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Cite this article: Hoyal Cuthill JF, Sewell KB, Cannon LRG, Charleston MA, Lawler S, Littlewood DTJ, Olson PD, Blair D. 2016

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Proc. R. Soc. B **283**: 20160585.

<http://dx.doi.org/10.1098/rspb.2016.0585>

Received: 14 March 2016

Accepted: 27 April 2016

Subject Areas:

evolution, taxonomy and systematics, ecology

Keywords:

invertebrates, phylogenetics, cophylogeny, symbionts, parasites, climate change, conservation

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Electronic supplementary material is available at <http://dx.doi.org/10.1098/rspb.2016.0585> or via <http://rspb.royalsocietypublishing.org>.

Australian spiny mountain crayfish and their temnocephalan ectosymbionts: an ancient association on the edge of coextinction?

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Australian spiny mountain crayfish (*Euastacus*, Parastacidae) and their ecotymbiotic temnocephalan flatworms (Temnocephalida, Platyhelminthes) may have co-occurred and interacted through deep time, during a period of major environmental change. Therefore, reconstructing the history of their association is of evolutionary, ecological, and conservation significance. Here, time-calibrated Bayesian phylogenies of *Euastacus* species and their temnocephalans (*Temnohaswellia* and *Temnosewellia*) indicate near-synchronous diversifications from the Cretaceous. Statistically significant cophylogeny correlations between associated clades suggest linked evolutionary histories. However, there is a stronger signal of codivergence and greater host specificity in *Temnosewellia*, which co-occurs with *Euastacus* across its range. Phylogeography and analyses of evolutionary distinctiveness (ED) suggest that regional differences in the impact of climate warming and drying had major effects both on crayfish and associated temnocephalans. In particular, *Euastacus* and *Temnosewellia* show strong latitudinal gradients in ED and, conversely, in geographical range size, with the most distinctive, northern lineages facing the greatest risk of extinction. Therefore, environmental change has, in some cases, strengthened ecological and evolutionary associations, leaving host-specific temnocephalans vulnerable to coextinction with endangered hosts. Consequently, the extinction of all *Euastacus* species currently endangered (75%) predicts coextinction of approximately 60% of the studied temnocephalans, with greatest loss of the most evolutionarily distinctive lineages.

1. Introduction

Evolutionary history is shaped both by biological interactions and the physical environment. Understanding these connections may be essential for biological conservation [1,2]. However, very few studies have reconstructed the shared histories of associated clades through deep time, during which extensive environmental change may have profoundly affected their evolution, both separately and jointly [3]. Furthermore, there is an urgent need for information on the specificity of interspecies associations in cases where the host is of conservation concern [4], because the extinction risk for symbionts (non-free-living organisms [5]) may be closely linked to that of their hosts [6–8]. Adaptation to new host species (host

switches) may reduce the risk of symbiont coextinction (in this case, defined as the extinction of a symbiont species owing to the extinction of its hosts [9,10]). However, host-switching dynamics are not yet well understood [11–13]. Furthermore, interactions between coextinction and long-term environmental change have been little studied, despite their potential importance in biodiversity loss [10,14]. Australian freshwater crayfish of genus *Euastacus* (the large and colourful spiny mountain crayfish) and their temnocephalan ectosymbionts (genera *Temnosewellia* and *Temnohaswellia*) provide an excellent model system for such a study.

Temnocephalans (Temnocephalida, Platyhelminthes) are specialized and distinctive ectosymbiotic flatworms, which show strong ecological associations with freshwater crustaceans [15]. They appear to be strongly dependent on their hosts for survival and reproduction in the wild: eggs are laid on the host, and development is direct. Most occur on the external surfaces, or within the branchial chambers, of freshwater crayfish of the family Parastacidae (electronic supplementary material, video S1). Although often regarded incorrectly as parasites [16], temnocephalans feed on small animals in the water surrounding their host and possibly on organisms fouling the host's branchial chamber [17], suggesting that their association with parastacids should be regarded as commensal to mutualistic [5,18].

The main radiations of both temnocephalans and parastacids [19–21] are on landmasses of Gondwanan origin; primarily Australia [22,23]. Thirty-two species of *Temnosewellia* and 13 of *Temnohaswellia* are now recognized from *Euastacus* hosts in Australia [24,25] (with the genus comprising 52 species [26]). Thus, the association between these two taxa is potentially both ancient and specific [19]. Fossil-calibrated, molecular-clock reconstructions indicate that *Euastacus* and its sister genus *Astacopsis* diverged in the Cretaceous, approximately 116 Ma [22]. Since then, Australia has experienced major environmental change associated with the break-up of Gondwana (which began around 165 Ma) [22,27]. From approximately 90 Ma, uplift of the great dividing range (GDR) [28] established the main river basins seen today (figure 1) [29]. During this period, Australia drifted northwards by approximately 30° (from 43 to 10° S) [29]. This was accompanied by broad climate warming and considerable drying, with a contraction of high rainfall biomes [29–31]. Consequently, it has been suggested that modern *Euastacus* (particularly the high-altitude species of northern Queensland) represent relicts of a cooler past climate [32,33].

The total range of *Euastacus* extends from South Australia and Victoria (around 39° S) to northern Queensland (around 16° S; figure 1). Species occur from sea level to altitudes of around 2000 m [34]. Most *Euastacus* have small geographical ranges. This is particularly evident in Queensland where populations are restricted to mountain-top streams in high rainfall areas with dense rainforest cover, and are likely to have narrow thermal tolerances and limited dispersal potential [34–37]. In the face of these narrow ranges, and possible anthropogenic threats, the majority of *Euastacus* have been assessed by the International Union for Conservation of Nature (IUCN) [38] as endangered or critically endangered, including all northern Australian species included in this study (figure 2). Concomitantly, these threats may apply to their temnocephalan symbionts [4] especially where alternative hosts are limited or unavailable. Coextinction risk for Australian temnocephalans has, however, never been formally assessed [7,38].

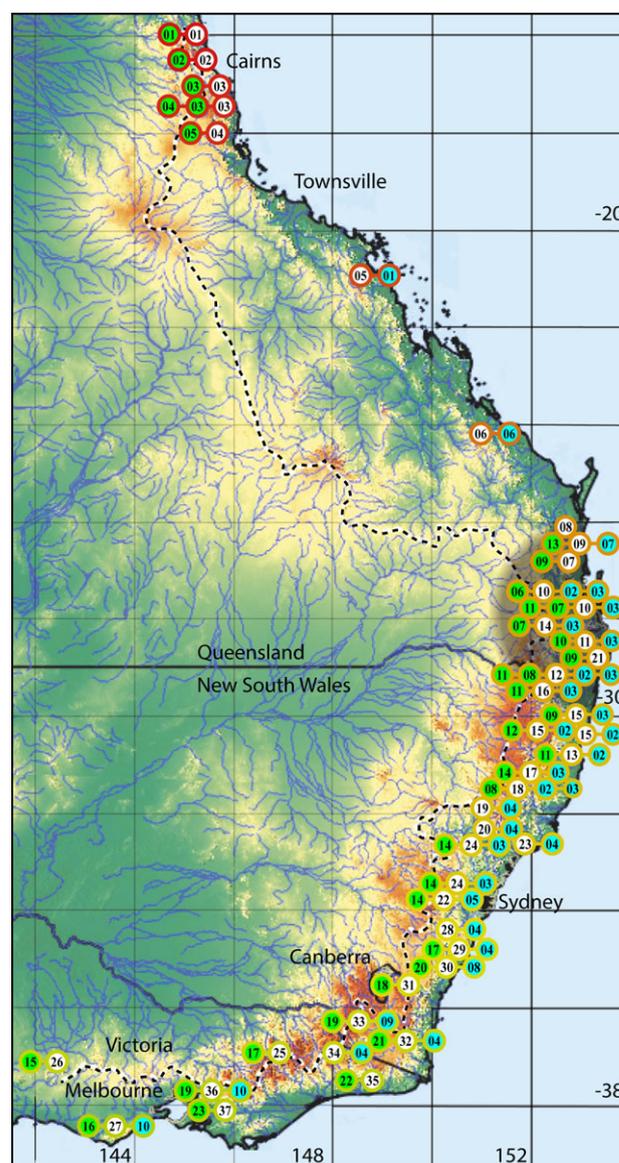


Figure 1. Geographical sampling locations for specimens of *Euastacus* crayfish (white-filled circles), and associated temnocephalan symbionts of genera *Temnosewellia* (green-filled circles) and *Temnohaswellia* (cyan-filled circles). Species numbers correspond to those in figure 2, outline colours correspond to sampled latitude (from red, 16° S in North Queensland, to green, 39° S in Victoria). The GDR is indicated by a dashed line. Shading indicates the MacPherson–Macleay overlap (MMO) zone at the Queensland/NSW border.

Here, we reconstruct ecological and evolutionary associations of the temnocephalan genera *Temnohaswellia* and *Temnosewellia* with *Euastacus* crayfish through deep time using Bayesian molecular phylogenetics, divergence dating, phylogeography, and cophylogeny. This enables us to assess long-term changes in distributions during a period of major environmental change, evaluate the extent of historical codivergence and host specificity, and ultimately to predict which temnocephalan lineages are at the greatest risk of coextinction.

2. Material and methods

(a) Sample collection

Thirty-seven *Euastacus* species were sampled (under appropriate state collecting permits) from locations across the entire geographical range (figure 1 and electronic supplementary material, table S1). Geographical gaps in sampling (e.g. mid-

northern latitudes, figure 1) were places where spiny mountain crayfish do not occur. Rarely, *Euastacus* species co-occur with other crayfish, particularly *Cherax* species, which might offer alternative hosts to temnocephalans (with relevance for coextinction risk). In these locations, any *Cherax* specimens found were collected and observed associations recorded (electronic supplementary material, table S1). Each crayfish collected was examined by eye and using a dissecting microscope to detect temnocephalans. Those observed were removed and preserved in cold 100% ethanol. Subsequently, anterior ends of representative worms were used for DNA analysis, whereas posterior ends (acting as hologenophores) were processed following [25] and used in that taxonomic study. Queensland Museum registration numbers of hologenophores are in the electronic supplementary material, table S1. Unless otherwise stated, analyses reported here used temnocephalans identified based on their morphology and for which sequences of both the nuclear 28S rRNA gene and the mitochondrial *cox1* gene were available (electronic supplementary material, table S1). Additional temnocephalan specimens, identified using morphological criteria, for which no (or only partial) sequence data were available often increased the known host and geographical ranges for the temnocephalan species (electronic supplementary material, table S1) and therefore contributed information for discussion.

(b) Molecular dataset

For *Euastacus* species, DNA was sequenced for four house-keeping genes: mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*), 12S and 16S, plus nuclear 28S [33]. The data obtained have previously been analysed and published [22,33]. For temnocephalans, portions of the nuclear 28S rRNA gene and the mitochondrial *cox1* were amplified using PCR and sequenced using the PCR primers. Primers used were '425F' (5'-GGNGCTAGNTCNATWTTAGGRGC-3') and 'new 1200R' (5'-CCCATTGAWAMNACATAATGAAAATG-3') for *cox1*, and 'Ltem180' (5'-GAAGTTCGCACGATTGCGG-3') and 'Ltem1000R' (5'-CACAAGCATAGTTCACC-3') for 28S. Sequences were aligned using the program MUSCLE [39] and processed with Gblocks to remove poorly aligned positions [31]. Temnocephalan DNA sequences were deposited in the GenBank database (accession nos. KX095257–KX095394, electronic supplementary material, table S1).

(c) Phylogenetic analyses

Bayesian phylogenetic analyses were conducted using the programs BEAST [40] to analyse concatenated genes and *BEAST [41] to conduct multi-locus population coalescent analyses (input xml files are provided in the Dryad Data Repository). For the multi-locus analyses, a linked tree was specified for the non-recombining mitochondrial loci. Bayesian Markov chain Monte Carlo (MCMC) chains used 400 million steps with a burn-in of 25%. Chain output was inspected to confirm convergence using the programs TRACER v. 1.5 (Rambaut and Drummond, 2003–2009) and FIGTREE (Rambaut, 2006–2012). To ensure that the phylogenetic results were robust across the different phylogenetic methods (programs BEAST, *BEAST, SPREAD (phylogeography) [42]), estimated topologies were compared using the percentage of shared clades, calculated in MESQUITE v. 2.75 [43]. Phylogenetic analyses of *Euastacus* used sequences from *Astacopsis tricornis* and *Paranephrops zealandicus* as outgroups. For *Temnosewellia*, outgroups were *Ts. minor* and *Ts. dendyi* from species of *Cherax*.

The evolutionary distinctiveness (ED) of each species was measured based on the species-level, time-calibrated molecular phylogenies, incorporating genetic diversity and evolutionary history (after [44]) using MESQUITE. ED is a measure of conservation priority, calculated by dividing the phylogenetic diversity [45] of each clade among its member species [44]. For each

crayfish species, we also calculated the evolutionarily distinct and globally endangered (EDGE) score [44], which weights ED by probability of extinction. Input extinction probabilities were based on the assessed IUCN Red List conservation status (least concern = 0.025, near threatened = 0.05, vulnerable = 0.1, endangered = 0.2, critically endangered = 0.4) following [44,46].

(d) Divergence dating

Substitution rate calibrations for the crayfish and temnocephalans used an uncorrelated relaxed molecular clock and an average invertebrate substitution rate of 0.0176 substitutions per site per Ma for mitochondrial *cox1* (based on [47]). A fossil node calibration of 116 Ma was also used for the divergence of crayfish family Parastacidae [22], applied as a normal prior on the age of the most recent common ancestor for the tree.

(e) Phylogeography

Phylogeographic histories of the studied clades were reconstructed based on a continuous spatial and temporal diffusion model in the program SPREAD [42]. This uses Bayesian inference to estimate both phylogeny and the geographical locations at the ancestral nodes of the tree, based on the input DNA sequences and the latitude and longitude at which each specimen was sampled (electronic supplementary material, table S1).

The geographical range of each *Euastacus* species (its total extent of occurrence) and minimum recorded altitude was extracted from the IUCN assessment report [36,38]. For each temnocephalan species, these data were then used to estimate the sum of recorded host ranges, average host range, and average host minimum altitude. These values were then compared with average sampled species latitudes (electronic supplementary material, table S1). Non-parametric Spearman rank-order tests were used to test correlations between these variables, with the program PAST [48], after Shapiro–Wilk normality tests typically showed non-normal distribution of data (electronic supplementary material, table S5).

(f) Cophylogeny

Cophylogeny analyses used the inferred phylogenies of *Euastacus*, *Temnosewellia*, and *Temnohaswellia*, plus observed associations between individual, gene-sequenced temnocephalans and their individual gene-sequenced hosts (electronic supplementary material, table S1). In a small number of cases, temnocephalans came from another individual of the same host species collected at the same locality.

Pairwise cophylogeny correlation tests [49] were conducted using TREEMAP v. 3 [50] to determine if the phylogenies of associated crayfish and temnocephalans were more similar than expected by chance. This tests the statistical significance of pairwise distance correlations between associated subtrees, by comparison against an expected random distribution (generated by repeatedly randomizing the symbiont tree). Unlike most cophylogeny methods, this test can accommodate multihost symbioses, as observed for 40% (4/10) of *Temnohaswellia* species and 30% (7/23) of *Temnosewellia* species.

To aid interpretation, event-based cophylogeny analyses were also performed on simplified trees (showing the relationships between the major clades of crayfish and temnocephalans) using the program JANE v. 4 [51], with the default cost regime (codivergence 0, duplication 1, duplication and host-switch 2, loss 1, failure to diverge 1).

(g) Host specificity

A Kruskal–Wallis test was used to compare the median number of symbionts hosted by northern plus far-northern, central, and southern *Euastacus* species (labelled in figure 2), based on all observed symbioses (electronic supplementary material, table S1).

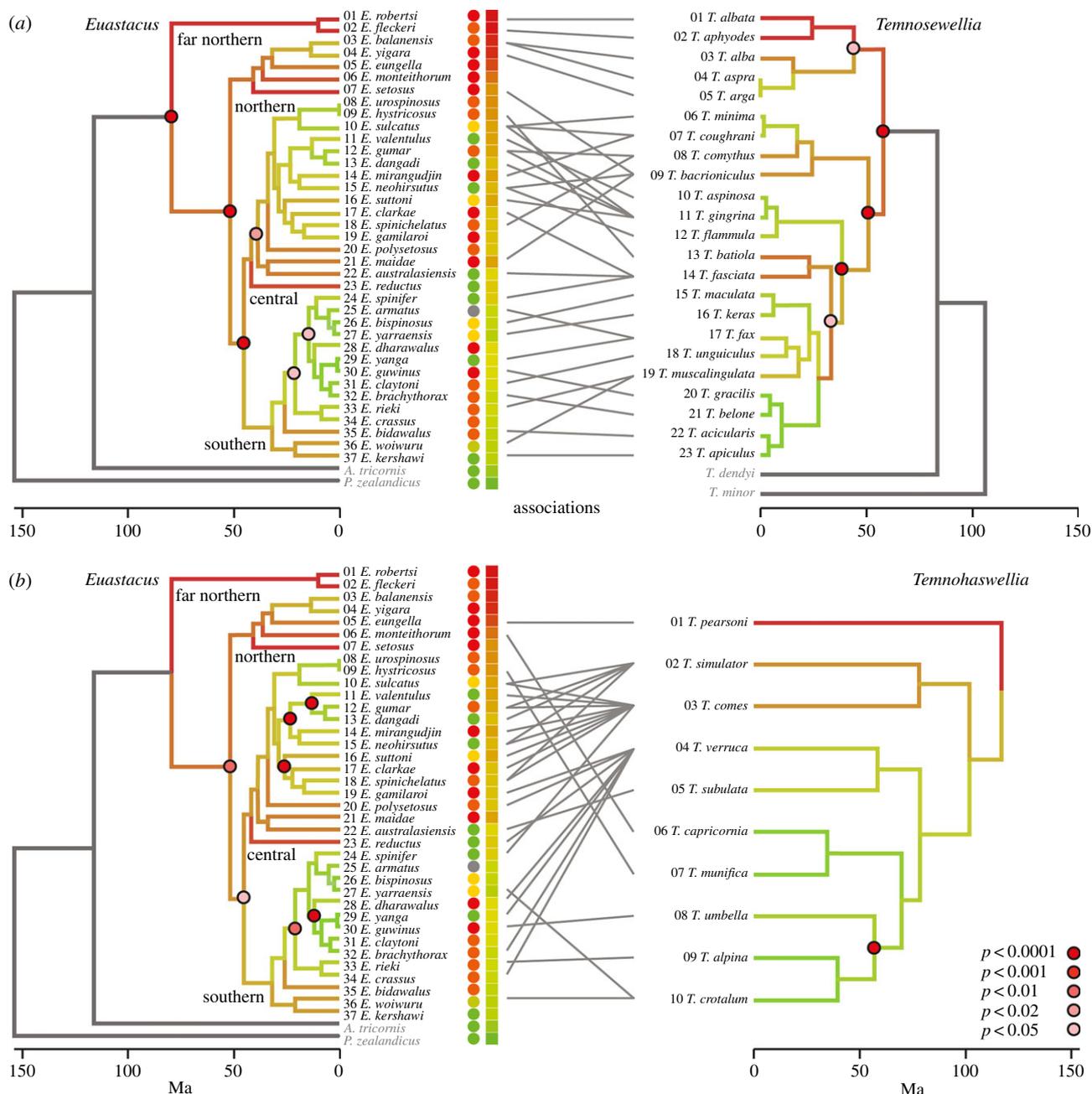


Figure 2. Cophylogeny correlations between the species-level Bayesian phylogenies of *Euastacus* and *Temnosewellia* (a) or *Temnohaswellia* (b). Grey lines: observed symbioses. Coloured circles at nodes: p -value of a pairwise cophylogeny correlation test (key to shading at bottom right). Coloured squares: average latitude of sampled crayfish specimens, north (red) to south (green). Coloured circles: conservation status of crayfish species (green, least concern; yellow-green, near threatened; yellow, vulnerable; orange, endangered; red, critically endangered; grey, not yet assessed) [38]. Colours of branches: evolutionary distinctiveness from most distinctive (red) to least distinctive (green).

This test is a non-parametric equivalent to ANOVA that can accommodate differences in sample sizes among test groups [48].

(h) Coextinction

The complete dataset of observed symbioses (electronic supplementary material, table S1) was used to construct an 'affiliation matrix' (following [9]), recording the presence (scored 1) versus absence (scored 0) of a given symbiont species on a given host species. We then estimated temnocephalan coextinction, given the extinction of a certain proportion of host species, based on repeated random sampling of hosts (without replacement and with 500 replicates) [9,11]. Because symbiont extinction risk may be affected by host extinction probabilities [4], patterns of coextinction were compared under equal versus weighted host extinction probabilities. Weighted probabilities were based on the assessed IUCN Red List conservation status, as described above. In each case, the loss of ED was also

estimated by taking the average of the calculated ED values for the temnocephalans remaining extant in each sampling replicate (i.e. the predicted loss of currently existing ED if certain species suffered coextinction). All coextinction analyses were performed using Matlab scripts written by J.F.H.C.

3. Results

(a) Phylogenetic results

Recognized temnocephalan and crayfish species were mostly confirmed to be monophyletic in our specimen-level analyses (electronic supplementary material, figures S1, S3, S5), with two exceptions discussed below (see also [33]). Consequently, further phylogenetic analyses were conducted at the species level using the Bayesian multi-locus coalescent model of *BEAST [41].

Reconstructed phylogenetic topologies were generally robust, regardless of phylogenetic method: with BEAST and *BEAST trees sharing 87% of clades for both *Euastacus* and *Temnosewellia*, and BEAST and SPREAD (phylogeography) trees sharing 92% (*Euastacus*), 80% (*Temnosewellia*), and 100% (*Temnohaswellia*) of clades (summarized in electronic supplementary material, table S2). For *Temnohaswellia*, however, the phylogenetic estimates based on specimen-level and species-level analysis shared only 50% of clades (figure 2 and electronic supplementary material, figure S17). The conflict relates to the relationships between the more southerly species (after the divergences of *Th. pearsoni*, *Th. simulator*, and *Th. comes*, placements of which are common to both trees). The very low posterior probabilities for relevant nodes suggest that there is comparatively little phylogenetic signal among the central and southern *Temnohaswellia* species (electronic supplementary material, figures S3 and S4).

The EDGE scores (electronic supplementary material, table S3) estimated on our phylogeny for *Euastacus* (figure 2) were compared against those recently estimated across a large synthetic phylogeny of global freshwater crayfish [46]. For 12 *Euastacus* species common to both studies, the ranks of the estimated EDGE scores were highly congruent (with a linear correlation coefficient of 92% and $p = 0.00002$).

(b) Cophylogeny

Cophylogeny correlation tests showed that associated phylogenies (of *Euastacus* and either *Temnosewellia* or *Temnohaswellia*), estimated at both the specimen level (electronic supplementary material, figure S17) and species level (figure 2), were more similar in topology than expected by chance. Specifically, these analyses identified multiple subtrees, indicated by coloured circles at internal nodes (figure 2), for which the associated taxa were more closely related to each other than expected by chance based on repeated randomizations of tree topology (with $p < 0.05$). Five of 22 internal nodes (23%) in the phylogeny of *Temnosewellia* showed statistically significant cophylogeny correlations with the *Euastacus* phylogeny (for which six out of 28, or 21%, of nodes showed significant cophylogeny correlations in return). For *Temnohaswellia*, only one out of nine internal nodes (11%) showed a statistically significant cophylogeny correlation, although seven out of 23 (30%) nodes in the corresponding *Euastacus* phylogeny showed significant p -values.

Two multihost species of *Temnohaswellia*, *Th. simulator* (associated with central clade crayfish), and *Th. verruca* (associated with southern crayfish) exhibit statistically significant cophylogeny correlations at their ancestral nodes on the specimen-level phylogeny (electronic supplementary material, figure S17b). This indicates that the hosts of each multihost temnocephalan species are more closely related than would be expected by chance, which is likely to have contributed to the significant cophylogeny correlations found at the base of some associated *Euastacus* clades (figure 2b and electronic supplementary material, figure S17b). No similar cophylogeny correlations are found for multihost species of the genus *Temnosewellia* (electronic supplementary material, figure S17a). However, the lower number of host species per multihost symbiont means that sample sizes for the detection of preferential host switching are also smaller.

(c) Divergence dating

Bayesian phylogenetic divergence dating indicated that *Euastacus* and both associated temnocephalan genera are Cretaceous in age, with overlapping Bayesian confidence intervals (BCIs) on the estimated ages of each most recent common ancestor (MRCA; electronic supplementary material, table S4 and figures S1–S7; figure 2). Initial analyses without codon partitioning of the *cox1* gene showed a younger age for *Temnosewellia* (26–29 Ma, electronic supplementary material, figure S6), with BCIs that did not overlap those for the genus *Euastacus*. However, partitioning of the mitochondrial *cox1* locus by codon position indicates an age for *Temnosewellia* (106 Ma) that is broadly compatible (given the associated confidence intervals) with the unpartitioned estimated origination ages for *Temnohaswellia* (118 or 132 Ma, respectively, for the coalescent and concatenated sequence analyses) and *Euastacus* (79 or 80 Ma; electronic supplementary material, figure S7 and table S6). The relative substitution rates estimated at the third codon position for *Temnosewellia* (substitutions per site per Ma: mean 2.867, BCI 2.779–2.944) were markedly higher than those for positions one (mean 0.122, BCI 0.047–0.207) and two (mean 0.011, BCI 0.004–0.020), suggesting this was the cause of the age discrepancy. A similar *cox1* codon-partitioned analysis for the genus *Temnohaswellia*, conducted for comparison, also indicated an older age of origination than the unpartitioned analysis, although with a large associated BCI which still overlapped that for *Euastacus* (224 Ma, BCI 83–519 Ma).

(d) Phylogeography and evolutionary distinctiveness

Figure 3 shows reconstructed phylogeographic histories for crayfish specimens and associated temnocephalans sampled from across the *Euastacus* range (figure 1). These reconstruct ancestral locations in the Murray–Darling river basin for the sampled species in each clade.

Non-parametric Spearman rank-order tests indicated a highly significant, negative correlation between the average sampled latitude for *Euastacus* species (with latitude increasing south to north) and their geographical range size ($D = 12\,435$, $p = 0.003$); a weakly significant, positive correlation between *Euastacus* latitude and minimum altitude ($D = 2\,005$, $p = 0.049$); and a significant, negative correlation between altitude and range ($D = 4\,945$, $p = 0.007$). For *Temnosewellia*, average host latitude also showed significant negative correlations with sum host range ($D = 2\,887$, $p = 0.043$) and average host range ($D = 2\,959$, $p = 0.028$), as well as a significant positive correlation with host altitude ($D = 7\,334$, $p = 0.007$). The host range and minimum altitude for *Temnohaswellia*, which included a smaller number of sampled species (10) and does not occur across the full *Euastacus* range, were not found to be significantly correlated with latitude (sum range $D = 226$, $p = 0.266$; average range $D = 254$, $p = 0.1$; altitude $D = 160$, $p = 0.346$).

Importantly, there was also a highly significant, positive correlation between *Euastacus* species latitude and ED ($D = 4\,087$, $p = 0.002$, figure 2 and electronic supplementary material, figure S11). The genus *Temnosewellia* showed a complementary significant, positive correlation between ED and average host latitude ($D = 854$, $p = 0.007$, figure 2a and electronic supplementary material, figure S12). By contrast, the genus *Temnohaswellia* did not ($D = 128$, $p = 0.519$, figure 2b and electronic supplementary material, figure S13).

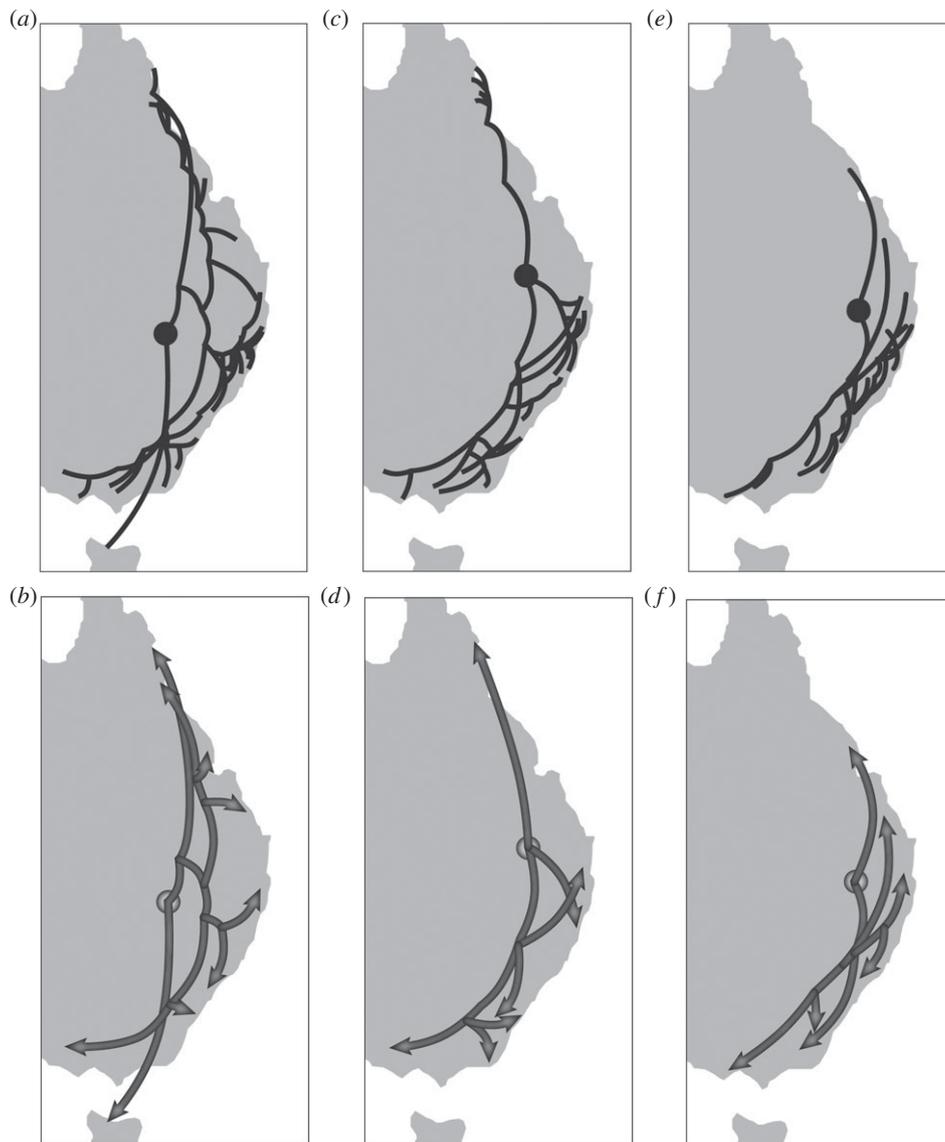


Figure 3. Detailed (top) and simplified (bottom) phylogeographic histories of *Euastacus* crayfish (a,b) and their *Temnosewellia* (c,d) and *Temnohaswellia* (e,f) symbionts. Circles indicate the reconstructed location of the MRCA of the species within each focal genus.

(e) Host specificity

Geographical variation is evident in patterns of observed host specificity. A Kruskal–Wallis test indicated significant differences in the number of symbiont species hosted by the northern plus far-northern, central, and southern *Euastacus* clades ($H = 7.4$, $p = 0.016$). Mann–Whitney pairwise comparisons indicated that the northern *Euastacus* species tend to host significantly fewer symbionts (median = 2) than members of the central (median = 3, $p = 0.07$) or southern clade (median = 3, $p = 0.006$), with no significant difference between the central and southern clades ($p = 0.97$). Most *Temnosewellia* and *Temnohaswellia* associated with northern *Euastacus* are specific to a single host species. Only one temnocephalan with multiple hosts (*Ts. bacrioniculus*) is associated with any member of the northern *Euastacus* clade, and that host species (*E. setosus*) is the most southerly of this group (figures 1 and 2). In contrast, 46% of temnocephalans associated with central and southern *Euastacus* species have more than one host.

Multihost species of *Temnohaswellia* have the greatest numbers of host species (with an average of 5.5 hosts among specimens used in the cophylogeny analyses, 12 when all records were included). The greatest number of

host species was eight in the case of *Temnohaswellia comes* (cophylogeny analyses), and this symbiont was identified on the basis of morphology and/or partial molecular data from a further 14 host species (electronic supplementary material, tables S1, S6, and figure S14). The host species were predominantly from the central clade, and virtually all members of that clade hosted *Th. comes*.

Multihost *Temnosewellia* species were associated with a lower number of hosts (2.1 on average, among specimens included in the cophylogeny analyses, three when all records were included). Three of the least host-specific species (*Ts. fasciata*, *Ts. bacrioniculus*, and *Ts. gingrina*) were associated primarily with the central *Euastacus* clade (figures 1–3). Given the paraphyletic nature of *Ts. bacrioniculus* in the specimen-level molecular phylogeny (electronic supplementary material, figures S5 and S17), this taxon might represent more than one species. *Temnosewellia gingrina* also presented as polyphyletic in the specimen-level molecular phylogeny and occurred on four host species in the central group (electronic supplementary material, figures S5 and S17 and table S1). In the molecular trees, *Ts. gingrina*, *Ts. flammula*, and *Ts. aspinosa* are very closely related (figure 2a). The three species occur in northern NSW/SE Queensland on

related host species (electronic supplementary material, table S1). Therefore, they might represent a species complex and the apparent polyphyly of *Ts. gingrina* may be resolved by recognition of cryptic species in the future.

(f) Coextinction

Computer-simulated coextinction analyses predict a curvilinear relationship between the proportion of temnocephalan symbionts undergoing coextinction and the proportion of *Euastacus* host species extinct (electronic supplementary material, figures S18–S20, S22). This is the expected relationship where at least some symbionts have more than one host species (whereas linear coextinction is predicted if each symbiont is associated with a single host [9]). Endangered *Euastacus* species (critically endangered, endangered, or vulnerable, following [9]) represent 75% (27/36) of IUCN-assessed species in our phylogenetic dataset (with one IUCN data-deficient species). If this proportion of *Euastacus* species were indeed to go extinct, our simulations predict that 60% (19/33) of the studied temnocephalan species would be lost to coextinction (electronic supplementary material, figure S19). Results for the complete affiliation matrix (electronic supplementary material, figure S18) and ingroup only (electronic supplementary material, figure S19) were highly similar, suggesting that the occasional observed presence on *Cherax* hosts may provide comparatively little protection from coextinction (with 58% temnocephalan coextinction predicted with 75% host extinction). When the two studied temnocephalan genera were analysed separately, predicted coextinctions were particularly high for *Temnosewellia* (74% of hosts endangered, predicting 62% coextinction; electronic supplementary material, figure S20) and somewhat lower for *Temnohaswellia* (71% of hosts endangered, predicting 46% coextinction; electronic supplementary material, figure S22).

With equal probabilities of extinction for all host species, the average ED across the surviving symbionts actually increases as a greater proportion of hosts are knocked out (electronic supplementary material, figures S21 and S23). This is to be expected, because the ED distributions are positively skewed (because most species have comparatively low ED and a few have high ED). However, when simulated host extinction probabilities are weighted more realistically [52], according to the IUCN assessment, the average ED for surviving *Temnosewellia* species declines progressively as host species go extinct (electronic supplementary material, figure S21). In contrast, ED values for the smaller *Temnohaswellia* dataset are more similar for equal and weighted extinction probabilities, although slightly lower ED values were also observed with weighted host extinction (electronic supplementary material, figure S23).

4. Discussion

(a) An ancient association between spiny mountain crayfish and their temnocephalan symbionts

Overall, the results suggest that several processes have been involved in producing the pattern of host–symbiont relationships, with important roles for both codivergence and host switching. The statistically significant topological similarities found between phylogenies of *Euastacus*, *Temnosewellia*, and *Temnohaswellia* (figure 2 and electronic supplementary

material, figure S17) indicate linked cophylogenetic histories for these symbionts and their hosts. Correspondingly, our time-calibrated Bayesian phylogenies are compatible with near-synchronous diversifications since the Cretaceous, indicating ancient associations between these genera (electronic supplementary material, table S4 and figure 2). This also applies to major subclades within each genus that have statistically significant cophylogeny correlations (figure 2).

For *Temnosewellia*, five deep nodes in the species-level phylogeny, associated with the major far-northern, northern, central, and southern crayfish clades, show significant cophylogeny correlations (figure 2a). The most basal of these nodes, dated to 58 Ma (BCI: 34–104 Ma, electronic supplementary material, figure S6) and with a highly significant cophylogeny correlation *p*-value of less than 0.0001 (figure 2a, right), marks the divergence between northern *Temnosewellia* species and the remainder. This is comparable to the ages of the far-northern and northern crayfish clades (figure 2 and electronic supplementary material, figure S17) dated to 79 Ma (BCI 56–106 Ma) and 50 Ma (BCI 35–68 Ma, electronic supplementary material, figures S1 and S2) respectively, and is compatible with an ancient codivergence event (electronic supplementary material, figure S8). There is some evidence for preferential [50] (or ‘clade-limited’ [53]) host switching (a phylogenetically and geographically limited host switch) between the two major northern *Euastacus* lineages (electronic supplementary material, figure S8), because the *Temnosewellia* species associated with these lineages form a monophyletic group, whereas the northern and far-northern *Euastacus* clades constitute a paraphyletic grade (figure 2 and electronic supplementary material, figure S17). Topologically similar patterns of divergence between more southerly *Euastacus* and *Temnosewellia* species are compatible primarily with codivergence events (figure 2a).

In contrast, the species-level cophylogeny analysis for *Temnohaswellia* (figure 2b) found only one symbiont subtree with a statistically significant cophylogeny correlation ($p < 0.0001$). This is the southernmost clade (*Th. umbella*, *Th. alpina*, and *Th. crotalum*: MRCA BCI 22–96 Ma, figure 2b, electronic supplementary material, figure S4). The age of the MRCA of the associated (southern) clade of *Euastacus* species (22–44 Ma) is compatible with codivergence (electronic supplementary material, figure S2). However, the additional influence of preferential host switching is suggested by the lack of perfectly isomorphic phylogenetic relationships between the associated species (electronic supplementary material, figure S10).

(b) Geographic influences on dispersal, host specificity, and host switching

The inferred mid-range location of the ancestor for both *Euastacus* and associated temnocephalans (figure 3) is compatible with linked phylogeographic radiations, largely owing to early and sequential separation of northern species (with continental movement and warming). It is possible, however, that the true ancestral location for the genus *Euastacus* was further south (the sister genus, *Astacopsis*, occurs only in Tasmania), but not recoverable because of subsequent events (geological, climatic, and/or biogeographic).

The phylogeographic reconstructions for both *Temnosewellia* and *Temnohaswellia* indicate that the biogeographic histories of species associated with central and

southern crayfish were more complex than those for the comparatively isolated northern lineages (figure 3c–f). Examples of dispersal are evident among central and southern temnocephalans, sometimes involving invasions of biogeographic regions already occupied by other temnocephalan lineages. *Temnohaswellia* has also undertaken a secondary incursion into the northern region (albeit its most southerly part) in the form of *Th. capricornia*. The closely related *Th. munifica* is associated with *E. hystriocosus*, a comparatively northerly member of the central crayfish clade. Both associations are compatible with a long-distance host switch by this temnocephalan lineage (electronic supplementary material, figure S9).

Host switching is a likely concomitant of dispersal and, in our study, is most commonly seen in the southern half of the latitudinal range. This suggests that host switching by temnocephalan species (as well as some intraspecies populations associated with different hosts) has been geographically as well as phylogenetically preferential. In this system, host relatedness and geographical proximity (both factors affecting host-switching opportunities [54]) are linked, as a result of the major latitudinal component in the radiation of *Euastacus* (figures 1 and 2).

The crayfish species hosting the largest number of temnocephalan species were *E. sulcatus* and *E. valentulus* (figure 2 electronic supplementary material, tables S1 and S6), both from the Queensland/NSW border region (figure 1). Therefore, these species are likely to represent key hosts for the conservation of temnocephalan biodiversity [52]. This is a region of high biodiversity, the MacPherson–Macleay overlap (MMO) zone, where temperate and tropical Australian biota overlap [55,56]. Many species of *Euastacus* occur in close proximity here, likely facilitating exchange of temnocephalans and thereby reducing coextinction risk [10]. Tallebudgera creek and its tributaries, located at the Queensland/NSW border, contain three species of *Euastacus* (*E. madae*, *E. valentulus*, and *E. sulcatus*) with several temnocephalan species in common. Elsewhere, sympatry of different *Euastacus* species is less common and generally involves species of different subgroups within the genus (as reviewed in [57]). Surprisingly, *E. armatus*, the crayfish species with by far the greatest geographical range [58], is only known to host two *Temnosewellia* and three *Temnohaswellia* species.

With regard to the potential for taxonomically wider host switching, most *Euastacus* are not found in sympatry with *Cherax* [25], the most widespread genus of Australian crayfish. In three cases, however, *Temnosewellia* species (*Ts. alбата*, *Ts. argeta*, and *Ts. cestus*—electronic supplementary material, table S1) were recovered from *Cherax* in locations where *Euastacus* species are known (or very likely) to occur. This indicates a potential for opportunistic host switching between genera, even if this is only of local and short-term evolutionary significance, though this may be unlikely to significantly lessen coextinction risk for the genus as a whole.

(c) Geographical trends in extinction risk for *Euastacus* and their temnocephalans

The more northerly crayfish (see also [46]) and their associated *Temnosewellia* species exhibit greater ED owing to their early divergence, comparative geographical isolation and, correspondingly, high genetic distinctiveness. In addition, the geographical ranges of northerly *Euastacus* species (and their temnocephalans) tend to be smaller than those of

southern species and lie at higher altitudes (see also qualitative discussion in [33]). A recent study estimating EDGE scores across a large sample (60%) of global freshwater crayfish species found extremely high values for *Euastacus* species, with several (including the northernmost, *E. robertsi*) within the 10 highest estimated EDGE scores [46].

Geographical range size is an important predictor of extinction risk [59] and a key variable in conservation assessment [60], which may in turn be used to calculate combined metrics of distinctiveness and extinction risk such as EDGE and phylogenetic endemism [61]. Contrary to some previous examples from other taxa [59,62,63], our study demonstrates that ED and range size can be statistically non-independent, in this case owing to a shared dependence on latitude.

Because both ED and host extinction risk tend to be higher at northern latitudes (figure 2), coextinction is more probable among more distinctive lineages. Consequently, when host extinction risk is taken into account, coextinction simulations predict an increasing loss of ED in *Temnosewellia* as a greater proportion of *Euastacus* species go extinct (electronic supplementary material, figure S21).

(d) Conclusions: an ancient invertebrate association on the edge of coextinction?

Together, these results indicate considerable geographical variation in the extent and strength of long-term ecological and evolutionary associations, with significant implications for relative coextinction risk. Both cophylogeny (linked phylogenetic histories) and host specificity (clade specificity and number of hosts) indicate consistent latitudinal patterns, which we attribute to the differential effects of long-term climate drying and habitat fragmentation (see also [32]). In particular, our results suggest that opportunities for host switching have been (and likely remain) far greater in central and southern Australia, in line with larger host species ranges, higher range overlap, and the greater host diversity [54]. In contrast, temnocephalan lineages (particularly *Temnosewellia*) associated with more geographically isolated, northern crayfish show greater host specificity and a stronger signal of ancient evolutionary association. These geographical, ecological, and evolutionary differences have a major effect on the relative extinction risks both for *Euastacus* species and their associated temnocephalans. In particular, northern temnocephalans observed to be associated with only a single geographically restricted and isolated crayfish host species (figures 1–3) are particularly vulnerable to coextinction with their hosts, all of which (in the northern and far-northern groups) are currently endangered or critically endangered [38,64]. These distinctive northern lineages, isolated during much of the Tertiary by environmental change, represent approximately 80–100 Myr (electronic supplementary material, table S4) of shared ecological and evolutionary history, but are now increasingly threatened by ongoing climate warming, drying, and habitat fragmentation [65]. Further south, however, many other species of *Temnohaswellia* and *Temnosewellia* also occur only on a single host species which is itself listed as endangered or critically endangered (figure 2). Notable among these are *Th. umbella* and *Ts. gracilis* unique to *E. guwinus*, a crayfish species occurring in an area of perhaps 10 km² [36] and classed by the IUCN as critically endangered. Consequently, current *Euastacus* endangerment (at approx. 75% of species) predicts

high levels of symbiont extinction (62% and 46%, respectively, for *Temnosewellia* and *Temnohaswellia*). Furthermore, when current IUCN host extinction probabilities are considered, weighted coextinction simulations predict the preferential and progressive loss of the most distinctive *Temnosewellia* lineages, representing the most ancient and specific, symbioses (electronic supplementary material, figure S21).

Since the collections of crayfish and temnocephalans reported here were collected in 2004 and earlier, several additional species of *Euastacus* have been described, mostly from the biodiverse region of NE NSW/SE Queensland [66]. Most of these are relatively small and inconspicuous species with very restricted ranges. These are likely to be of high conservation concern and nothing is yet known about their temnocephalan symbionts. Assessment of extinction risk for all temnocephalan ectosymbionts of endangered crayfish should therefore be a priority for future efforts to extend IUCN Red List criteria [60] to invertebrates.

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