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# Tri-locus sequence data reject a "Gondwanan origin hypothesis" for the African/South Pacific crab genus *Hymenosoma*

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ABSTRACT

Crabs of the family Hymenosomatidae are common in coastal and shelf regions throughout much of the southern hemisphere. One of the genera in the family, *Hymenosoma*, is represented in Africa and the South Pacific (Australia and New Zealand). This distribution can be explained either by vicariance (presence of the genus on the Gondwanan supercontinent and divergence following its break-up) or more recent transoceanic dispersal from one region to the other. We tested these hypotheses by reconstructing phylogenetic relationships among the seven presently-accepted species in the genus, as well as examining their placement among other hymenosomatid crabs, using sequence data from two nuclear markers (Adenine Nucleotide Transporter [ANT] exon 2 and 18S rDNA) and three mitochondrial markers (COI, 12S and 16S rDNA). The five southern African representatives of the genus were recovered as a monophyletic lineage, and another southern African species, *Neorhynchoplax bovis*, was identified as their sister taxon. The two species of *Hymenosoma* from the South Pacific neither clustered with their African congeners, nor with each other, and should therefore both be placed into different genera. Molecular dating supports a post-Gondwanan origin of the Hymenosomatidae. While long-distance dispersal cannot be ruled out to explain the presence of the family Hymenosomatidae on the former Gondwanan land-masses and beyond, the evolutionary history of the African species of *Hymenosoma* indicates that a third means of speciation may be important in this group: gradual along-coast dispersal from tropical towards temperate regions, with range expansions into formerly inhospitable habitat during warm climatic phases, followed by adaptation and speciation during subsequent cooler phases.



## 1. Introduction

The genus *Hymenosoma* is one of 17 genera in the brachyuran family Hymenosomatidae MacLeay, 1838, a group of crabs that tend to be important components of the macrozoobenthic faunas of estuarine, coastal and shelf regions throughout much of southern and eastern Africa, the South Pacific region, South-, East- and Southeast Asia, as well as South America (Lucas, 1980; Ng and Chuang, 1996). The type species of the genus *Hymenosoma* belongs to the southern African representative *H. orbiculare* Desmarest, 1823, and until recently, only two other species were recognised, namely *H. depressum* Hombron and Jacquinot, 1846 from New Zealand and *H. hodgkini* Lucas, 1980 from eastern Australia. Ng et al. (2008) list an obscure fourth species as *?Hymenosoma gaudichaudii* Guérin, 1831.

### 1.1. Taxonomy of Hymenosoma

The taxonomic history of *Hymenosoma* is chequered, and uncertainties remain concerning which species should be placed in this genus. Recently, genetic analyses have shown much potential in resolving long-disputed taxonomic issues by clarifying the taxon-

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omy of its African representative (Edkins et al., 2007). It has long been suspected that *H. orbiculare* may comprise several distinct species (e.g. Lucas, 1980) given its great amount of morphological variation and its occurrence in a wide variety of habitats (including the southern African continental shelf, shallow-water coastal habitats, estuaries and even a relict estuarine lake). Granulose specimens from deeper-water habitats of south-western Africa were originally described as *H. geometricum* Stimpson, 1858, but this species was subsequently treated as a junior synonym of *H. orbiculare* by Stebbing (1914), as it was considered to fall morphologically within the variability of the latter. Genetic data have now shown that specimens that are morphologically intermediate between *H. geometricum* and *H. orbiculare* in fact belong to a third, undescribed species, a discovery that resulted in the re-establishment of *H. geometricum* as a valid species name (Edkins et al., 2007). The genetic data further showed that small-bodied specimens from South Africa's southeast and east coast represent two more undescribed species. Phylogenetic relationships among the five southern African species remained unresolved.

The taxonomic history of *H. depressum* has been as confused as that of *H. orbiculare*. This species was originally assigned to the genus *Hymenosoma* (its original designation being *Hymenosoma depressa* Jacquinot, 1835), but Montgomery (1931) pointed out that it cannot belong to this genus because of the presence of an epistome (which is absent in *H. orbiculare*) and proposed to place it into the genus *Hombronia*. Gordon (1966) considered this generic name to be invalid because it is a junior synonym of *Halicarcinus* White, 1846, and Melrose (1975) then proposed placing the species into a new genus, *Cyclohombronia*. As a result of the discovery of the Australian species *H. hodgkini* (which, like *H. orbiculare*, lacks an epistome, but which also differs from this species in a number of morphological features in which it resembles *H. depressum*), Lucas (1980) restored *H. depressum* to the genus *Hymenosoma*.

### 1.2. Biogeographic hypotheses

The prevalence of hymenosomatid crabs in Africa, Australasia and South America could be an indication that this family is of Gondwanan origin, and the presence of the genus *Hymenosoma* on two of the former Gondwanan land-masses suggests that its African and South Pacific lineages may have diverged as long ago as the break-up of Western and Eastern Gondwanaland (comprising Africa/South America and Madagascar/India/Antarctica/Australia, respectively) during the Middle Jurassic (~165 MYA according to Rabinowitz et al. (1983) or ~175 MYA according to Schettino and Scotese, 2005). Gondwanan origin hypotheses have been suggested to explain the disjunct distributions of various groups of closely related temperate marine species that, like *Hymenosoma*, are represented on former Gondwanan land-masses (e.g. Paine and Suchanek, 1983; Williams et al., 2003; Wood et al., 2007). Using molecular dating, Williams et al. (2003) showed that the distribution of the snail genus *Austrolittorina* was well supported by such a hypothesis, whereas Wood et al. (2007) rejected it for the mussel genus *Perna*. In the case of the genus *Hymenosoma*, the notion that its species are Gondwanan relics is challenged by palaeontological data. Although no fossil Hymenosomatidae are known, the family is often considered to be closely associated with the Majidae Samouelle, 1819 (Guinot and Richer de Forges, 1997), the oldest fossils of which have been found in Eocene deposits (~50–60 MYA; Spears et al., 1992). Post-Gondwanan dispersal scenarios represent alternatives explaining the distribution of the genus. The fact that these crabs have abbreviated larval development (Lucas, 1980) suggests that planktonic dispersal may be limited, but long-distance dispersal of adults seems feasible. Although some are strong swimmers over short distances, none of the species of *Hymenosoma* are capable of sustained swimming, but may cling to floating seaweeds (McLay and Teske, pers. obs.) and therefore be able to disperse over large distances using floating objects as rafts. A further explanation for the observed distribution pattern is gradual along-coast dispersal from one region to the other via Southeast- and South Asia. The hymenosomatid crab genera *Neorhynchoplax* Sakai, 1938 and *Elamena* H. Milne Edwards, 1837, are present in all these regions (e.g. Ng and Chuang, 1996), but the absence of *Hymenosoma* from Asia indicates that this is an unlikely scenario.

To test these hypotheses, we aimed to resolve phylogenetic relationships among all presently-accepted members of the genus *Hymenosoma* using sequence data from three independently-evolving loci. We further intended to determine whether these data support a sister-taxon relationship between the Hymenosomatidae and the Majidae, and used molecular dating to determine whether the origin of either the genus *Hymensoma* or the family Hymenosomatidae could predate the break-up of Gondwanaland.

## 2. Materials and methods

### 2.1. Sample collection and extraction

Hymenosomatid crabs of the genera *Hymenosoma* Desmarest, 1823, *Amarinus* Lucas, 1980, *Elamena* H. Milne Edwards, 1837, *Halicarcinus* White, 1846, *Neorhynchoplax* Sakai, 1938 and *Neohymenicus* Lucas, 1980 were collected in southern Africa, eastern Australia and New Zealand (see Table 1 for names of sampling sites and Fig. 1 for their locations) either by hand or by sieving soft-bottom sediment with a D-frame net (estuarine samples), or by towing a dredge net behind a speedboat (samples from the continental shelf of South Africa). In most cases, samples were preserved in 70% ethanol upon collection, and DNA was extracted using the CTAB method (Doyle and Doyle, 1987) or the DNEasy Tissue Kit (Qiagen). In the case of small-bodied specimens (*H. hodgkini*, *Neohymenicus pubescens* and the undescribed species of *Hymenosoma* from the southeast and east coasts of South Africa), it was found that molecular markers were more likely to amplify if DNA had been extracted from living specimens. Whenever it was not possible to carry out CTAB extractions because of the distance to the nearest laboratory, DNA was extracted in the field using the Chelex® method (Walsh et al., 1991).

### 2.2. Amplification and sequencing

Phylogenetic relationships among the seven presently-accepted species in the genus *Hymenosoma*, and their placement among other genera in the family Hymenosomatidae, were investigated using two nuclear markers (a portion of the Adenine Nucleotide Transporter [ANT] gene and the complete 18S rDNA) and three mitochondrial markers (partial COI, 12S rDNA and 16S rDNA).

Details about the primers used are listed in Table 2. PCR profiles consisted of an initial denaturation step (3 min at 94 °C), 35 cycles of denaturation at 94 °C (30 s), annealing (45 s at marker-specific temperatures, see Table 2) and extension at 72 °C (45 s), followed by a final elongation step (72 °C for 10 min).

### 2.3. Phylogenetic reconstruction

#### 2.3.1. Phylogenetic trees reconstructed using sequence data from individual loci

To compare the phylogenetic signal provided by each individual locus, we constructed phylogenetic trees using parsimony analysis in MEGA v4 (Tamura et al., 2007). Positions containing alignment gaps or missing data were excluded, and default settings were specified for all remaining parameters. Support for nodes was as-

**Table 1**
Species collected, sampling localities, collectors, museum collection numbers (when available) and GenBank accession numbers of hymenosomatid crabs and three outgroup species.

| Species | Locality | Collector | Collection No. | GenBank Accessions | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | ANT gene | 18S rDNA | 16S rDNA | 12S rDNA | COI gene |
| *Hymenosoma orbiculare* Desmarest, 1823 | Rietvlei[SA] | Teske | SAM A45647 | FJ432032 | FJ812331 | FJ812313 | FJ812295 | DQ351395 |
| *H. geometricum* Stimpson, 1858 | False Bay[SA] | Edkins | SAM A45593 | FJ432029 | FJ812332 | FJ812314 | FJ812296 | EF198478 |
| *H.* sp. 1 | False Bay[SA] | Edkins | SAM A45593 | FJ432033 | FJ812333 | FJ812315 | FJ812297 | EF198483 |
| *H.* sp. 2 | Qolora[SA] | Papadopoulos | SAM A45648 | FJ432035 | FJ812334 | FJ812316 | FJ812298 | DQ351416 |
| *H.* sp. 3 | Mzingazi[SA] | Newman | SAM A45649 | FJ432037 | FJ812335 | FJ812317 | FJ812299 | DQ351421 |
| *H. hodgkini* Lucas, 1980 | Hawkesbury[AU] | Sandoval-Castillo | AM P80026 | FJ432030 | FJ812345 | FJ812327 | FJ812309 | FJ812291 |
| *H. depressum* Hombron and Jacquinot, 1846 | Little Akaloa Bay[NZ] | McLay | MNZ CR.13701 | FJ432028 | FJ812339 | FJ812321 | FJ812303 | FJ812285 |
| *Elamena producta* Kirk, 1879 | Kaikoura[NZ] | McLay | MNZ CR.13697 | FJ432022 | FJ812337 | FJ812319 | FJ812301 | FJ812283 |
| *Halicarcinus ovatus* Stimpson, 1858 | Narrabeen[AU] | Teske | AM P80514 | FJ432025 | FJ812342 | FJ812322 | FJ812304 | FJ812286 |
| *H. cookii* Filhol, 1885 | Kaikoura[NZ] | McLay | MNZ CR.13687 | FJ432023 | FJ812341 | FJ812324 | FJ812306 | FJ812288 |
| *H. varius* (Dana, 1851) | Kaikoura[NZ] | McLay | MNZ CR.13690 | FJ432026 | FJ812343 | FJ812325 | FJ812307 | FJ812289 |
| *H. innominatus* Richardson, 1949 | Kaikoura[NZ] | McLay | MNZ CR.13684 | FJ432024 | FJ812340 | FJ812323 | FJ812305 | FJ812287 |
| Amarinus paralacustris (Lucas, 1970) | Dee Why[AU] | Beheregaray | AM P80515 | FJ432020 | FJ812344 | FJ812326 | FJ812308 | FJ812290 |
| *Neohymenicus pubescens* (Dana, 1851) | Kaikoura[NZ] | McLay | MNZ CR.13686 | FJ432038 | FJ812338 | FJ812320 | FJ812302 | FJ812284 |
| *Neorhynchoplax bovis* (Barnard, 1946) | Haga Haga[SA] | Teske | SAM A45650 | FJ432039 | FJ812336 | FJ812318 | FJ812300 | EF198477 |
| *Outgroup:* | | | | | | | | |
| *Leptomithrax sternocostulatus* (H. Milne Edwards, 1851) | Chowder Bay[AU] | McCracken | AM P80226 | FJ812282 | FJ812346 | FJ812328 | FJ812310 | FJ812292 |
| *Paragrapsus laevis* (Dana, 1851) | Hawkesbury[AU] | Teske | AM P80219 | FJ432010 | FJ812348 | FJ812330 | FJ812312 | FJ812294 |
| *Portunus pelagicus* (Linnaeus, 1758) | Sydney Fish Market[AU] | Teske | — | FJ432042 | FJ812347 | FJ812329 | FJ812311 | FJ812293 |

*Abbreviations:* AU, Australia; AM, Australian Museum; MNZ, Te Papa Museum, Wellington; NZ, New Zealand; SA, South Africa; SAM, Iziko Museums of Cape Town.

sessed by generating 10,000 bootstrap replications. For the outgroup, we used the three brachyuran species *Leptomithrax sternocostulatus* (Majidae), *Paragrapsus laevis* (Varunidae) and *Portunus pelagicus* (Portunidae).

All sequences for which length differences were identified were aligned using the program BALI-PHY v2.0.2 (Suchard and Redelings, 2006). This program simultaneously estimates alignment and phylogeny and in this way avoids problems associated with poor starting trees. Alignment of the two mitochondrial rDNA regions was particularly challenging. In order to incorporate as much phylogenetic signal as possible and generate alignments associated with the best possible trees, we created two data-sets, each of which comprised sequences of either 12S or 16S rDNA, combined with all of the parsimony informative sites of the COI sequences (for which no alignment was necessary). The length of these data-sets remained sufficiently small to allow the program to run efficiently. The GTR+I+G model (Rodríguez et al., 1990) was specified and a total of 100,000 iterations were performed. Following examination of log-likelihood scores using TRACER v1.4 (available from http://beast.bio.ed.ac.uk/Tracer) to ensure that stationarity was reached after a burn-in of the first 10% of iterations, maximum *a posteriori* trees and associated alignments were produced. In each case, the procedure was repeated twice to check for consistency of results.

*2.3.2. Phylogenetic trees reconstructed using combined sequence data*

Phylogenetic analyses of combined data-sets can reveal hidden support even for phylogenetic relationships that are in conflict among phylogenies constructed using individual markers (Gatesy et al., 1999). We therefore combined sequence data from the three loci.

Phylogenetic trees for the combined data-set were reconstructed using parsimony as described for the individual loci, as well as the minimum evolution method (Rzhetsky and Nei, 1992) and Bayesian inference. The minimum evolution tree was constructed using pairwise deletion of missing data in MEGA. Evolutionary distances were estimated using the Maximum Composite Likelihood method (Tamura et al., 2004), and positions containing alignment gaps or missing data were deleted in a pairwise fashion. Support for nodes was assessed by generating 10,000 bootstrap replications. Bayesian inference was conducted using MRBAYES v3.1.2 (Ronquist and Huelsenbeck, 2003). The GTR+I+G model was specified for all partitions, and each partition was allowed to have its own rate. Two independent Bayesian inferences were run simultaneously for 3 million generations, each with four Markov Chain Monte Carlo chains (one cold chain and three heated chains). Trees were sampled every 100 generations. Likelihood scores were examined in TRACER to ensure that stationarity was reached after a burn-in of 10%. A 50% majority rule consensus tree was constructed from all remaining trees, and posterior probability values were obtained with the 'sumt' command. To check for consistency of results, posterior probability values and likelihood scores during stationarity were compared for the two runs, and analyses were repeated twice to check for consistency.

*2.4. Molecular dating*

We used the Bayesian method implemented in the program MULTIDIVTIME (Thorne and Kishino, 2002) to estimate divergence times among hymenosomatid crab lineages. MULTIDIVTIME estimates divergence times under a relaxed clock model, and calibration points can be specified as upper or lower bounds on node ages. As no fossil Hymenosomatidae are known and no other divergence events in the phylogeny could be used as calibration points, we combined sequence data of the hymenosomatid crabs with previously published brachyuran sequences from two studies that used molecular dating. The first of these (Porter et al., 2005) used fossil calibration and a phylogeny that included representatives from several major brachyuran families. Molecular dating in the second study (Schubart et al., 1998) was based on the assumption that the divergence between West Atlantic and East Pacific representatives of the grapsid genus *Sesarma* was linked to the well-documented closure of the Central American Seaway.

Fossils used for calibration were *Eocarcinus praecursor* (Withers, 1932), the oldest known fossil of a true crab (specified as 1.9–1.95, i.e. 190–195 MYA) and *Notocarcinus sulcatus* (Schweitzer and Feldmann, 2000), the oldest known fossil representative of the Cancri-

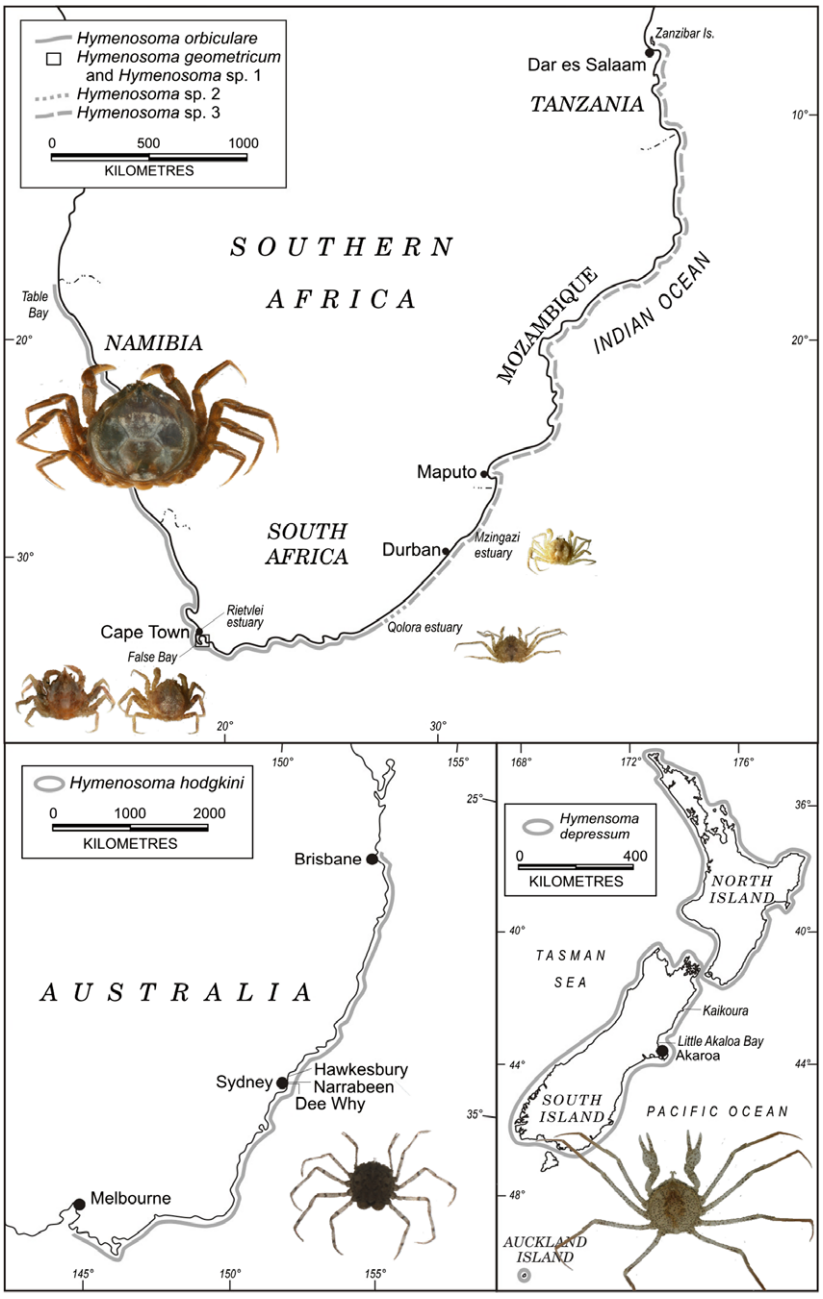


**Fig. 1.** Maps of Africa, eastern Australia and New Zealand showing distribution ranges of the seven presently-accepted members of the genus *Hymensoma*, as well as sampling sites.

nae (0.413–0.49, i.e. 41.3–49 MYA). Upper and lower bounds of calibration points based on divergence of transisthmian sister lineages of *Sesarma* were set to 0.02 (2 MYA) and 0.031 (3.1 MYA), respectively. The latter date is the time of the final closure of the Central American Seaway (Coates et al., 1992), but Schubart (pers. comm.) pointed out that overland dispersal may still have been possible in these species after the closure of the seaway through brackish-water habitats, and gene flow among the *Sesarma* lineages may only have completely ceased more than a million years later.

The program MULTIDIVTIME requires a tree topology, but the placement of the Hymenosomatidae among the brachyurans used in the previous two studies is not certain. Guinot and Richer de Forges (1997) hypothesised that the Hymenosomatidae are more closely related to the Majoidea than to any other brachyuran group, based on morphological similarities between the Hymenosomatidae and the majoid family Inachoididae Dana, 1851. Ng et al. (2008) considered this taxonomic relationship provisional, although they concurred that morphological and larval data do indeed support such a placement. To resolve this issue, we constructed a 50% majority rule consensus tree in MRBAYES using three partitions for which sequence data are available for all of the brachyurans included by Porter et al. (2005), as well as the Hymenosomatidae: 18S, 16S and COI. We aligned 18S and 16S sequences using BALI-PHY as described previously, and then used GBLOCKS v0.91b (Castresana, 2000) to eliminate poorly aligned regions. Default parameters were specified at the GBLOCKS Server (http://molevol.ibmb.csic.es/Gblocks_server.html). Accordingly, we also removed third character positions of the COI sequences to reduce the effect of multiple mutations at the same sites. Test phylogenies reconstructed using the minimum evolution method in MEGA revealed that this did not diminish phylogenetic

**Table 2**
Molecular markers sequenced and primers used.

| Markers | Primer names | Primer sequences | References |
|---|---|---|---|
| ANT[1,4] | DecapANT-F | 5′-CCTCTTGAYTTCGCKCGAAC-3′ | Teske and Beheregaray (2009) |
| | DecapANT-R | 5′-TCATCATGCGCCTACGCAC-3′ | |
| 18S rDNA[2,4,6] | 5′F | 5′-TYCCTGGTTGATYYTGCCAG-3′ | Weekers et al. (1994), Samraoui et al. (2003) |
| | 557F | 5′-GCCAGCAGCCGCGGT-3′ | |
| | 1262R | 5′-GGTGGTGCATGGCCGTY-3′ | |
| | 3′R | 5′-TGATCCATCTGCAGGTYCACCT-3′ | |
| COI[1,3] | CrustCOI-F | 5′-TCAACAAATCAYAAAGAYATTGG-3′ | Teske et al. (2006) |
| | DecapCOI-R | 5′-AATTAAAATRTAWACTTCTGG-3′ | |
| 12S rDNA[1,3] | 12Sai | 5′-AAACTAGGATTAGATACCCTATTAT-3′ | Kocher et al. (1989) |
| | 12SMB | 5′-GAGAGTGACGGGCGATGTGT-3′ | |
| 16S rDNA[1,5] | 16SarL | 5′-CGCCTGTTTATCAAAAACAT-3′ | Palumbi (1996) |
| | 16SbrH | 5′-CGGGTCTGAACTCAGATCACGT-3′ | |

Annealing temperatures: [1]50 °C and [2]54 °C.
$MgCl_2$ concentrations: [3]6 mM, [4]3 mM and [5]2 mM.
[6]Two portions of the 18S rDNA were amplified separately using primer combinations 5′F – 1262R and 557F – 3′R.

signal: phylogenies based on first and second character positions had the same topology as trees constructed from combined sequence data, whereas phylogenies constructed using only third character positions did not recover the Hymenosomatidae as a monophyletic lineage. In MRBAYES, 18S rDNA, 16S rDNA, COI first codon positions and COI second codon positions were each specified as a separate partition. The method was otherwise identical to that used in Section 2.3.2. For the outgroup we used *Homarus* sp.

Three different analyses were conducted in MULTIDIVTIME to compare the effects of specifying different calibration points and using different combinations of molecular markers. The first included both fossil calibration points and calibration points based on the divergence of the species of *Sesarma*. Only 18S and 16S rDNA sequences were used for molecular dating in this case, because the partial COI sequences of *Sesarma* spp. generated by Schubart et al. (1998) are from a different portion of the gene. No 18S rDNA sequences are available for this genus either, but in this case we considered it appropriate to use the sequence of the closely related *Pachygrapsus marmoratus* for all six *Sesarma* species. Given that 16S rDNA sequences are much more variable than 18S rDNA sequences, and that the 16S rDNA sequences of these species are very similar (maximum divergence: 5%), we considered it reasonable to assume that the signal potentially provided by 18S rDNA would be insignificant relative to that of 16S rDNA at this taxonomic level. The second method excluded the species of *Sesarma* and included the reduced COI data-set used for phylogenetic reconstruction. The third method used calibration points based on the divergence of the species of *Sesarma* only and included sequence data from a single marker, 16S rDNA.

Sequences used for phylogenetic placement of the Hymenosomatidae among other brachyurans, and those used for molecular dating, are listed in Table 3. Settings in MULTIDIVTIME were as follows: *rttm* (prior expected number of time units between tip and root): 1.9 (190 MYA; values of *rttm* should be between 0.1 and 10); *rtrate* (mean of the prior distribution for the rate at the root node: 0.5; *brownian*: 0.8; *bigtime*: 100. The magnitude of the standard errors was set equal to that of the parameters *rttm*, *rtrate* and *brownian*. The Markov chain was sampled 50,000 times, with 100 cycles between each sample and a burn-in of 50,000 cycles. A second run was then carried out with 100,000 samples and a burn-in of 100,000 cycles. Congruence between the two runs was considered to indicate that the program had been run for sufficiently long to obtain reliable estimates. We then repeated all three runs by doubling all priors to rule out the possibility that the choice of priors affected the results.

**Table 3**
Sequences of taxa used for molecular dating and to determine the placement of the Hymenosomatidae among other brachyurans.

| Species | 18S rDNA | 16S rDNA | COI | Reference |
|---|---|---|---|---|
| *Cancer pagurus* | DQ079743 | DQ079708 | | Porter et al. (2005) |
| *C. productus* | | | DQ882044 | Costa et al. (2007) |
| *Carcinus maenas* | DQ079744 | DQ079709 | | Porter et al. (2005) |
| | | | DQ523686 | Roman (2006) |
| *Geothelphusa* sp. | DQ079750 | DQ079715 | | Porter et al. (2005) |
| *G. tawu* | | | AB266297 | Shih et al. (2007) |
| *Homarus gammarus* | DQ079749 | DQ079714 | | Porter et al. (2005) |
| *H. americanus* | | | DQ889104 | Costa et al. (2007) |
| *Maja squinado* | DQ079758 | DQ079723 | | Porter et al. (2005) |
| | | | EU000835 | Sotelo et al. (2008) |
| *Necora puber* | DQ079759 | DQ079724 | | Porter et al. (2005) |
| | | | DQ480362 | Pan et al. (2008) |
| *Pachygrapsus marmoratus* | DQ079763 | DQ079728 | | Porter et al. (2005) |
| *P. crassipes* | | | AY952139 | Cassone and Boulding (2006) |
| *Sesarma reticulatum* | DQ079763[a] | AJ225867 | | Schubart et al. (1998) |
| *S. rhizophorae* | DQ079763[a] | AJ225851 | | Schubart et al. (1998) |
| *S. curacaoense* | DQ079763[a] | AJ225870 | | Schubart et al. (1998) |
| *S. crassipes* | DQ079763[a] | AJ225869 | | Schubart et al. (1998) |
| *S. sulcatum* | DQ079763[a] | AJ225853 | | Schubart et al. (1998) |
| *S. aequatoriale* | DQ079763[a] | AJ225874 | | Schubart et al. (1998) |

[a] No 18S rDNA sequence is available for the grapsid genus *Sesarma*; a sequence of the closely related *Pachygrapsus marmoratus* was used instead.

## 3. Results

### 3.1. Characterisation of sequence data

All sequences generated in this study were submitted to GenBank (Table 1), and characteristics of the five markers used are listed in Table 4. As the ANT gene has not previously been used in crustacean phylogenetics, we explored it in more detail. The primer annealing site of forward primer DecapANT-F was located close to the intron (whenever one was present), and most trace files therefore did not contain readable exon sequences at their 5′ ends. The second exon region, on the other hand, was readily recognisable in all sequences and followed an AG element indicating

**Table 4**
Characterisation of molecular markers.

| | | Molecular marker | | | | |
|---|---|---|---|---|---|---|
| | | ANT exon 2 | 18S rDNA | 16S rDNA | 12S rDNA | COI gene |
| Sequence length (bp) | | 246 | 1736 | 534 | 375 | 598 |
| Variable sites | | 62 | 115 | 257 | 281 | 252 |
| Parsimony informative | | 42 | 49 | 192 | 154 | 219 |
| Per codon position | 1 | 10 | | | | 45 |
| | 2 | 4 | | | | 9 |
| | 3 | 26 | | | | 165 |
| Percent informative sites | | 17 | 3 | 14 | 18 | 37 |
| Mean nucleotide composition (%) | T: | 28.3 | 24.4 | 37.1 | 39.8 | 37.5 |
| | C: | 24.1 | 23.8 | 15.1 | 8.4 | 18.0 |
| | A: | 17.5 | 24.9 | 37.8 | 38.4 | 27.0 |
| | G: | 30.1 | 26.9 | 10.0 | 13.4 | 17.2 |
| Transitions/transversions | | 1.1 | 1.4 | 0.8 | 0.7 | 1.0 |

Statistics were calculated in MEGA based on BALI-PHY alignments of all 15 ingroup taxa.

the end of the intron. Introns were found in all species except *Amarinus paralacustris* (the longest being >834 bp in length in *Hymenosoma hodgkini*), but in most cases, these were considered too divergent to be alignable. We limited the use of the ANT sequences to the second exon region. Following removal of ambiguous sites from the 3′ end of the sequences, this partition was 246 bp in length.

### 3.2. Phylogenetic reconstructions

#### 3.2.1. Phylogenetic trees reconstructed using sequence data from individual loci

Phylogenies reconstructed using individual loci (Fig. 2) were largely congruent and recovered the following patterns: (a) monophyly of the southern African representatives of *Hymenosoma*, (b) monophyly of *H. geometricum* and *H.* sp. 1, (c) monophyly of the South Pacific species *Elamena producta*, *Neohymenicus pubescens*, *Hymenosoma depressum* and the four representatives of *Halicarcinus*, and (d) monophyly of the species of *Halicarcinus*. The placement of *Hymenosoma hodgkini* and *Amarinus paralacustris* varied. In Fig. 2a, their position among the other Hymenosomatidae was unresolved, whereas in Fig. 2b and c, they were recovered as sister taxa, and basal to the other South Pacific species, with high bootstrap support.

#### 3.2.2. Phylogenetic trees reconstructed using combined sequence data

All three methods of phylogenetic reconstruction using combined sequence data from all three loci recovered congruent trees (Fig. 3), except that the exact placement of species differed within the cluster comprising *Hymenosoma depressum* and the genera *Neohymenicus*, *Elamena* and *Halicarcinus*. While the relationship between these genera remained unresolved, their monophyly was strongly supported. All three methods further recovered the monophyly of the southern African representatives of *Hymenosoma* and their sister-taxon relationship with *Neorhynchoplax bovis*, the monophyly of all South Pacific species, and the monophyly of *Hymenosoma hodgkini* and *Amarinus paralacustris*. Within the southern African *Hymenosoma* cluster, phylogenetic relationships were well resolved with high nodal support, with the exception of the sister-taxon relationship of *Hymenosoma* sp. 2 and the cluster comprising *H. orbiculare*, *H. geometricum* and *H.* sp. 1.

#### 3.2.3. Molecular dating

A 50% majority rule consensus tree of phylogenetic trees sampled using MRBAYES recovered the Hymenosomatidae as a monophyletic lineage (PP [posterior probability]: 1.00) and in this case, phylogenetic relationships among all the African species were well resolved. *Geothelphusa* sp. was recovered as the sister taxon of the

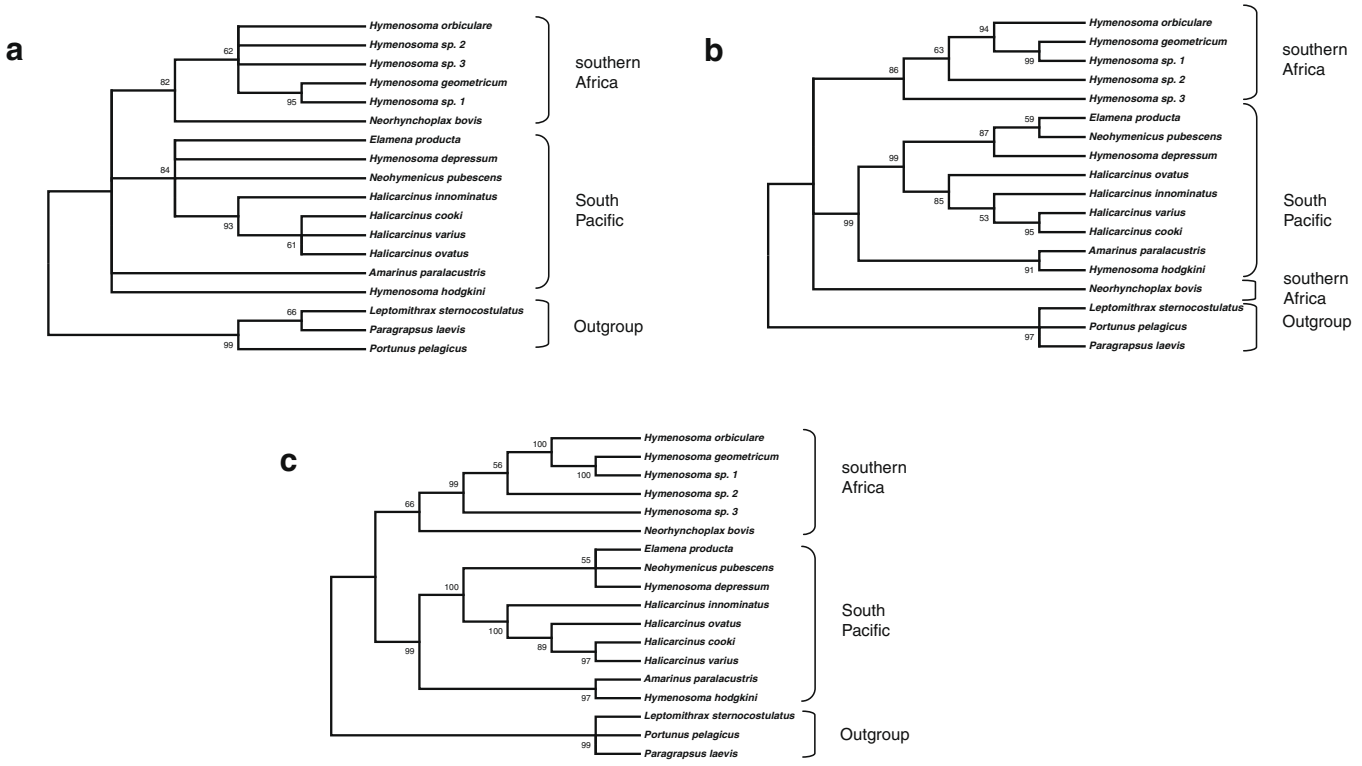


**Fig. 2.** Phylogenies of hymenosomatid crabs from southern Africa and the South Pacific (Australia and New Zealand) from three independently-evolving loci inferred using parsimony analysis; (a) nuclear ANT exon 2; (b) nuclear 18S rDNA; (c) mitochondrial DNA (16S rDNA, 12S rDNA and COI gene). Nodal support ($\geqslant$50%) from 10,000 bootstrap replications is indicated on the left of some clades.

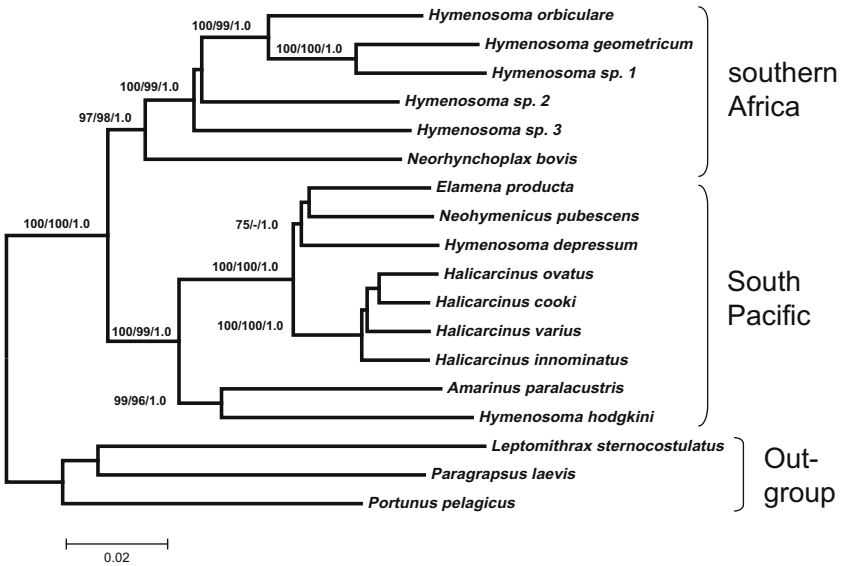


**Fig. 3.** A minimum evolution tree of combined sequence data of hymenosomatid crabs from southern Africa and the South Pacific (Australia and New Zealand). Partitions included nuclear ANT exon 2 and 18S rDNA, as well as mitochondrial 16S rDNA, 12S rDNA and COI. Strong support for nodes (bootstrap values ⩾75% and posterior probabilities ⩾0.95) from three methods of phylogenetic reconstruction (minimum evolution, parsimony and Bayesian inference) are indicated on the left of some clades.

Hymenosomatidae (PP: 0.99), whereas *Maja squinado* was in a basal position in this tree. With the exception of a node supporting the monophyly of *Carcinus* and *Necora* (PP: 1.0), nodes associated with older divergence events were not strongly supported (PP < 0.95). The topology used for molecular dating was consequently based on the brachyuran subtree published by Porter et al. (2005), with the Hymenosomatidae placed as the sister lineage of *Geothelphusa* sp., and *Sesarma* spp. placed as sister to *Pachygrapsus* sp. (Fig. 4).

Divergence time estimates of the first two methods of molecular dating were almost identical, irrespective whether the divergence events of the *Sesarma* species were included or excluded as calibration points, and whether or not COI sequences were included (Table 5). The origin of the Hymenosomatidae was placed during the Middle Oligocene (mean ± SD: 29 ± 7 and 30 ± 8 MYA) and that of the African and South Pacific lineages during the Late Oligocene (African: 24 ± 8 and 25 ± l7; South Pacific: 27 ± 8 and 25 ± 7 MYA). Estimates for the third method, which were based on a single marker (16S rDNA) and excluded fossil calibration points, were consistently lower, although 95% confidence intervals overlapped.

## 4. Discussion

Even though hymenosomatid crabs are widespread throughout the southern hemisphere and some species are the most abundant soft-bottom brachyurans in their region of occurrence (Poore, 2004; Teske, pers. obs.), they have attracted little scientific attention, possibly because many have a small body size and cryptic colouration. To our knowledge, the work on hymenosomatid crabs from southern Africa and the South Pacific presented here is the first successful attempt to resolve phylogenetic relationships within the family. In most cases, the sequence data employed were suitable to resolve these relationships with high support, and long-established taxonomic notions based on misleading morphological characters could be rejected.

### *4.1. Rejection of the monophyly of Hymenosoma*

Phylogenies reconstructed from three independently-evolving loci recovered the southeast African species *Neorhynchoplax bovis* as the sister-taxon of the southern African representatives of *Hymenosoma*. This strongly suggests that the South Pacific species presently placed in the genus *Hymenosoma* should be assigned to other genera. *Hymenosoma depressum* from New Zealand was found to be closely related to species of the genera *Elamena*, *Neohymenicus* and *Halicarcinus*. Genetic distances between these were lower than those among southern African representatives of *Hymenosoma*. Despite this, we consider the morphological differences evident among these taxa, as well as the much lower genetic distances among the different representatives of the genus *Halicarcinus*, to be sufficient to justify maintaining each as a separate genus. We consequently consider it most appropriate to return *Hymenosoma depressum* to the formerly-proposed genus *Cyclohombronia* (Melrose, 1975). The absence of an epistome prompted Lucas (1980) to conclude that "*H. hodgkini* n. sp. is clearly a *Hymenosoma* species". However, support for a sister-taxon relationship of *H. hodgkini* with *Amarinus paralacustris* (which was particularly strong in the combined genetic analyses) indicates that the importance of this morphological character was overestimated. *H. hodgkini* and *A. paralacustris* are nonetheless genetically very different from each other, and for that reason, we consider it appropriate that *H. hodgkini* be placed into a new genus.

### *4.2. Biogeography and evolution of the southern African species of Hymenosoma*

Phylogenetic relationships among the southern African representatives of *Hymenosoma* were particularly well resolved and allow the reconstruction of the group's evolutionary history. The basal taxon in this lineage is the small-bodied species from the African east coast (*Hymenosoma* sp. 3), a species whose distribution ranges from the South African east coast to possibly Zanzibar (although it is not yet known whether additional lineages occur in this region). The fact that the small-bodied species from South Africa's southeast coast (*Hymenosoma* sp. 2) is the next basal species in the group (i.e. it has a sister-taxon relationship with the remaining species rather than with the morphologically very similar *Hymenosoma* sp. 3, a relationship that was particularly well supported in the data-set used for molecular dating that excluded poorly aligned regions) indicates that the small body size and shallow water habit of these species may be representative of the

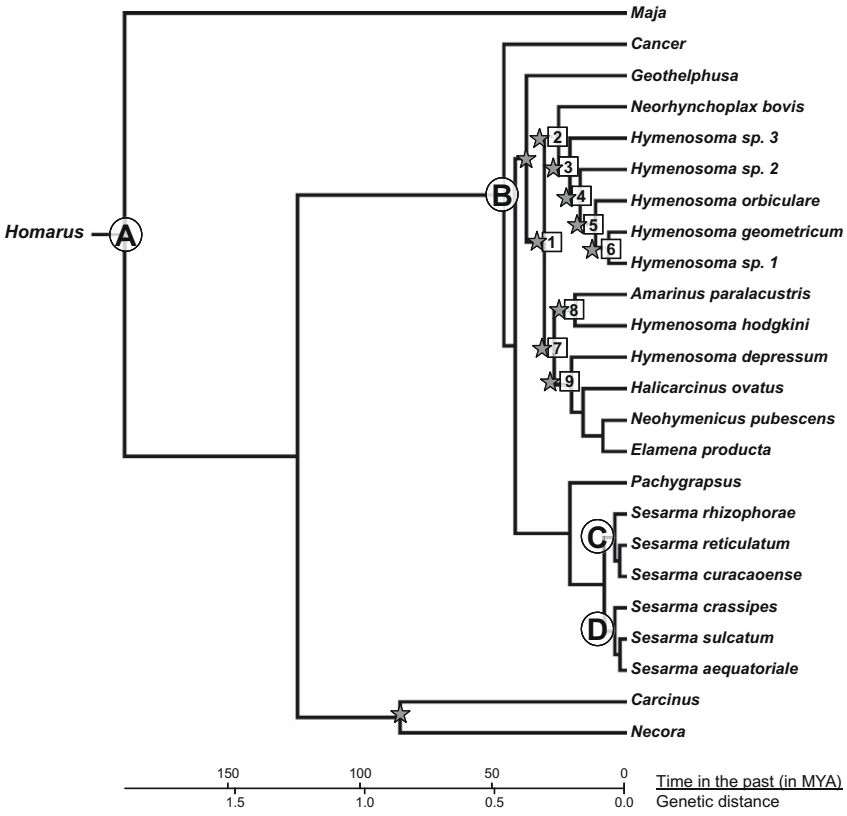


**Fig. 4.** A MULTIDIVTIME chronogram depicting divergence times of hymenosomatid crab species in relation to other brachyurans, based on 18S and 16S rDNA sequences. The topology was based on that in Porter et al. (2005), and the placement of the Hymenosomatidae among other brachyurans was obtained by reconstructing phylogenetic relationships in MRBAYES using 18S and 16S rDNA, as well as COI. Grey stars indicate nodes that were strongly supported in this analysis (posterior probability $\geqslant 0.99$; the phylogeny did not include the *Sesarma* spp. because the corresponding portion of the COI gene was not available). Calibration points are indicated as white circles, and letters within these indicate the following: (A) age of *Eocarcinus praecursor*, the earliest known true crab species (Withers, 1932); (B) age of *Notocarcinus sulcatus*, the oldest known representative of the Cancrinae (see Schweitzer and Feldmann, 2000); (C and D) divergence of geminate *Sesarma* spp. present on either side of the isthmus of Panama; it was assumed that these diverged as a result of the final closure of the Central American Seaway, between 2.0 and 3.1 MYA. Divergence events of interest are indicated as white squares, and numbers within these are listed in Table 5. The scale bar indicates F84 distances (the evolutionary model implemented in the program MULTIDIVTIME) at the bottom and corresponding divergence time in million years ago on top. The chronogram depicted was obtained by specifying upper and lower bounds for each calibration point. See Table 5 for standard deviations and confidence intervals, as well as for estimates obtained using alternative methods.

ancestral condition in the genus. This hypothesis is further supported by the fact that one of the remaining three species (*H. orbiculare*) is also confined to shallow water, and that its juveniles are morphologically very similar to the adults of *H.* sp. 2 and 3 (Edkins et al., 2007). The derived position of the two monophyletic deeper-water species (*H. geometricum* and *H.* sp. 1) among the southern African representatives of *Hymenosoma* indicates that the genus established itself on the South African continental shelf comparatively recently.

The present-day distribution patterns of the African species of *Hymenosoma* indicate a range expansion from the tropical Western Indian Ocean southwards into South Africa. Using molecular dating, this was estimated to have occurred during the Early Miocene and could be explained by the fact that southern Africa experienced considerably warmer climatic conditions during that time (Flower and Kennett, 1994). This and subsequent divergence time estimates are based on the results from Methods 1 and 2, which we consider to be more precise than those of Method 3 because they incorporate more sequence data. Moreover, a number of studies have shown that most of the geminate species pairs present on either side if the Isthmus of Panama diverged prior to the complete closure of the seaway (e.g. Duda and Rolán, 2005), and the estimates from Method 3 are therefore likely to postdate the nodes' true ages. The first split in the phylogeny of the African species of *Hymenosoma* (approx. $20 \pm 8$ MYA) may have resulted from climatic cooling in southern Africa, as the populations present on the south-east coast and farther west became physiologically adapted to the new conditions. The next divergence event (a split between lineages on the southwest coast vs. the southeast coast that was estimated to have occurred between $16 \pm 7$ and $17 \pm 6$ MYA may have been linked to the onset of cold-water upwelling on South Africa's west coast (Siesser, 1980), again resulting in adaptation of populations affected by the new conditions. Teske et al. (2007) found a comparable divergence scenario in the estuarine snail *Nassarius kraussianus* (Dunker, 1846) in this region. This species underwent a range expansion into south-western Africa during the previous interglacial, but unlike most other warm-water molluscs whose fossil record also indicates a range expansion during this time (Tankard, 1975), its range did not contract during subsequent cooling. This suggests that the western populations became adapted to colder water. Such physiological adaptation to differences in water temperature among sister lineages of southern African coastal invertebrates have been reported for the mudprawn *Upogebia africana* (Teske et al., 2008) and the mussel *Perna perna* (Zardi et al., unpublished data).

The establishment of *Hymenosoma* in deeper waters of the south-west coast was estimated to have occurred between $10 \pm 0.03$ and $11 \pm 6$ MYA during the Early Late Miocene (Tortonian), followed by a further speciation event between $5 \pm 0.02$

**Table 5**
Divergence time estimates among lineages of hymenosomatid crabs as calculated in MULTIDIVTIME.

| Node | Divergence time estimate in MYA (±SD) [95% confidence interval] | | |
|---|---|---|---|
| | Method 1 | Method 2 | Method 3 |
| 1 | 30 (±8) | 29 (±7) | 15 (±9) |
| | [12–43] | [16–42] | [5–39] |
| 2 | 24 (±8) | 25 (±7) | 13 (±8) |
| | [7–39] | [12–39] | [4–35] |
| 3 | 20 (±8) | 20 (±7) | 11 (±7) |
| | [6–35] | [8–34] | [3–30] |
| 4 | 16 (±7) | 17 (±6) | 9 (±6) |
| | [4–31] | [7–31] | [2–24] |
| 5 | 11 (±6) | 10 (±5) | 6 (±4) |
| | [2–24] | [3–22] | [1–17] |
| 6 | 6 (±4) | 6 (±4) | 3 (±3) |
| | [0.3–17] | [1–15] | [0–11] |
| 7 | 27 (±8) | 25 (±7) | 12 (±8) |
| | [10–40] | [13–38] | [4–33] |
| 8 | 19 (±7) | 18 (±6) | 7 (±6) |
| | [6–34] | [8–32] | [0–22] |
| 9 | 20 (±7) | 15 (±6) | 9 (±6) |
| | [7–35] | [6–30] | [2–25] |
| A | **192 (±1)** | **192 (±1)** | |
| | **[190–195]** | **[190–195]** | |
| B | **46 (±2)** | **46 (±2)** | |
| | **[42–49]** | **[42–49]** | |
| C | **2.6 (±0.3)** | | **2.6 (±0.3)** |
| | **[2.0–3.1]** | | **[2.0–3.1]** |
| D | **2.6 (±0.3)** | | **2.6 (±0.3)** |
| | **[2.0–3.1]** | | **[2.0–3.1]** |

Node numbers and letters correspond to those depicted in Fig. 4. A maximum of four calibration points were specified. Nodes A and B were calibrated on the basis of fossil ages, whereas nodes C and D were based on the divergence between geminate sister lineages of the brachyuran genus *Sesarma* that are believed to have diverged as a result of the closure of the Central American Seaway. Three different methods of calibration were used; nodes used in each case are indicated in bold.
Method 1: All calibration points included, data-set included 18S and 16S rDNA, and the 18S rDNA sequence of *Pachygrapsus* was used for all *Sesarma* species.
Method 2: Fossil calibration points only, data-set included 18S and 16S rDNA, as well as COI; *Sesarma* species were excluded.
Method 3: No fossil calibration points, data-set included 16S rDNA only.

and 6 ± 4 MYA during the Late Late Miocene (Messinian). Little is known about microhabitat preferences of the two deeper-water species, but the fact that *H. geometricum* collected in dredge samples tended to be associated with the large ascidian *Pyura stolonifera* (Heller, 1878), while the other species was not (Edkins et al., 2007), suggests that the larvae of the two preferentially settle in different habitats. There is some evidence that hymenosomatid crab species of the genus *Elamena* may be symbiotically associated with echinoderms and abalone (genus *Haliotis*; Poore, 2004), and it is likely that a similar relationship exists between *H. geometricum* and *P. stolonifera*. Too little ecological and phylogeographic information on the two species is presently available to determine conclusively what drove their divergence, but it appears that the parapatric speciation event that gave rise to the ancestor of the two deeper-water species may have been followed by what could be considered sympatric speciation. The shared distribution of the deeper-water species is particularly unusual when compared with the strict geographic separation of the shallow-water species (Fig. 1).

### 4.3. Phylogenetic relationships of the southern temperate Hymenosomatidae

The hymenosomatid crabs studied here were divided into two distinct regional clusters that were each associated with components of the former Gondwanan supercontinent. However, divergence time estimates of ~30 MYA (Oligocene) for Methods 1 and 2 and ~15 MYA (Miocene) for Method 3 considerably postdate the break-up of Eastern and Western Gondwanaland during the mid-Jurassic (~175–165 MYA) hypothesised to have resulted in the divergence of the African and South Pacific representatives of the Hymenosomatidae. The idea of a Gondwanan origin of the two lineages of hymenosomatid crabs is further weakened by the fact that most of the South Pacific genera are also common in regions that were not part of Gondwanaland (including East- and Southeast Asia), and that the African species *Neorhynchoplax bovis* has congeners in India, Sri Lanka, China and elsewhere (Ng and Chuang, 1996). The presence of hymenosomatid crabs in remote regions such as Mauritius, the Maldives Archipelago, New Caledonia and Palau Island indicates that despite their abbreviated larval development, some of these crabs have a reasonably high dispersal potential. This supports the feasibility of post-Gondwanan dispersal scenarios. Specimens from unsampled regions may eventually be useful to understand how hymenosomatid crabs established themselves in their region of occurrence and where their centre of origin was located. The markers used here are likely to prove useful to resolve the phylogeny of the family.

### 4.4. Phylogenetic placement of the family Hymenosomatidae

The Hymenosomatidae are often considered to be closely related to the Majoidae, and together with the Parthenopidae and Mimilambridae, these families have been grouped in the section Oxyrhyncha (Felder et al., 1985). Our genetic data did not support the monophyly of this group, but instead recovered *Geothelphusa* in a strongly-supported sister-taxon relationship with the Hymensomatidae. It must be noted, however, that sequences of all three markers used to determine the phylogenetic placement of the Hymenosomatidae (18S rDNA, 16S rDNA and COI) have been generated for relatively few brachyuran genera, and the sister-taxon relationship of these two morphologically very different groups is likely to be merely an artefact of incomplete taxon sampling.

In a study investigating phylogenetic relationships among representatives of the Majoidea, Hultgren and Stachowicz (2008) found little support for the monophyly of several families, and suggested that some of the morphological characters used to classify the majoids may be subject to convergent evolution. The placement of the Hymenosomatidae among these was not explored, and a phylogenetic tree constructed using two mitochondrial markers that were used both in their study and ours (16S and COI) was largely unresolved (not shown). A representative of the Inachidae (*Podochela hemiphillii*) was recovered in a sister-taxon relationship with the Hymenosomatidae, but with low bootstrap support (54%), and as in our phylogeny used for molecular dating, *Maja* was recovered in a basal position in the tree. In another recent phylogenetic study of brachyurans based on 18S rDNA sequences, Ahyong et al. (2007) recovered the hymenosomatid crab included in their study (*Amarinus paralacustris*) as the sister taxon of *Dorippoides facchino*, and both were closely related to the majoid *Schizophrys aspera*. These phylogenetic relationships were, however, poorly supported in terms of jackknife values and Bayesian posterior probabilities. We therefore suggest that the molecular markers employed in our study and in these two previous studies are insufficient to resolve phylogenetic relationships among the Hymenosomatidae and other brachyuran lineages, and consider our conclusion that the Hymenosomatidae are not part of the Majoidea to be preliminary. The genetic data presented here and elsewhere need to be complemented with additional samples of the taxa presently grouped under the Majoidea, and additional nuclear markers that contribute phylogenetic signal towards resolving older divergence events (e.g. those used in Tsang et al., 2008 and Mahon and Neigel, 2008) need to be sequenced.

## 5. Conclusion

Sister-taxon relationships of species present in regions that were formerly part of the Gondwanan supercontinent are usually attributed to allopatric speciation, either in the form of vicariance (e.g. van Bocxlaer et al., 2006) or transoceanic dispersal (e.g. Waters and Roy, 2004). The presence of the genus *Hymenosoma* in Africa and the South Pacific region cannot be explained by either hypothesis because of a flawed taxonomy. However, the evolutionary history of the African species of *Hymenosoma* indicates that parapatric speciation linked to climatic oscillations may represent a third mode of speciation, and phylogeographic data from southern Africa (Teske et al., 2007; Teske et al., 2008) indicate that it may play a role in establishing biogeographic patterns in coastal invertebrates in general. The range expansion from tropical to temperate regions identified in the African species of *Hymenosoma* may have analogues among hymenosomatid crab genera represented in the temperate South Pacific region, as several of these have sister taxa in tropical regions to the north (Ng and Chuang, 1996). Extending the sampling range to include hymenosomatid crabs from Asia may therefore greatly enhance our understanding of how the members of this family established a range that reaches from the tropics to cool-temperate regions of the southern hemisphere, and elucidate the relative importance of vicariance events, long-distance dispersal and climate-driven range expansions in establishing these patterns.

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