oculatus* + *C. o. gilloglyi* formed a strongly supported clade (PP = 0.99), whereas *C. o. cheesmani* was found to have a weakly supported relationship with *C. dimidiatus* (PP = 0.56). Within *C. o. gilloglyi*, both western and eastern clades were well supported (both with PP = 1), however, the node linking the two clades did not have strong support (PP = 0.47). The sister taxa of *C. o. oculatus* + *C. o. gilloglyi* were not resolved with any degree of support.

Unlike ML analyses, the members of the *Mythorax* subgenus were resolved as monophyletic (PP = 0.9), however the other subgenera were not monophyletic, and *Euparea* and *Urophorus* are nested within *Carpophilus*. The latter has strong support for a relationship with *C. obsoletus* (PP = 0.93), while the former has moderate support for being in a clade that includes *C. marginellus*, *C. lugubris*, *C. bakewelli*, *C. discoideus* and *C. antiquus* (PP = 0.63).

### 3.3.2. 28S rDNA
Maximum likelihood inferred a single tree with a –ln likelihood of 3603.59084 from a GTR + F model with nucleotide frequencies and rate parameters as shown in Table 3.

The 28S ML analysis showed resolution at the subgeneric level, but did not adequately resolve lower relationships (Fig. 3). The monophyly of the *C. oculatus* group was supported by a bootstrap of 85%; *C. o. cheesmani* was sister to the other two subspecies, the separation supported by a bootstrap of 84.3%, while *C. o. gilloglyi* was paraphyletic with respect to *C. oculatus* and did not show the geographic structure found in the mitochondrial data. Beyond this, resolution within the genus was extremely poor. *C. gaveni*, *C. maculatus*, *C. schoediei* and *C. robustus* were identical in their 28S sequences and were barely different from *C. davidsoni*, *C. mutilatus* and *C. nepos*. *Carpophilus dimidiatus* was shown to have an extremely divergent 28S sequence, and a relationship with *Euparea signata* was supported with a bootstrap of 73%.

28S results are much more congruent with subgeneric classification than COI, though subgenera were not strictly monophyletic in all cases. Species within *Carpophilus* *s. str.* came out as monophyletic, though it also included *U. humeralis*. The single species within *Semocarpalus*, *C. marginellus*, was sister to, but strongly differentiated from the *Urophorus–Carpophilus* *s. str.* clade. *Ecromorphus* was paraphyletic with respect to a monophyletic *Mythorax* (excluding *C. dimidiatus*).

### 3.3.3. ITS2
Analysis of 53 ITS2 sequences produced a tree with a ML of –ln = 1478.1793 using a GTR model (Table 3). Adding a gamma distribution to the model did not result in a different topology. ITS2 data was only gathered for select taxa within the *Mythorax* subgenus. At this level it showed good variation between species (Fig. 4). *Carpophilus o. cheesmani* was again shown to be a sister taxon to the other *C. oculatus* subspecies, supported by a bootstrap value of 78.6%. In contrast to the 28S data there was sufficient variation in the marker to show intra-specific genetic structure. Within *C. o. oculatus* there was found to be a lot of structuring, though no geographic influence is apparent with these analyses. These specimens were resolved as being paraphyletic with respect to a monophyletic and very homogeneous *C. o. gilloglyi* that shows no geographic structuring.

Unexpectedly, *C. schioedtei* came out within *C. oculatus*. It sits on a reasonably long branch within the group and may be a case of homoplasy, possibly combined with insufficient taxon sampling.

### 3.4. SH tests
SH-tests of topology were conducted on the COI dataset to determine the individual and combined significance of *Carpophilus* and *C. oculatus* non-monophyly. ITS2 was tested for *C. oculatus* monophyly only. Results show that constraining monophyly resulted in a significantly worse likelihood score for both markers (Table 5). The change in likelihood score of enforcing *C. oculatus* monophyly was little different from that of forcing *Carpophilus* monophyly, despite being the result of a single change in the tree, namely the change in position of *C. cheesmani*. Likewise, the combined changes to the tree were substantially different from the optimal, but little different from the individual changes.

### 3.5. GMYC analysis of COI
Running GMYC with a single threshold on the Bayesian MCC tree resulted in a significantly better fit than the null model (logL = 604.55, 2ΔL = 22.73; χ² test, d.f. = 4, P < 0). Using multiple thresholds did not result in a significantly better model (logL = 606.35, 2ΔL = 3.6; χ² test, d.f. = 12, P = 0.99), and the following results use the single threshold as the standard. The model with the highest likelihood inferred 34 entities, with a confidence interval of 14–45 entities. The entities estimated by the highest likelihood model were broadly congruent with the species boundaries accepted here, though it overestimated the number of entities within *C. maculatus* clade (Fig. 6; leftmost, green bar). *C. oculatus*, the best GMYC model recognised the eastern and western clades of *C. o. gilloglyi* as separate entities. When multiple thresholds were considered, GMYC greatly overestimated the number of distinct entities, resulting in dividing *C. maculatus* and the eastern clade of *C. o. gilloglyi* into six entities each, and *C. oculatus* into three.

An alternative method to reporting GMYC uncertainty is the model-weighting approach of Powell (2012). Using the results from both single and multiple-threshold GMYC runs, the model-weighted average number of entities is 32.5 with a variance of 63.11, from a total of 155 models. None of the models gave the exact taxonomic arrangement proposed here, but models that supported single *C. maculatus* and *C. o. oculatus* + *C. o. gilloglyi* entities had a sum of Akaike weights (Wₙ(comb)) of 0.056 (n = 4); compared with Wₙ(comb) = 0.087 (n = 7) where a *C. o. oculatus*/C. o. gilloglyi split was supported; and Wₙ(comb) = 0.26 (n = 10) where *C. o. gilloglyi* was further subdivided into the eastern and western clades and *C. maculatus* is split into an Australian and a Pacific clade.

Results from the 2000 randomly selected trees were very variable. The number of clusters estimated using a single threshold ranged from 2 to 103. The number most commonly inferred was 34, with 141 trees having this number of clusters. The range of the number of clusters inferred using multiple thresholds was smaller than the single threshold, from 2 to 84, but the inter-quartile range was greater (21 vs 19.25) (Fig. 5). The number most commonly inferred was 48, with 70 trees having this number of clusters. The number of entities covered by the MCC confidence

### Table 4

| COI mean pairwise distances between *C. oculatus* subspecies. Upper triangle: p-distances; Lower triangle: ML distances; Diagonal: ML distance/PD-distance. |
|-----------------|-----------------|-----------------|
| *C. o. oculatus* | *C. o. gilloglyi* | *C. o. cheesmani* |
| 0.0091/0.0037   | 0.0669           | 0.0921           |
| 0.2063          | 0.0910/0.0415    | 0.1137           |
| 0.3959          | 0.4662           | 0.0/0            |

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interval accounted for a large proportion of the variation in the sampled trees. The proportion of the single threshold results within the interval was 0.697, while 0.5615 of the variation in the multiple threshold models was within the interval. Likelihood ratio tests between single and multiple threshold models showed that multiple thresholds did not result in significantly better models in all but two cases.

4. Discussion

Data presented here demonstrate that the subspecies of *C. oculatus* are genetically well differentiated from each other, with COI model-corrected distances ranging between 20–46%. In all analyses, *C. o. oculatus* and *C. o. gilloglyi* emerged as sister taxa. Beyond that however, relationships are unclear. *Carpophilus o. chee smani*
was closely related to the other two subspecies according to the nuclear datasets, but was placed well outside the oculatus–gilloglyi clade in COI analyses. C. o. oculatus and C. o. gilloglyi are reciprocally monophyletic in the NT-coded COI dataset, however this monophyly breaks down with the modification of the COI dataset, and both nuclear genes show paraphyly of these two taxa.

Removal of third-codon positions and RY coding are usually undertaken to resolve deeper phylogenetic nodes as they reduce noise from faster-evolving sites and eliminate transition bias (Philips et al., 2004). In this instance they were not successful, with no deeper nodes being resolved with any greater certainty than in the full NT-coded dataset. However, modified datasets did resolve C. o. gilloglyi as being paraphyletic with respect to C. o. oculatus, an observation that is consistent with 28S data. This paraphyly hints

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**Fig. 4.** ML tree of ITS2 sequences. Bootstrap values are shown above nodes with greater than 50% support. Geographic codes as for Fig. 2.

**Table 5** SH test results for COI and ITS2 datasets.

<table>
<thead>
<tr>
<th>Tress</th>
<th>log likelihood (ln L)</th>
<th>Difference (ln L)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Most likely</td>
<td>-7788</td>
<td>0</td>
<td>0.5483</td>
</tr>
<tr>
<td>C. oculatus monophyly</td>
<td>-9118</td>
<td>1330</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Carpophilus monophyly</td>
<td>-9125</td>
<td>1337</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Both</td>
<td>-9120</td>
<td>1332</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>ITS2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Most likely</td>
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<td>0</td>
<td>0.4870</td>
</tr>
<tr>
<td>C. oculatus monophyly</td>
<td>-1529</td>
<td>38</td>
<td>0.0046</td>
</tr>
</tbody>
</table>
that incomplete lineage sorting may be affecting the gene trees presented here, or suggests an ancestor–descendant relationship between *C. o. gilloglyi* and *C. o. oculatus*. Removal of third-codon positions also led to a decrease in genetic structuring within *C. gilloglyi* and *C. o. oculatus*, showing that the bulk of the differences between these two taxa are in the faster-evolving third-codon position, while differences between these taxa and *C. o. cheesmani* include first and second codon positions, confirming that *C. o. cheesmani* is an older lineage than the other two taxa.

Despite having genitalia and colouration very similar to *C. o. gilloglyi*, *C. maculatus* was not inferred as being the sister taxon to *C. oculatus*. COI analyses did not show any support for nodes beyond species-level groupings, while analysis of 28S resolved a sister group relationship with a clade including *C. maculatus*, *C. robustus*, *C. schoedeti* and *C. gaveni*.

While the sample sizes and geographic coverage of other *Carphophilus* species are not large enough to make definitive conclusions regarding their evolutionary history, it is interesting to note the comparative amounts of variation in *C. nepos*, *C. mutilatus* and *C. maculatus*. *Carphophilus nepos* shows distinct differences in COI between the three specimens from Tahiti, New Caledonia and from a laboratory culture in the USA, with average intra-specific ML distances of 4.2%. This degree of genetic variation suggests that this species may have been present in the Pacific for some time. By comparison, *C. mutilatus* specimens showed a much lower level of variation with an average ML intra-specific distance of 0.42%, despite specimens being collected over a similarly large geographic area. This much lower level of genetic variation suggests that *C. mutilatus* may be a relatively recent arrival in the Pacific compared with *C. nepos*, or that it may be more readily dispersed, resulting in greater genetic connectivity between populations. *Carphophilus maculatus* shows genetic structure between island groups and within single islands, suggesting that this species has been present in these areas for a substantial period of time. The greatest degree of structuring exists between a clade representing two Australian specimens and a clade composed of Pacific Islands specimens; these two groups differing by a genetic distance of over 10%. This separation is not shown by the nuclear genes sampled, and no other evidence currently exists that would justify considering these populations different species. The level of genetic structuring may represent long isolation of the Australian and Pacific populations of *C. maculatus*.

Species assigned to the subgenus *Myothorax* formed a monophyletic group in most analyses, though in COI data it was present with low support. 28S was reasonably congruent with subgeneric classifications, but did not resolve subgenera as being strictly monophyletic. These preliminary results suggest that the classification of Kirejtschuk (2008) may represent natural clades, however more comprehensive species sampling within these subgenera is required before this can be confirmed.

Our molecular data consistently resolve *Urophorus* and *Epuraea* within *Carphophilus sensu lato*. While the first of these genera was originally described as a subgenus of *Carphophilus*, the second has always been considered to be distinct, usually being placed in a different subfamily to *Carphophilus*. *Urophorus* was elevated to a full genus by Gillogly (1962), but later workers have debated the validity of this elevation and have continued to consider it as a subgenus (e.g. Ewing and Cline, 2005). Though the structure of the male terminalia in *U. humeralis* looks similar to that in *Carphophilus*, closer investigation shows that the 8th tergite makes up the entire pygidium in *U. humeralis*, as opposed to the small, ventrally situated button-like structure found in *Carphophilus*. *Urophorus* possesses a round patch with significantly different sculpture in the same location as the 8th tergite in *Carphophilus*, which completes the illusion of similarity. This morphological feature provides significant evidence against the placement of *Urophorus* within *Carphophilus* as inferred from our molecular data. Greater taxon sampling within *Urophorus* and other *Carphophilus* subgenera and the use of a genetic marker with a conservative rate of evolution is needed to investigate this situation further.

Despite being proposed as a nuclear equivalent of the COI region for the identification of species (Sonnenberg et al., 2007), the 28S D1–D2 region proved to be unreliable for distinguishing between species-level taxa in this research. This was particularly the case within the *Myothorax* subgenus, members of which were distinctly different in their COI sequences. However, 28S did show promise for the detection of higher-level phylogenetic relationships. The placement of *U. humeralis* and *Epuraea* within *Carphophilus* may be explained by insufficient taxon sampling or long branch attraction. The level of divergence of *C. dimidiatus* is very high. More specimens of this species are required to confirm whether this divergence is an error. As a member of the *Myothorax* subgenus, it is expected that the species would have been placed with the remainder of species in that subgenus.

GMYC results were largely congruent with morphological species. In some instances the model with the highest likelihood over-estimated the number of species when intra-specific genetic structuring was evident, i.e. within *C. o. oculatus* + *C. o. gilloglyi* and *C. maculatus*. However, models within the confidence interval of the analysis were more inclusive. The GMYC confidence interval of the MCC tree adequately reflected the variability in the sample, as reflected by the confidence interval encompassing the bulk of the density of the distribution of the results on the sampled trees. The use of the AIC and model weights extends the use of GMYC from its current use as comparing two models, to enabling the weight of evidence for different scenarios to be considered in taxonomic decisions. In this instance, while there is more evidence from GMYC for recognising the East/West split in *C. o. gilloglyi* and the Australia/Pacific split in *C. maculatus*, the evidence ratios against recognising the subspecies (*Epuraea subsp* = 2.981), or having a combined *C. o. oculatus* + *C. o. gilloglyi* clade (*Epuraea clade* = 4.604) is not so high as to be beyond the realms of possibility. GMYC was originally developed to provide estimates of species numbers as part of large-scale community ecology projects where in-depth taxonomic expertise was not available and not necessary. In this context it can be a useful tool (Monaghan et al., 2009; Pinzon-Navarro et al., 2010); however its
Fig. 6. Bayesian maximum clade credibility tree of NT-coded COI sequences. Posterior probabilities are shown above all nodes. 95% highest posterior density error bars for node heights are given for nodes with a posterior probability greater than 0.5. Geographic codes are the same as in Fig. 2. Bars to the right of the tree show the composition of clusters estimated by single-threshold GMYC models (from left to right): highest likelihood GMYC model (green), the lower confidence interval (light blue), and the upper confidence interval (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
estimates are very broad, thereby limiting its utility in the context of a systematic treatment of a taxon.

In the light of the morphological and genetic differences between the subspecies of *C. oculatus*, it is justifiable to elevate *C. o. cheesmani* to a full species while retaining *C. o. gilloglyi* and *C. o. oculatus* as subspecies. In all analyses, *C. o. cheesmani* is monophyletic and substantially different from other *C. oculatus* subspecies, and can separated by the shape of male genitalia, and by the differences in pronotal punctuation (Dobson, 1993b). The elevation of this subspecies to full species follows the precedent of a Californian tiger beetle, *Cicindela lunalonga* Schaupp, which was elevated from being a subspecies of *C. terricola* following molecular evidence of strict monophyly and a high pairwise divergence in phylogenetic analyses (Woodcock et al., 2007). The two other subspecies of *C. oculatus* are retained at that rank as they show paraphyly, and as an indication that this complex has some interesting biology worthy of further study.

There may be some justification for the species determination of the *C. o. gilloglyi* geographic clades. However, no lines of evidence outside of COI currently point to species-level differences between the two clades. Specimens from these areas, which appear to be identical morphologically, have been found in the same habitat and appear to have the same ecological niche. It is possible that these populations are in the early stages of allopatric speciation. It is surprising that these two clades are so distinct considering the high frequency of human movement between Fiji and Tonga. It is even more puzzling when the large geographical distances between islands inhabited by the eastern clade are considered, over which it appears to have maintained recent gene flow. Because of the limited sampling in Tonga, more focused collecting throughout Tonga and the Lau archipelago would be needed to determine whether the apparent geographic separation of the two *C. o. gilloglyi* clades is real, or if there is some overlap in the ranges of these clades and to what extent that overlap occurs. More detailed specimen sampling, particularly with the use of rapidly evolving genetic markers such as microsatellites, may provide clues to the creation and persistence of this deep divergence within *C. o. gilloglyi*. Should the two clades be found to exist in sympatry, the proportion of individuals belonging to each clade plus the genetic diversity of these two populations and extent to which their distributions overlap may offer clues as to the processes (e.g. invasion) that currently influence the distribution of each clade.

5. Conclusion

This research set out to resolve the taxonomic questions surrounding the subspecies of *C. oculatus*, and to determine the degree of similarity between *C. oculatus* and *C. maculatus*. Consistent evidence from three gene regions reveal that *C. maculatus* and *C. o. cheesmani* are distinct species, while *C. o. oculatus* and *C. o. gilloglyi* are closely related taxa that show paraphyletic structure with genetic markers. These species therefore need to be treated as such in biosecurity operations. While this distinction will necessitate increased vigilance by biosecurity organisations, the level of genetic structuring between islands of the South Pacific suggests that *C. oculatus* and *C. maculatus* may not be as invasive as previously assumed.

At a higher level, this study suggests that *Carpophilus* may not be monophyletic, potentially incorporating the genera *Urophorus* and *Epuraea*. This is unexpected as it runs against several lines of evidence from morphology, but is to be taken with caution as few specimens and species of *Urophorus* and *Epuraea* were sampled for this study, and these taxa appear with the representatives of a poorly sampled subgenus of *Carpophilus*. A formal investigation of this possibility will require representative samples of all three genera. It will also need to use genetic markers that have a slower rate of evolution, as COI showed saturation at the subgenus level within *Carpophilus*. A somewhat more robust result is that the subgenus *Myothorax* appears to be monophyletic. Unfortunately, no other *Carpophilus* subgenera showed any indication of monophyly, but the sampling scheme was not designed to explicitly test this hypothesis.

Finally, this study further confirms that the South Pacific region is a dynamic region, and shows that widespread species within the region can have intriguing biology and can be as interesting and informative as single island endemics.

5.1. Taxonomy of *C. maculatus* and *C. oculatus* sensu lato

The length of specimens were measured from the anterior margin of the pronotum to the posterior margin of the elytra at the elytral suture, as the abdomen in preserved specimens can be significantly distended or contracted. Specimens were received on loan from the Oxford University Museum of Natural History (Oxford, Britain) (OUMNH), New Zealand Arthropod Collection (Auckland, New Zealand) (NZAC), MAF Biosecurity New Zealand (Auckland, New Zealand) (MAF), Queensland Museum (Brisbane, Australia) (QM), the University of the South Pacific (Suva, Fiji) (USP) and the Hunterian Museum (Glasgow, Scotland) (HM).

5.1.1. *Carpophilus maculatus* (Murray, 1864)

*Diagnosis*: L: 1.6–2.2 mm, W: 1–1.4 mm. Colour variable from yellow–brown to dark brown, often with lighter, T-shaped marking on the elytra. Pronotum length/width ratio 1:1.5, punctured with reniform punctures (Fig. 7a). Prosternal process expanded behind procoxae. Female pygidium truncate.

*Distribution*: Bismarck Archipelago (Gillogly, 1969, LUNZ), Caroline Islands (Gillogly, 1962, Cook Islands (NZAC, Dobson unpubl., LUNZ), Easter Island (NM), Fiji (MAF, Dobson unpubl., NZAC, LUNZ), Gilbert Islands (Dobson unpubl., Gillogly, 1962), Guam (Gillogly, 1962), Hawaii (Fiji (MAF, Dobson unpubl., NZAC, LUNZ), Kiribati (Tarawa) (NZAC, Marquesas Islands (Dobson unpubl.), Mariana Islands (Gillogly, 1962), Marshall Islands (Gillogly, 1962), Nauru (LUNZ), New Caledonia (NZAC, LUNZ, Dobson unpubl., QM), Niue (NZAC, Dobson unpubl., HM), Palau (Gillogly, 1962), Papua New Guinea (Dobson unpubl.), Samoa (NZAC, LUNZ, Dobson unpubl., HM), Society Islands (LUNZ, Dobson unpubl.), Solomon Islands (Dobson unpubl., Tokelau (NZAC, HM), Tonga (NZAC, MAF, Dobson unpubl.), Tuamotu Archipelago (Dobson unpubl.), Austral Islands (Dobson unpubl., LUNZ), Tuvalu (NZAC, LUNZ, HM), Vanuatu (Dobson unpubl., OUMNH, NZAC, LUNZ), Christmas Is., Australia, Philippines, Cuba, Cocos-Keeling Is, Indonesia, Nicobar Islands, Mollucas.

*Comments*: Abundant throughout the Pacific and found on a wide variety of fruit and vegetables. Very variable in colouration. This species be distinguished from *C. oculatus* and *C. cheesmani* by the wider pronotum. Illustration of the male genitalia can be found in Dobson (1955).


*Diagnosis*: L: 1.8–2.4 mm, W: 1.0–1.4 mm. Colour brown to black, with lighter, reddish ring on each elytron. Pronotum length/width ratio 1:1.3, coarsely and densely punctured with reniform punctures (Fig. 7b), microsculpture minutely reticulate coriaceous. Prosternal process rounded. Female pygidium truncate.


*Comments*: Restricted to Vanuatu where it is sympatric with *C. o. oculatus* from which it can be differentiated by the sculpture of the pronotum. The two species can be found syntopically, however *C. cheesmani* is the rarer of the two (SDJ Brown pers.
obs.). This species has been collected from the rotting fruit of breadfruit (Artocarpus altilis), oranges (Citrus sp.) and guava (Psidium guajava), and sweet potato (Ipomoea batatas) tubers.

5.1.3. Carpophilus oculatus oculatus (Murray, 1864)

Diagnosis: L: 1.8–2.6 mm, W: 1.0–1.6 mm. Colour variable, from light brown to black, usually with lighter ring on each elytron. Pronotum length/width ratio 1:1.3, pronotal punctures sparse, round and fine on disc, becoming reniform and coarser toward margin (Fig. 7c), no impunctate medial line; microsculpture minutely reticulate coriaceous; usually less pubescent. Female pygidium truncate.

Distribution: Cook Islands (Rarotonga) (Dobson, 1993b, NZAC), Fiji (Quisenberry, Taveuni, Kadavu, Viti Levu, Vanua Levu) (Dobson, 1993b, LUNZ, MAF, USP, NZAC), Hawaii (Dobson, 1993b), Marquesas Islands (Fatu Hiva) (Dobson, 1993b), Nauru (LUNZ), New Caledonia (Grande Terre) (QM, NZAC), Niue (Niue) (NZAC), Samoa (Upolu, Tutuila) (HM, NZAC), Society Islands (Bora Bora, Tahiti) (Dobson, 1993b, LUNZ), Tokelau (Nukunono) (NZAC), Tonga (Tongatapu) (Dobson, 1993b, NZAC, MAF, HM, LUNZ), Tuvalu (Rotuma) (LUNZ), Vanuatu (Espiritu Santo, Efate) (LUNZ).

Comments: Widespread throughout the South Pacific and found on a wide variety of fruit and vegetables. While the ranges of C. o. oculatus and C. o. gilloglyi overlap over a significant part of their range, they are rarely found syntopically (SDJ Brown pers. obs.).

5.1.4. Carpophilus oculatus gilloglyi Dobson 1993b

Diagnosis: L: 1.5–2.4 mm, W: 0.9–1.4 mm. Colour variable, from light brown to black, usually with lighter ring on each elytron. Pronotum length/width ratio 1:1.3, pronotal punctures on vertex and disc of pronotum close and reniform (Fig. 7d), impunctate median line anterior of scutellum; microsculpture reticulate coriaceous, coarser than in other subspecies. Female pygidium emarginate medially.

Distribution: Caroline Islands (Ponape Island, Truk) (Dobson, 1993b), Cook Islands (Rarotonga, Atiu) (Dobson, 1993b, NZAC, MAF), Easter Island (NZAC), Fiji (Vanua Levu, Taveuni, Viti Levu, Kadavu, Ovalau, Lakeba) (LUNZ, Dobson, 1993b, USP, NZAC), Kermadec Islands (Meyer Island, Raoul Island, Chanters Islets) (Dobson, 1993b, NZAC, LUNZ), Niue (Dobson, 1993b, NZAC), Samoa (Upolu, Savai’i) (NZAC, HM), Society Islands (Bora Bora, Moorea, Tahiti) (Dobson, 1993b, LUNZ), Tonga (Tongatapu, Vava’u, Eua, Niuafo’ou) (Dobson, 1993b, NZAC, MAF, HM, LUNZ), Austral Islands (Rapa, Rimatara) (LUNZ), Tuvalu (Rotuma) (LUNZ).

Comments: Widespread throughout the South Pacific and found on a wide variety of fruit and vegetables. While the ranges of C. o. oculatus and C. o. gilloglyi overlap over a significant part of their range, they are rarely found syntopically (SDJ Brown pers. obs.).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.04.018.
References


