

Phylogenetic analysis of lineage relationships among hyperiid amphipods as revealed by examination of the mitochondrial gene, *cytochrome oxidase I (COI)*

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Synopsis Among metazoans, crustaceans display the greatest disparity between body plans and are second only to the insects in overall species diversity. Within the crustaceans, the Amphipoda rank as one of the most speciose extant orders. Amphipods have successfully invaded a variety of ecosystems, including the pelagic midwater environment. Despite their abundance in varied and dissimilar habitats, and the use of traditional morphological and systematic comparative analyses, phylogenetic relationships among amphipods remain uncertain. The pelagic amphipods, hyperiids, have highly divergent life histories and morphological attributes in comparison to more familiar benthic, nearshore, intertidal, and terrestrial amphipods. Some of these adaptations are likely correlated with their pelagic life history and include features such as hypertrophied olfactory and visual systems, duplications of the eyes, and an array of modifications to the appendages. Many of these morphological features may represent homoplasies, thus masking the true phylogenetic relationships among extant hyperiid amphipods. Here, we sample a wide range of amphipod taxa for the *COI* gene and present the first preliminary molecular phylogeny among the hyperiids.

Introduction

Amphipoda [Crustacea; Malacostraca; Peracarida] is a monophyletic, species-rich, assemblage (Schram 1986; Schmitz 1992). They occur in nearly all known marine, freshwater, and brackish water environments as well as in highly humid terrestrial ecosystems (Barnard and Karaman 1991; Van Dover 1992; Vinogradov et al. 1996; Poltermann et al. 2000; Takhteev 2000; Serejo 2004). This ecological diversity is reflected in similarly high levels of morphological variation. The unique structure and arrangement of the posterior-most three pairs of appendages (uropods) represent the exclusive, synapomorphic, characters that unite the amphipods as a natural, monophyletic group. They are further distinguished by a combination of additional features including lateral compression of the body, presence of sessile compound eyes, the general orientation of the thoracomere appendages (pereopods) to the body axis, and the close arrangement of the anterior gnathal appendages, including the maxillipeds, into a basket-like shape around the mouth to form a compact buccal mass. The amphipods have traditionally been organized into four groups, the largely

benthic taxa Gammaridea, Caprellidea, Ingolfiellidea, and the exclusively pelagic midwater taxon Hyperiidea (Martin and Davis 2001). The pelagic hyperiid amphipods are a major constituent of crustacean zooplankton (Bowman and Gruner 1973) and in some regions their swarming behavior leads to their being a primary food source for large planktivores (Vinogradov et al. 1996).

The phylogenetic relationships among the hyperiids remain a mystery. In fact, the relationships among, and within, all four of the major amphipod taxonomic groups remain poorly resolved due to conflicting suites of morphological characters currently in use by systematists (Martin and Davis 2001). This presents a conundrum in which we have no large-scale phylogenetic hypotheses for the Amphipoda, a highly successful monophyletic group that has high biological diversity coupled with evolutionary radiations in dissimilar environments. Although there have been recent advances in morphological and molecular analyses among some of the gammaridean groups of amphipods (Meyran et al. 1997; Englisch et al. 2003; Myers and Lowry 2003; Lörz and Held 2004; Serejo 2004; Davolos and Maclean 2005; Macdonald et al. 2005), there have

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been no molecular phylogenetic studies and few comparative morphological analyses among the hyperiids (Pirlot 1932; Coleman 1994; Zeidler 1999, 2003a, 2003b, 2004). This is surprising given that many descriptions of hyperiid species date from the 19th century and nearly all midwater plankton tows, at any depth, recover representatives from the group. Some of the major biological and evolutionary questions that rely on a hypothesis of lineage relationships, and thus remain unaddressed in this group include how patterns of biological diversity arise, how biological form and function are linked to evolutionary radiations, and in the particular case of the pelagic hyperiids, what aspects of their body plan are required for successful colonization and radiation within midwater niches. Phylogenetic analysis can ultimately address whether an ancestral benthic amphipod stem group invaded the pelagos, or conversely, whether an ancestral pelagic amphipod stem group invaded the benthos, or whether there has been a more complex interplay between hyperiid lineages with regard to colonization of oceanic midwater habitats.

An accurate phylogenetic assessment using purely morphological features is problematic. Thus far, there is no single synapomorphy known for the hyperiids. As a consequence the notion of hyperiid polyphyly has been suggested in some of the major taxonomic works (reviewed by Vinogradov et al. 1996). This suggests extensive convergence, or homoplasy, of groups of morphological attributes required for a pelagic existence and for their frequently observed associations with gelatinous zooplankton.

Convergent evolution and associated character homoplasy are one of the central themes in evolutionary biology and arguably one of the most difficult problems facing phylogenetic reconstruction of relationships within and among lineages. Character homoplasy can easily mask relationships between related lineages (examples among amphipods include structural simplifications such as reductions of the maxilliped, the pleopods, and the uropods). Environmental factors play a strong role in the evolutionary shaping of morphologies through time. Since all hyperiid taxa are pelagic, it is instructive to consider some of the major features affecting organisms in the marine midwater environment. Volumetrically, the oceanic midwater is massive, accounting for ~90–99.8% of the habitable space on the planet (Cohen 1994). Although vast, the oceanic midwater environment is far from being featureless and many biologically relevant partitions

can, and should be, taken into account when considering organismal diversity in the midwater.

One of the most critical parameters affecting midwater organisms is light (reviewed by Warrant and Locket 2004). The average depth of the open ocean is 3800 m. Down-welling ambient light (sun, moon, and starlight) penetrates as deep as 1000 m. Long wavelength light is rapidly absorbed within the first ~150 m of the water column, the epipelagic zone, leaving only shorter wavelength light to illuminate the mesopelagic zone (~150–1000 m depth). The remaining ~75% of midwater habitat, the bathypelagic (~1000–2500 m depth) and abyssopelagic (~2500 m depth and beyond) zones, is characterized by an absence of down-welling surface light. In the bathypelagic and abyssopelagic zones down-welling light is completely replaced by omnidirectional bioluminescent point light sources. Many hyperiids participate in well-characterized cyclical, vertical, diel migrations driven by light, in which zooplankton move upward in the water column at dusk and downward at dawn. This includes many hyperiid species described as being associated with the mesopelagic and bathypelagic midwater zones (Vinogradov et al. 1996). Two additional biologically important parameters of consequence at mesopelagic, bathypelagic, and abyssopelagic depths are dissolved oxygen content and temperature. Between 400 and 1000 m is an oxygen poor water layer of variable thickness, the hypoxic oxygen minimum layer (OML), a niche in which there are particular adaptations such as reduced metabolic rates, improved oxygen uptake, and increased dependence on anaerobic metabolism (Childress and Seibel 1998). Additionally, these depths are characterized by constant temperatures near 4°C.

Another significant physical attribute of the midwater environment is its inherent patchiness. This patchiness, or granularity, creates substantial 3D surfaces with which small clinging planktonic organisms, in particular, can interact. This patchiness is a combination of both the planktonic organisms themselves, as well as a host of other organic aggregates collectively known as “marine snow” (Silver et al. 1978; Robison et al. 2005). Among the Amphipoda, modifications of morphologies associated with a clinging benthic life style could play a role in exploiting the variety of 3D surfaces present in midwater habitats (Laval 1980).

The exclusively pelagic life style of the hyperiids has historically made detailed studies of these amphipods difficult. As *in situ* observations of hyperiids continue to emerge, it is clear that most species have complex life histories involving obligate

commensalism or parasitic relationships with a wide variety of midwater gelatinous zooplankton such as siphonophores, salps, ctenophores, and cnidarians (Harbison et al. 1977; Madin and Harbison 1977; Harbison et al. 1978; Laval 1980; Gasca and Haddock 2004; Gasca et al. 2007). Thus, the availability of surfaces in midwater appears to play an important ecological role among hyperiid amphipods and morphological specializations in different hyperiid lineages may derive from interactions with preferred surfaces.

We have used recent advances in SCUBA technology and modern techniques for collecting in midwater in combination with traditional netting to sample a diversity of hyperiid amphipods for the mitochondrial gene, *cytochrome oxidase I* (*COI*), for a preliminary molecular assessment of hyperiid phylogenetic relationships. *COI* encodes for a subunit of cytochrome oxidase, an enzymatic protein complex absolutely required for aerobic metabolism (Castresana et al. 1994). *COI* was chosen for preliminary analysis for several reasons including maternal inheritance of the mitochondrial genome, reduced occurrence of mitochondrial gene recombination, extensive use of *COI* in phylogenetic reconstructions, and the availability of related sequences from publicly curated databases (Avice 1986; Avice et al. 1987; Simon et al. 1994; Simon et al. 2006).

Materials and methods

Specimen collection

Amphipod specimens were collected using a variety of techniques. Intertidal amphipods were collected by hand. Sub-surface benthic amphipods were collected via snorkeling, open-circuit (OC), and closed-circuit rebreather (CCR) SCUBA (Ambient Pressure Diving Ltd., UK). Pelagic amphipods were collected using a combination of snorkeling, OC, and CCR SCUBA blue-water diving (Hamner 1975; Hamner et al. 1975; Haddock and Heine 2005), remotely operated underwater vehicles (MBARI), ring nets, and opening-closing trawling nets (Childress et al. 1978). Physical vouchers exist for all specimens and are housed at the Kewalo Marine Lab (Honolulu, HI, USA). Specimens of *Parhyale hawaiiensis* came from a breeding colony maintained at the Kewalo Marine Laboratory (Browne et al. 2006) and those of *Jassa slatteryi* came from a breeding colony maintained at the University of California at Berkeley (Patel). Specimens of hyperiid amphipods were identified using the taxonomic keys of Bowman and Gruner (1973), Vinogradov et al. (1996), and Zeidler

(1999, 2003a, 2003b, 2004). Thirteen samples included in this analysis were not identified to species level. They have been assigned temporary names indicating their affinity to described species.

Sequence cloning

Genomic DNA was isolated with DNeasy Tissue Kit (Qiagen, Inc.) from isolated pleopod and/or dissected and isolated trunk muscle tissue. *COI* PCR was completed using conserved primers LCO1490 5-GGT CAA CAA ATC ATA AAG ATA TTG G-3 and HCOoutout 5-GTA AAT ATA TGR TGD GCT C-3 (Folmer et al. 1994; Schwendinger and Giribet 2005). PCR products of the appropriate size were direct sequenced by MacroGen, Inc (South Korea).

Phylogenetic analysis

Relevant *COI* sequences from 94 gammarids and two isopod outgroups were selected and added to the analysis from Genbank. Sequences were initially aligned and edited using Sequencher (Gene Codes Corporation). Multiple sequence alignments were generated using T-Coffee (Notredame et al. 2000). Alignments were then manually viewed and adjusted in MacClade v4.08 (Maddison and Maddison 2005).

Bayesian phylogenetic inference analysis was executed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The molecular evolution model, GTR+I+ Γ , was selected using the Akaike Information Criterion (AIC) as implemented in Model-Test 3.7 (Posada and Crandall 1998). Four independent searches were run from 30 million generations to 40 million generations, and trees were sampled every 100 generations. Likelihoods were visualized with Tracer v1.3 (Rambaut and Drummond 2003) to determine the number of generations to burn-in and to assess convergence of data sets. Consensus trees from the independent runs were compared to assess convergence and topology congruence of data sets. A consensus tree from the combined Bayesian runs was constructed and represents a total of 130 million generations from the four independent runs with trees sampled every 1000 generations and an initial burn-in of 5 million generations. Consensus trees were rooted with the isopod *Armadillidium vulgare* *COI* sequence. Resulting trees were visualized with TreeView X (Page 1996) and FigTree (<http://evolve.zoo.ox.ac.uk/software.html?id=figtree>).

Maximum likelihood (ML) analysis was executed with RAxML-VI-HP (Stamatakis 2006). The model GTRMIX was selected for executing four initial runs, each with 250 searches. Searches from the ML runs

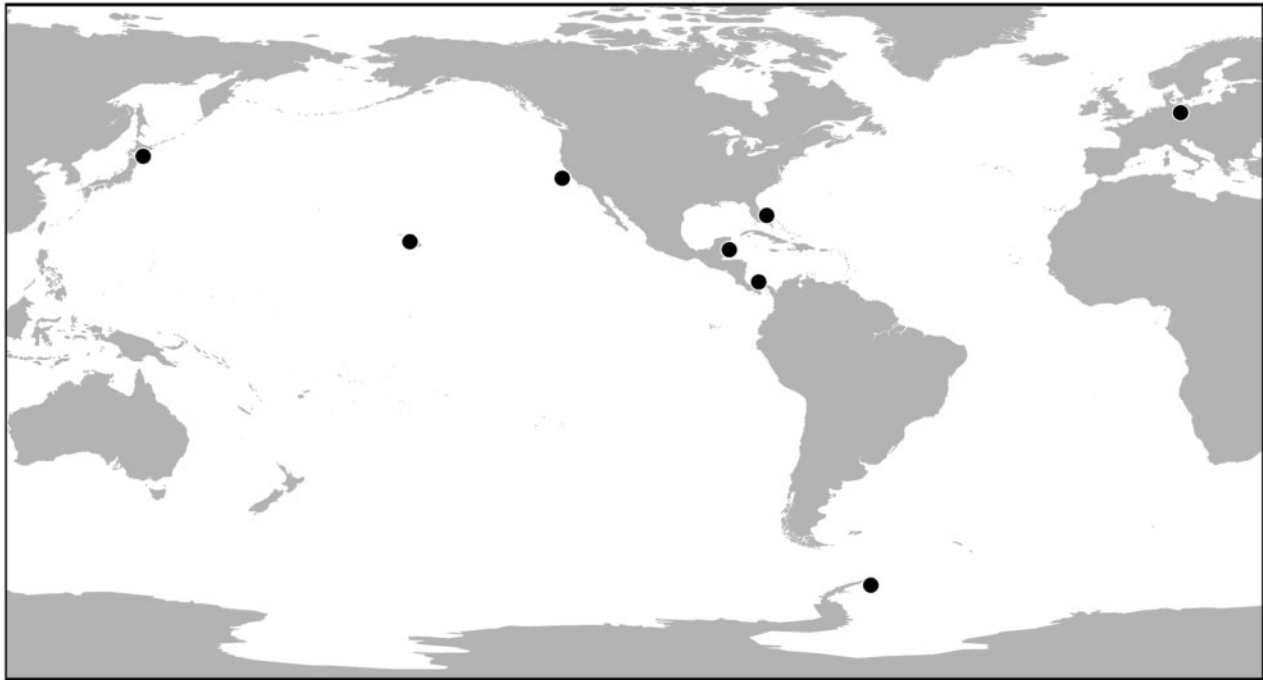


Fig. 1 Map of localities at which collections were made. Black circles indicate areas from which amphipods were collected for the present study (data appear in Table 1).

were combined and the consensus from the top 500 trees was visualized to assess convergence of topologies. Bootstrapping of ML analysis was executed with Seqboot (Felsenstein 1989) followed by 20 independent searches for the most likely tree per bootstrap iteration with RAxML-VI-HPC. The best tree was retained for each of the 129 iterations that were executed. Resulting trees were visualized with FigTree (<http://evolve.zoo.ox.ac.uk/software.html?id=figtree>).

A mutational saturation plot (Philippe et al. 1994; Philippe and Forterre 1999) was generated using a maximum parsimony (MP) analysis with 10 random sequence addition MP heuristic searches. The best tree was used to generate a patristic distance matrix. Individual pair-wise values were compared on *X–Y* scatter plots. The *X*-axis was used for the inferred substitution values and the *Y*-axis was used for the observed substitution values.

Results

Phylogenetic analysis

A diversity of amphipods from the Northeast, Northwest, and Central Pacific, the western edge of the Atlantic Gulf Stream, the Caribbean Sea, Germany, and the Weddell Sea were collected and sampled for ~815bp of the *COI* gene (Fig. 1, Table 1). The 72 new amphipod *COI* sequences

obtained for this study have been deposited with Genbank (Table 1). In an effort to test the hyperiids as a monophyletic group within Amphipoda, additional nonhyperiid amphipod *COI* sequences available from Genbank were incorporated into our phylogenetic analyses (Table 2). Our *COI* analysis included 168 taxa of which 52 represent a broad sampling among known hyperiidean forms.

Bayesian phylogenetic inference was used to infer *COI* gene lineage relationships. The number of generations required to stability (burn-in) was as high as 4.2 million, with an average burn-in of 3.5 million generations across four independent runs. This was due, in part, to the large number of taxa analyzed. Our combined bayesian *COI* analysis recovered three clades of hyperiid amphipods (Figs. 2 and 4, Supplementary data). The hyperiids comprising clade 1 appear to branch early among the taxa included in this analysis. However, the relationship between hyperiids comprising clades 2 and 3, as defined in this analysis, remain unclear. Although independent runs always recovered the same topologies among hyperiid taxa within each of the three clades, the relationship between clade 2 and clade 3 varied. The clade 2 hyperiids alternate between sister to clade 3 hyperiids (Supplementary data) and sister to the iphimiid gammarid clade (Supplementary data); however, in both cases the resolution at these deep nodes is low. ML was also used to infer *COI*

Table 1 List of specimens collected

Taxon	Collection Locality	Latitude	Longitude	Depth (m)	COI Accession
<i>Acanthoscina acanthodes</i>	Fort Pierce, FL, USA	27.34°N	79.54°W	0–100	EF989700
<i>Brachyscelus cruscum</i>	Kona coast, HI, USA	19.42°N	156.07°W	0–1	EF989658
<i>Brachyscelus globiceps</i>	Kona coast, HI, USA	19.35°N	156.00°W	0–1	EF989660
<i>Brachyscelus rapax</i>	Green Bay, PAN	9.14°N	82.14°W	0–2	EF989659
<i>Calamorrhynchus pellucidus</i>	Oahu, HI, USA	21.12°N	158.19°W	0–77	EF989649
<i>Capprellid KBH</i>	Kewalo Basin Harbor	21.17°N	157.51°W	0–1	EF989681
<i>Cilicæa</i> sp.	Kewalo Basin Harbor	21.17°N	157.51°W	0–1	EF989646
<i>Cranocephalus scleroticus</i>	Kona coast, HI, USA	19.35°N	156.00°W	0–1	EF989648
<i>Cylopus lucasii</i>	Weddell Sea	60.94°S	53.12°W	0–353	EF989691
<i>Cylopus magellanicus</i>	Weddell Sea	60.94°S	53.12°W	0–353	EF989690
<i>Cyphocaris</i> sp.	California, USA	36.33°N	122.90°W	300–700	EF989702
<i>Cystisoma gershwinæ</i>	California, USA	36.58°N	122.50°W	1144	EF989675
<i>Cystisoma pellucida</i>	California, USA	36.43°N	124.07°W	0–400	EF989676
<i>Gammarid KML</i>	KML seatables	21.17°N	157.51°W	N/A	EF989703
<i>Gammarid Tib750</i>	California, USA	36.26°N	122.59°W	2764	EF989706
<i>Gammarid Tib844</i>	California, USA	35.50°N	123.87°W	3591	EF989707
<i>Glossoccephalus milneedwardsi</i>	Pelican Cayes, BZ	16.40°N	88.11°W	0–3	EF989654
<i>Glossoccephalus</i> sp.	California, USA	36.60°N	122.37°W	541	EF989655
<i>Hyperia macrocephala</i>	Weddell Sea	60.56°S	52.81°W	0–328	EF989666
<i>Hyperietta parviceps</i>	Kona coast, HI, USA	19.35°N	156.00°W	0–1	EF989686
<i>Hyperioides longipes</i>	Fort Pierce, FL, USA	27.34°N	79.54°W	0–100	EF989685
<i>Hyperoche capucinus</i>	Weddell Sea	60.56°S	52.81°W	0–328	EF989665
<i>Hyperoche martinezi</i>	California, USA	36.37°N	122.10°W	100–200	EF989668
<i>Hyperoche medusarum</i>	California, USA	35.80°N	122.85°W	403	EF989667
<i>Iulopsis loveni</i>	Fort Pierce, FL, USA	27.34°N	79.54°W	0–100	EF989669
<i>Jassa slatteryi</i>	UC Berkeley culture	N/A	N/A	N/A	EF989682
<i>Lanceola loveni</i>	California, USA	36.37°N	122.09°W	1000	EF989693
<i>Lanceola pacifica</i>	California, USA	35.50°N	123.87°W	1324	EF989697
<i>Lanceola sayana</i>	California, USA	36.43°N	124.07°W	0–400	EF989696
<i>Leptocotis tenuirostris</i>	Fort Pierce, FL, USA	27.34°N	79.54°W	0–100	EF989653
<i>Lestrignonus schizogeneios</i>	Kona coast, HI, USA	19.35°N	156.00°W	0–1	EF989684
<i>Lycaea nasuta</i>	Kona coast, HI, USA	19.42°N	156.07°W	0–1	EF989647
<i>Lysianassoid</i>	California, USA	36.33°N	122.90°W	300–700	EF989712
<i>Microphasma agassizi</i>	California, USA	36.43°N	124.07°W	0–400	EF989692
<i>Mimonectes loveni</i>	California, USA	36.60°N	122.38°W	500–700	EF989698
<i>Monoculodes</i> sp.	Fort Pierce, FL, USA	27.34°N	79.54°W	0–100	EF989705
<i>Orchestia cavimana</i>	Tegeler See, GER	52.34°N	13.15°E	0	EF989708
<i>Oxycephalus clausi</i>	Oahu, HI, USA	21.29°N	158.23°W	BW	EF989652
<i>Paraoroides</i> sp.	Kewalo Basin Harbor	21.17°N	157.51°W	0–1	EF989711
<i>Paraphronima gracilis</i>	California, USA	36.43°N	124.07°W	0–400	EF989674
<i>Parapronoe cambelli</i>	Oahu, HI, USA	21.12°N	158.19°W	0–77	EF989657
<i>Parhayle hawaiiensis</i>	KML culture	N/A	N/A	N/A	EF989709
<i>Phronima bucephala</i>	California, USA	36.43°N	124.07°W	0–400	EF989680
<i>Phronima sedentaria</i>	California, USA	36.33°N	122.90°W	300–700	EF989679
<i>Phronimella elongata</i> ATL	Fort Pierce, FL, USA	27.34°N	79.54°W	0–100	EF989677

(continued)

Table 1 Continued

Taxon	Collection Locality	Latitude	Longitude	Depth (m)	COI Accession
<i>Phronimella elongata</i> PAC	Oahu, HI, USA	21.26°N	158.20°W	0–680	EF989678
<i>Phronimopsis spinifera</i>	Fort Pierce, FL, USA	27.34°N	79.54°W	0–100	EF989683
<i>Phrosina semilunata</i> ATL	Fort Pierce, FL, USA	27.34°N	79.54°W	0–100	EF989670
<i>Phrosina semilunata</i> PAC	Oahu, HI, USA	21.27°N	158.14°W	0–50	EF989671
<i>Platyscelus serratus</i>	Fort Pierce, FL, USA	27.34°N	79.54°W	0–100	EF989662
<i>Primno brevidens</i>	California, USA	36.37°N	122.10°W	100–200	EF989672
<i>Primno evansi</i>	Fort Pierce, FL, USA	27.34°N	79.54°W	0–100	EF989673
<i>Rhabdosoma whitei</i>	Oahu, HI, USA	21.13°N	158.16°W	0–258	EF989650
<i>Rhachotropis</i> sp.	California, USA	36.33°N	122.90°W	300–700	EF989704
<i>Scina borealis</i>	California, USA	36.33°N	122.90°W	300–700	EF989699
<i>Scypholanceola aestiva</i>	California, USA	36.43°N	124.07°W	0–400	EF989694
<i>Scypholanceola</i> sp.	California, USA	35.48°N	123.86°W	1399	EF989695
<i>Stenothoidae</i>	Waikiki, HI, USA	21.15°N	157.50°W	36	EF989710
<i>Streetsia challengerii</i>	Oahu, HI, USA	21.27°N	158.14°W	0–50	EF989651
<i>Synopia</i> sp.	Carrie Bowe Caye, BZ	16.47°N	88.04°W	0–10	EF989701
<i>Themsito japonica</i>	Hokkaido, JN	42.00°N	141.00°E	0–500	EF989663
<i>Themsito pacifica</i>	California, USA	36.43°N	124.07°W	0–400	EF989664
<i>Thyropus sphaeroma</i>	Oahu, HI, USA	21.12°N	158.19°W	0–77	EF989661
<i>Tryphana malini</i>	California, USA	36.80°N	121.80°W	0–100	EF989656
<i>Vibilia antarctica</i>	Weddell Sea	60.94°S	53.12°W	0–353	EF989689
<i>Vibilia propinqua</i>	California, USA	35.46°N	122.50°W	BW	EF989687
<i>Vibilia viatrix</i>	California, USA	35.30°N	123.52°W	BW	EF989688

The BW abbreviation in the depth column indicates specimens collected by bluewater diving (depths between 1–30 m).

gene lineage relationships and bootstrap values were mapped to the most likely ML tree. Our combined ML COI analysis recovers three clades of hyperiids nearly identical to those from our Bayesian results (Figs. 2, 3, and 4, Supplementary data). The most likely ML tree unites the three hyperiid clades. However, there is no bootstrap support for this result (Fig. 3, Supplementary data). With a high degree of support, we also recovered monophyletic caprellids (from the small number included in this study) as branching from within the gammariids (Figs. 2 and 3, Supplementary data). An internal assessment of our results was by recapitulation of the two Lake Baikal gammariid radiations (Macdonald et al. 2005) and recapitulation of the Antarctic radiations of the *Epimeria* and Iphimediid gammariids (Lorz and Held 2004) (Supplementary data).

Although, we found many amphipod crown-group relationships to be highly supported, the majority of the deep nodal relationships among the Amphipoda are poorly supported and remain largely unresolved from COI sequencing alone (Supplementary data). Interestingly, most hyperiid COI sequences have long branches relative to the other amphipod

sequences examined. This is indicative of a differential rate of change occurring among hyperiid COI sequence clades relative to the other amphipod COI sequences examined.

Morphological homoplasy among the Amphipoda is a well-characterized phenomenon that has historically confounded taxonomic descriptions (Barnard and Karaman 1991; Vinogradov et al. 1996), and the use of molecular data for phylogenetic methodologies is not immune from the effects of homoplasy. At the level of DNA sequences, homoplasy can manifest as site-specific variation that escapes detection due to two proximate causes (1) inadequate taxon sampling which thereby misses phylogenetically informative sequence variation, and (2) a rapid rate of sequence change among the taxa being examined which eliminates evidence of past, phylogenetically useful, sequence variation. To assess the potential influence of sequence homoplasy and to further characterize the variation in branch lengths that we observed among the taxa sampled, we generated a mutation saturation plot. We used maximum parsimony to generate a patristic distance matrix (Philippe and Forterre 1999). In a mutation saturation plot,

Table 2 List of additional amphipod taxa and *COI* sequences from Genbank used in this study

Taxon	<i>COI</i> Accession	Taxon	<i>COI</i> Accession
Abyssorchomene_sp	U92669	Gammaracanthus_lacustris	AY061796
Acanthogammarus_brevispinus	AY926651	Gammarus_aequicaudus	AY926667
Acanthogammarus_flavus	AY061800	Gammarus_annulatus	AY926668
Acanthogammarus_victorii	AY926652	Gammarus_daiberi	DQ300255
Amphithoe_longimana	AY926653	Gammarus_duebeni	AY926669
Armadillidium_vulgare	AF255779	Gammarus_lacustris	AY926671
Asellus_aquaticus	AY531826	Gammarus_tigrinus	DQ300242
Brandtia_lata	AY926654	Gmelinoides_fasciata	AY926675
Chaetogammarus_marinus	AY926655	Gnathiphimedia_mandibularis	AF451353
Chaetogammarus_obtusatus	AY926656	Gnathiphimedia_sexdentata	AF451354
Chydaekata_sp	DQ838028	Hakonboekia_strauchii	AY926676
Crangonyx_floridanus	AJ968911	Hirondellea_dubia	AY183359
Crangonyx_pseudogracilis	AJ968903	Hyale_nilssoni	AF520435
Crangonyx_serratus	AY926658	Hyalella_azteca	DQ464719
Cyamus_erraticus	DQ095139	Hyalella_montezuma	AY152807
Cyamus_gracilis	DQ095105	Hyalella_muerta	DQ464603
Cyamus_ovalis	DQ095047	Hyalella_sandra	DQ464682
Dikerogammarus_haemobaphes	AY529049	Hyalella_simplex	AF520434
Dikerogammarus_villosus	AY529048	Ipshimediella_cyclogena	AF451348
Echiniphimedia_echinata	AF451352	Ipshimediella_georgei	AF451349
Echiniphimedia_hodgsoni	AF451350	Ipshimediella_rigida	AF451347
Echiniphimedia_waagelei	AF451351	Maarrka_wollii	DQ838034
Echinogammarus_ischnus	AY326126	Macrohectopus_branickii	AY926677
Echinogammarus_trichiatus	AY529051	Megomaera_subtener	AY926678
Eogammarus_confervicolus	AY926659	Melita_nitida	AY926679
Eogammarus_oclairei	AY926660	Micruropus_crassipes	AY926680
Epimeria_georgiana	AF451341	Micruropus_fixseni	AY926681
Epimeria_macrodonata	AF451343	Micruropus_glaber	AY926682
Epimeria_reoproi	AF451342	Micruropus_wahli	AY926683
Epimeria_robusta	AF451344	Molina_pleobranchos	DQ255962
Epimeria_rubriques	AF451345	Monoculodes_antarcticus	AF451356
Epimeria_similis	AF451346	Monoporeia_affinis	AY926684
Eulimnogammarus_cruentus	AY926661	Niphargus_fontanus	DQ064702
Eulimnogammarus_cyaneus	AY061801	Niphargus_rhenorhodanensis	DQ064703
Eulimnogammarus_inconspicuous	AY926662	Niphargus_virei	DQ064749
Eulimnogammarus_maacki	AY926663	Obesogammarus_crassus	AY189482
Eulimnogammarus_viridulus	AY926664	Odontogammarus_calcaratus	AY926685
Eulimnogammarus_vittatus	AY926666	Ommatogammarus_albinus	AY926686
Eurythenes_gryllus	U92660	Orchestia_uhleri	AY152751
Eusirus_cf_perdentatus	AF451355	Pallasea_cancellus	AY926687
Eusirus_cuspidatus	AY271852	Paramelitidae_sp	DQ838036
Euxinia_maeoticus	AY529038	Paramphithoe_hystrix	AY271847
Exhyalella_natalensis	AF520436	Perthia_sp	DQ230097
Gammaracanthus_aestuariorum	AY061798	Pilbarus_millsi	DQ490125
Gammaracanthus_caspicus	AY061797	Poekilogammarus_pictoides	AY926690

(continued)

Table 2 Continued

Taxon	COI Accession	Taxon	COI Accession
Pontogammarus_abbreviatus	AY926691	Rhachotropis_inflata	AY271854
Pontogammarus_obesus	AY529041	Synurella_sp	AJ968914
Pontogammarus_robustoides	AY529047	Uroctena_sp	DQ230128
Rhachotropis_aculeata	AY271853	Ventiella_sulfuris	U92667

the presence of a curve with a mutational plateau indicates that progressive saturation for mutational changes is occurring and thus there is a loss of the phylogenetic signal necessary for accurately resolving deep branching nodes (Philippe et al. 1994; Philippe and Forterre 1999).

Our scatter plot of observed versus inferred *COI* substitutions has an atypical, wide, cloud-like distribution (Fig. 5A). In general, there were fewer observed substitutions than predicted to have occurred between *COI* sequence pairs. No plateau corresponding to *COI* mutational saturation could be easily discerned from the full data set. Sampling subsets of *COI* sequence, however, revealed three distinguishable patterns of *COI* gene variation segregating among the taxa in this study (Fig. 5B–D). This indicates divergent, nonuniform, patterns of sequence change among *COI* gene lineages in the Amphipoda.

Pair-wise comparisons among the three hyperiid clades demonstrates that among these taxa there is a saturation curve containing many observed substitutions that are relatively close to inferred substitutions as well as an expected mutational plateau (Fig. 5B). Therefore, although the hyperiid *COI* branches are generally longer than the other amphipod sequences considered, they should contain sufficient sequence variation to reliably resolve many crown-group relationships (Fig. 4). Each of the three detected hyperiid *COI* clades independently retains the general shape of this curve (Supplementary data).

Superimposing pair-wise comparisons between the largest recovered monophyletic gammariid *COI* clade exclusive of hyperiid sequences (Fig. 2) reveals a cloud-like cluster (Fig. 5C). The cluster of substitution values does not display a discernable curve and plateau trend. The cluster departs strongly from the ideal observed/inferred substitution ratio, and a significant proportion of pair-wise values exhibit low inferred substitution values coupled with low observed substitution values. This supports the general observation of shorter branch lengths observed in this gammariid clade. The shape of this cluster strongly suggests that mutational saturation has removed most of the phylogenetic

signal associated with comparisons between these sequences. Indeed, a majority of the nodes in this clade are characterized by low Bayesian posterior probabilities and no ML bootstrap support, and the deep nodes in particular are not well supported (Fig. 2).

Finally, superimposing pair-wise comparisons between the hyperiids and all other amphipod taxa results in an additional informative shift in the cluster of substitution values (Fig. 5D). The distribution of the values observed in this case suggests that the majority of the pair-wise comparisons between gammariid and hyperiid *COI* sequences have particularly low phylogenetic signal, as observations of sequence substitution fall dramatically relative to inferred sequence substitutions. Consideration of the shape of clustered values in Fig. 5C and D confirms that mutational saturation for *COI* has occurred among the gammariid amphipods included in this analysis. The observation of homoplasy among gammariid versus gammariid and gammariid versus hyperiid *COI* sequences does not prevent reconstruction of lineage relationships within hyperiid clades. However, *COI* sequence homoplasy does prevent the accurate recovery of relationships between the three recovered hyperiid clades that contain deeper nodes that may interdigitate with ill-defined gammariid taxa.

Discussion

The phylogeny of pelagic midwater hyperiid amphipods

The histories of patterns of descent by modification are difficult to discern when shared attributes are the result of convergence. Among the Amphipoda, morphological convergence (homoplasy) is well documented. To help improve our understanding of uncertain relationships and the relative importance of morphological characters, we examined the *COI* gene among 168 amphipodan taxa including 52 hyperiids. Our results indicate that there may have been as many as three independent radiations among the hyperiids. In addition, our *COI* data suggest taxonomic affinities for several previously

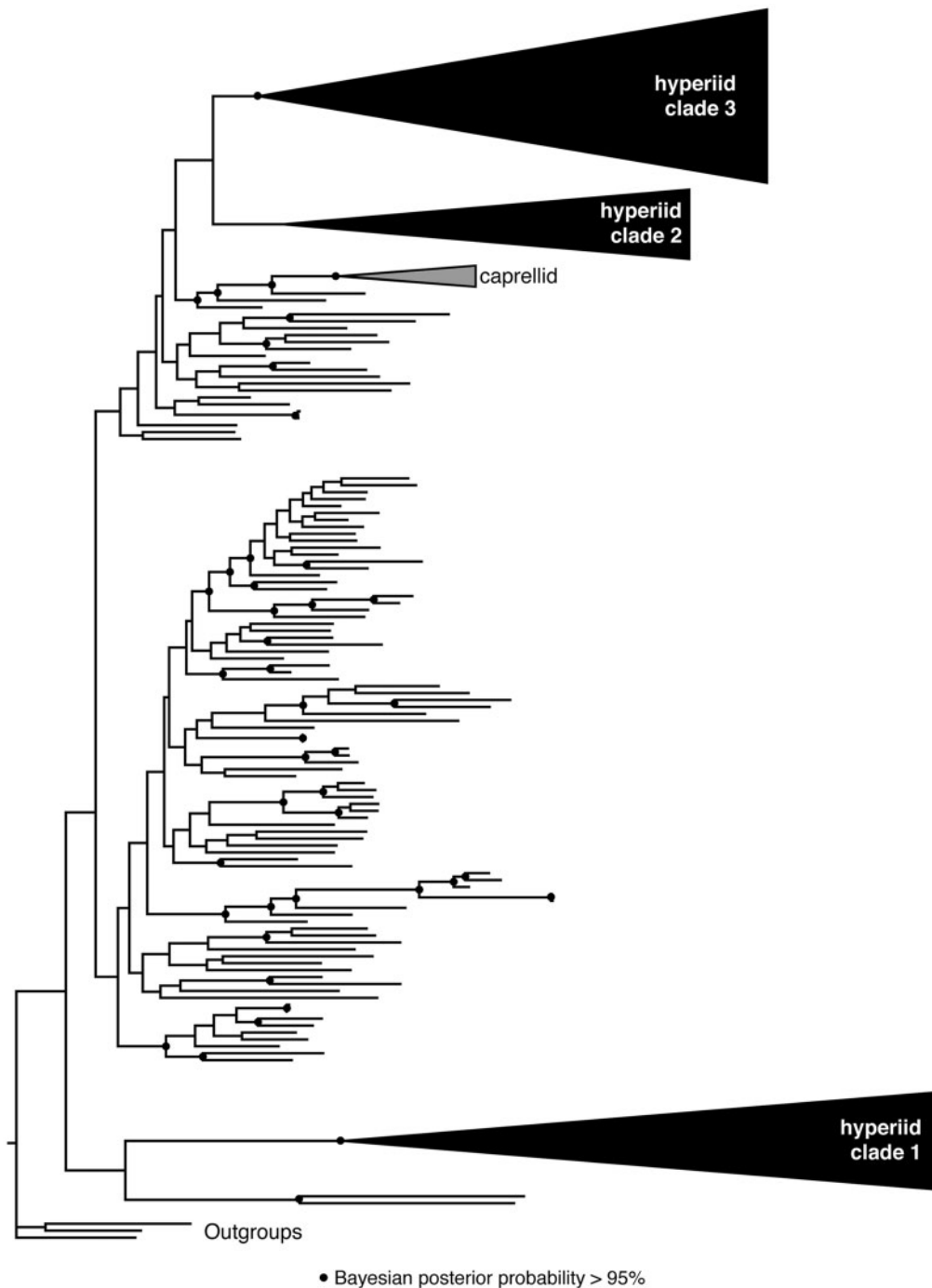


Fig. 2 *COI* Bayesian analysis combined consensus tree. Consensus tree is the post burn-in tree from four combined independent runs. Branch nodes with Bayesian posterior probability support greater than 95% are marked with black circles. Black triangles represent collapsed views of the three recovered hyperiid clades. Grey triangle represents collapsed view of the caprellid clade. Bayesian consensus tree topology proposes a sister group relationship between hyperiid clade 2 and hyperiid clade 3 but with low confidence. A majority of the lineage relationships between taxa within the hyperiid clades have high support values (Fig. 4). In contrast, the vast majority of deep branching node hypotheses are unsupported by *COI* sequence data.

enigmatic hyperiid taxa. Bayesian results suggest that at least one of the hyperiid radiations (hyperiid clade 1 in Fig. 2) may be an early diverging lineage within Amphipoda. Our Bayesian results also suggest that the hyperiids are likely to be polyphyletic (Fig. 2).

ML results unite the three hyperiid clades but with no bootstrap support (Fig. 3). We also find a highly supported monophyletic clade of caprellids from the *COI* sequence data included in this analysis. In our analyses, the caprellid clade is always sister to the

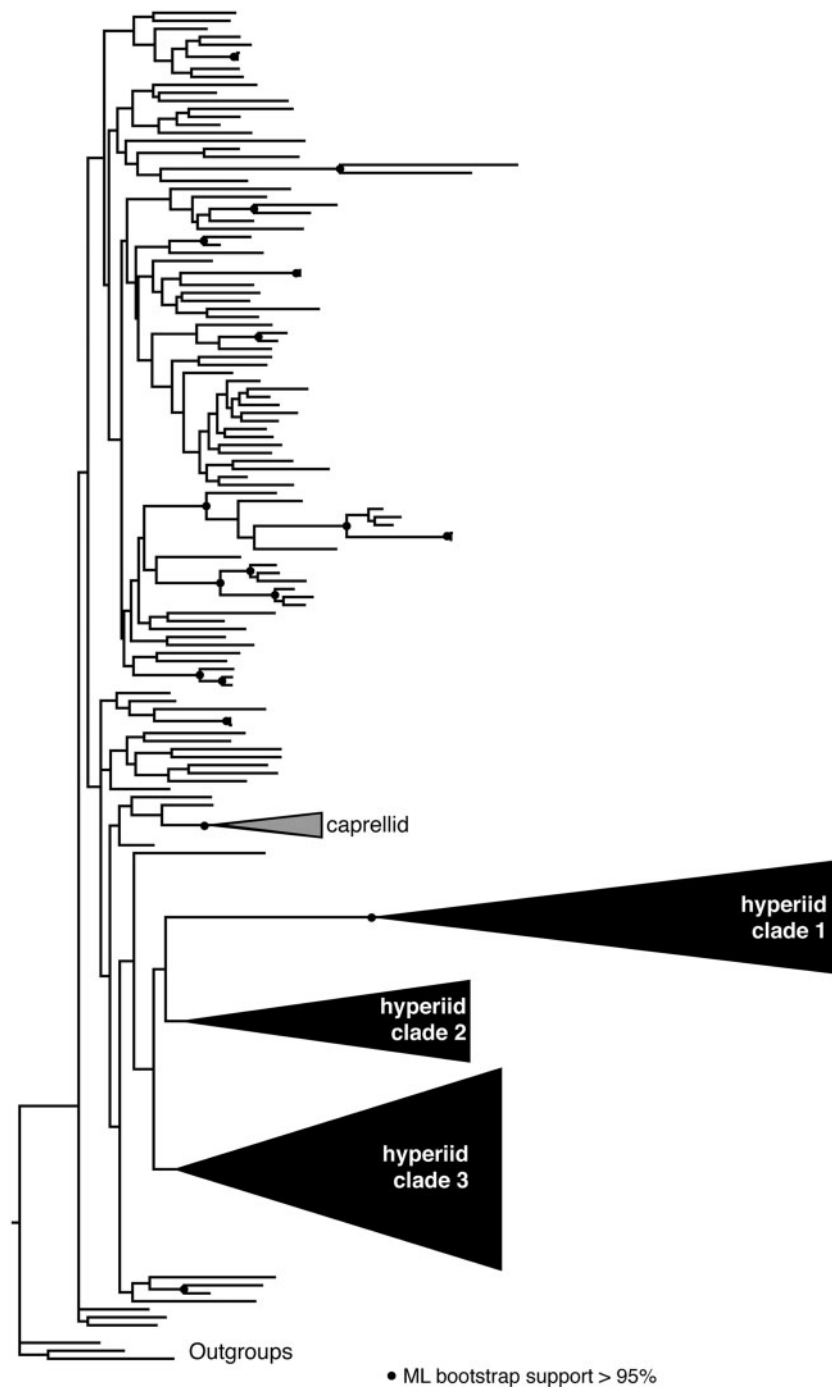


Fig. 3 COI Maximum likelihood tree. ML tree is the most likely tree from four independent runs. Branch nodes with bootstrap support greater than 95% are marked with black circles. Black triangles represent collapsed views of the three recovered hyperiid clades. Grey triangle represents collapsed view of the caprellid clade. The most likely ML tree topology proposes a sister group relationship among all three hyperiid clades. This relationship has no bootstrap support. Many lineage relationships between taxa within the hyperiid clades have high support values (Fig. 4). In contrast, the vast majority of deep branching nodes have no bootstrap support (Supplementary data).

lineage that includes the ischyrocerid *J. slatteryi* (Supplementary data). This result is in agreement with recent morphological analyses of corophiid and caprellid groups (Myers and Lowry 2003). COI sequence variation among the amphipod taxa

sampled does not, however, resolve either the relationships between separate hyperiid radiation events or the relationships of lineages among the majority of the gammarids due to inadequate resolution at deeper nodes.

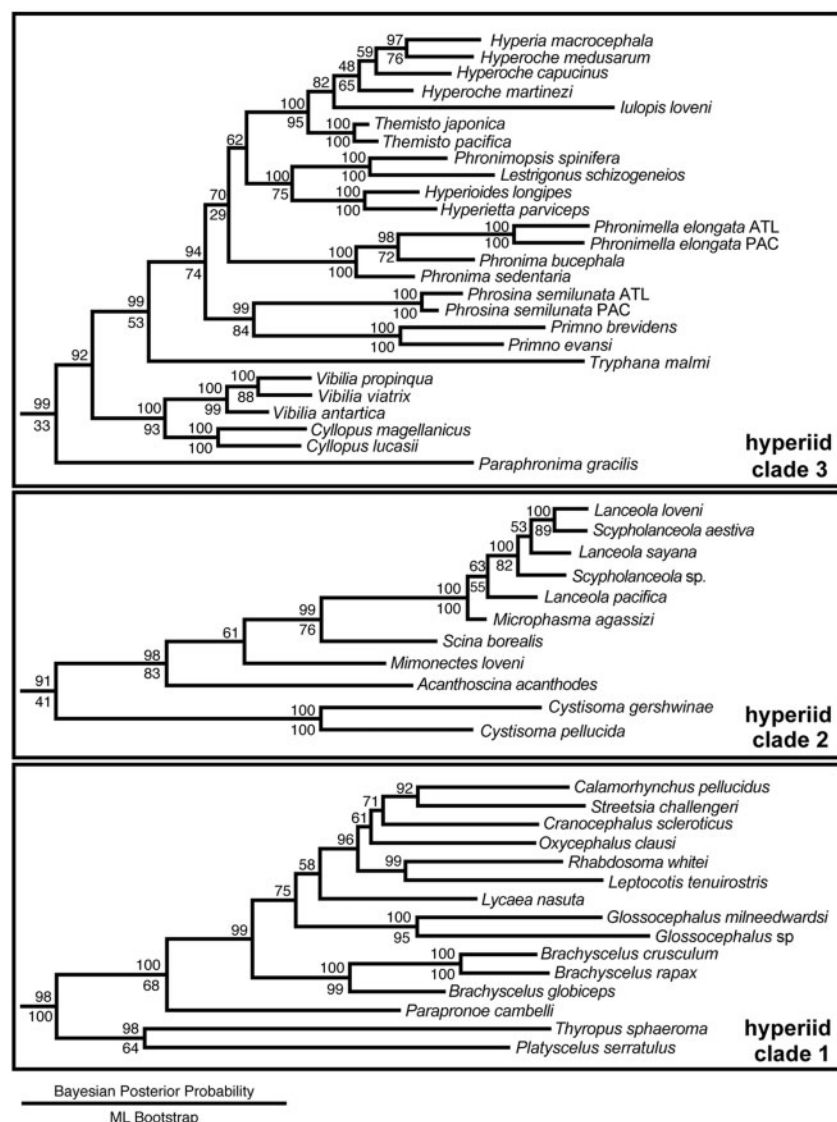


Fig. 4 Inferred hyperiid relationships from *COI* analyses. The three hyperiid clades are represented here from the Bayesian consensus tree shown in Fig. 2. Branch nodes show Bayesian posterior probability support above and ML bootstrap support value below. The clade topologies represented here are consistently recovered between independent Bayesian and ML runs (Supplementary data).

Several interesting observations from our preliminary molecular analysis of the hyperiids can be made. First, there is a fair amount of agreement between the clades recovered in our molecular results (Fig. 4) and traditional taxonomic groupings of hyperiids based on morphology alone (Vinogradov et al. 1996). For example, several monophyletic lineages composed of classical taxonomic groupings are recovered in our analyses including the infraorder Physosomata within clade 2, and the superfamilies Platysceloidea, Vibilioidea, and Phronimoidea within clade 1 and clade 3, respectively (Fig. 4, Supplementary data).

However, a number of interesting questions regarding hyperiid relationships arise from our

analyses. For example, traditional morphological examinations have been unable to confidently place the genus *Cystisoma* relative to other hyperiid taxa (Vinogradov et al. 1996; Zeidler 2003a). In our analyses, *Cystisoma* appears to diverge at the base of clade 2 (Fig. 4, Supplementary data) that includes representatives of the classically defined infraorder Physosomata (Vinogradov et al. 1996). Additional sampling of *Cystisoma* and other members of clade 2 are needed to further explore this inferred relationship. Among the representatives of clade 1, we consistently recovered, albeit with low support, *Lycaea nasuta* splitting the classically defined family Oxycephalidae (Fig. 4, Supplementary data) suggesting that Oxycephalidae, as currently

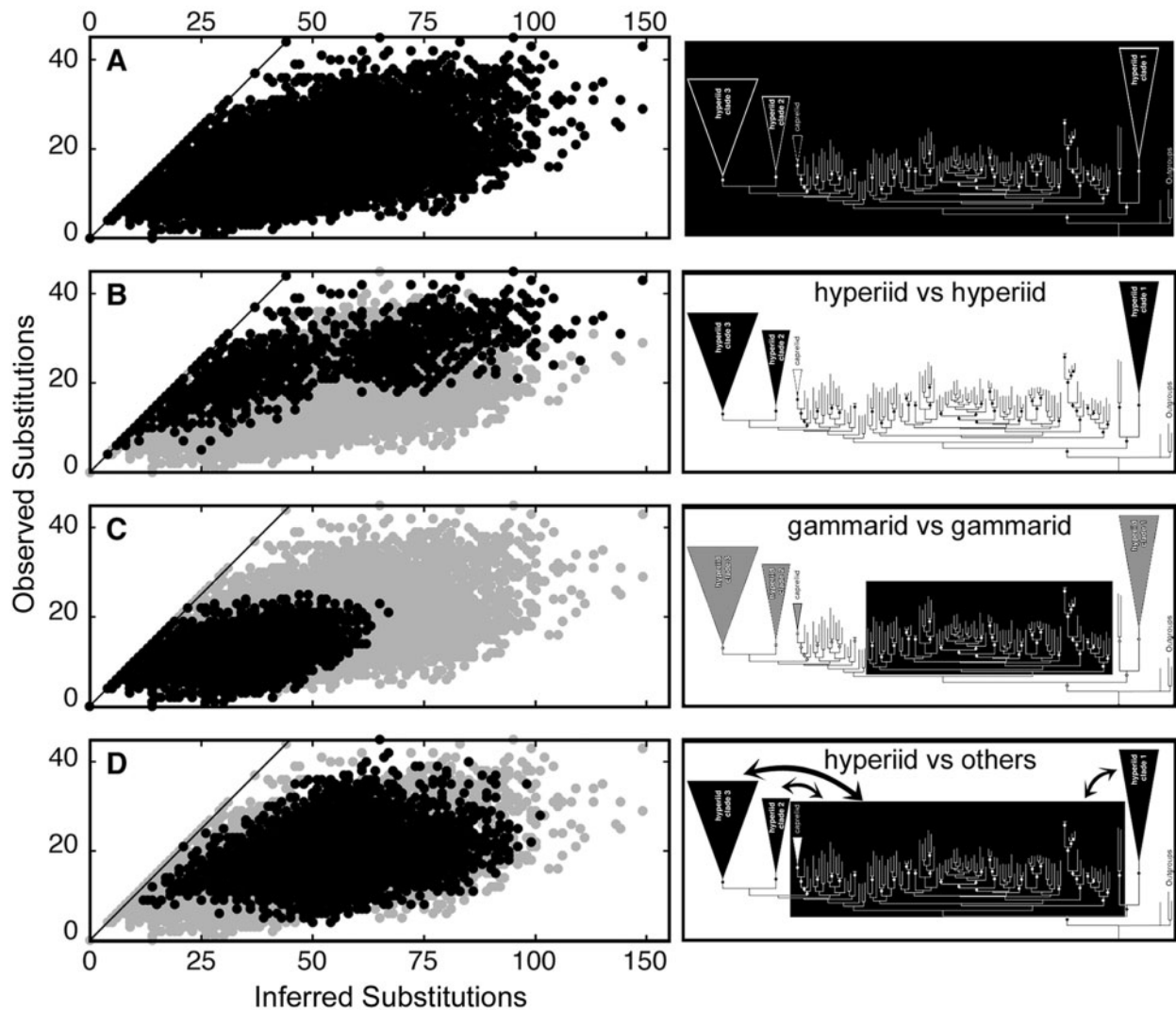


Fig. 5 Mutation saturation scatter plots. The Y-axis is the observed number of differences between pairs of *COI* sequences. The X-axis is the inferred number of differences between pairs of *COI* sequences determined using maximum parsimony methods. Each circle represents an observed versus inferred substitution value for a pair of *COI* sequences. The black line represents the ideal unsaturated case in which the number of observed substitutions equals the number of inferred substitutions. Diagrams on the right indicate which groups of taxa (black circles) are being compared in each panel. (A) Among Amphipoda sampled, observed versus inferred *COI* substitutions have a wide cloud-like distribution. In general, there are fewer observed substitutions than predicted to have occurred between *COI* sequence pairs. No plateau corresponding to *COI* mutational saturation can be easily discerned from the full dataset. (B) The black circles indicate pair-wise comparisons between hyperiid *COI* sequences. Each of the three detected hyperiid *COI* clades can be superimposed on this curve (Supplementary data). The distribution of hyperiid values is a well-defined subset showing a distinct plateau that indicates mutational saturation among the hyperiid *COI* sequences as the number of predicted substitutions increases in the absence of observed substitutions among the pair-wise comparisons. Additionally, the curve corresponds with the general observation of longer branch lengths associated with members of the three hyperiid *COI* clades relative to gammariid *COI* clades. (C) The black circles indicate pair-wise comparisons between the largest monophyletic gammariid *COI* clade exclusive of hyperiid sequences. The distribution of gammariid values reflect shorter branch lengths among gammariid *COI* sequences. The shape of the cloud-like cluster suggests that these sequences have low relative phylogenetic signal relative to *COI* sequence comparisons between hyperiids. (D) The black circles indicate pair-wise comparisons between gammariid and hyperiid *COI* sequences. The distribution of the values observed in this case suggest that the majority of the pair-wise comparisons between gammariid and hyperiid *COI* sequences have low phylogenetic signal as observations of sequence substitution fall dramatically relative to inferred sequence substitutions.

recognized (Vinogradov et al. 1996), maybe polyphyletic. Further sampling between *Lycaea* and other members of the Oxycephalidae would be informative with regard to this tentative result. We also consistently recovered *Tryphana malmi* as a well-supported member of clade 3, whereas morphological analysis alone suggests *Tryphana* would be affiliated with the Platyscloidea (and thus clade 1 in our analyses). This is a rather surprising result that suggests further analyses of *Tryphana* relative to members of both clade 1 and clade 3 are warranted.

The presence of significant intraspecific variation in *COI* has been used among some amphipod groups to discriminate between populations of closely related species (Meyran et al. 1997; Witt et al. 2003; Kaliszewska et al. 2005; Lefebure et al. 2006; Witt et al. 2006). In our analysis we sampled two monotypic genera, *Phronimella elongata* and *Phrosina semilunata*, from both the Central Pacific and the Atlantic Gulf Stream. Both are members of hyperiid clade 3 (Fig. 4). We recovered surprisingly long branch-lengths between Pacific and Atlantic isolates. Two alternative scenarios can be considered. The *COI* sequence divergence could indicate cryptic speciation within these two genera that have historically been considered monotypic. Alternatively, it could represent substantial isolation and divergence of the pan-oceanic populations of these two species. Further comparisons between populations of these taxa from both the Atlantic and the Pacific oceans, are warranted.

One of the main mechanisms contributing to sequence homoplasy and the loss of phylogenetic signal is site-specific mutational saturation (Philippe et al. 1994; Philippe and Forterre 1999). Our test for mutational saturation among the *COI* sequences used in this analysis indicates saturation (loss of phylogenetic signal) among the majority of gammarid *COI* sequences (Fig. 5C). Comparisons between hyperiid *COI* sequences demonstrate differential evolution of this gene and the retention of phylogenetically significant information among these sequences (Fig. 5B, Supplementary data). However, the low signal present among the majority of the gammarid sequences included prevent the recovery of lineage relationships outside of the three well-supported hyperiid clades.

Morphological and molecular homoplasy play a central role in evolutionary biology and represent major impediments to understanding links between organismal genotype and phenotype. Improving support for deep branching nodes within Amphipoda is crucial to hypothesizing credible

evolutionary relationships of the hyperiid radiations reported here to other amphipodan faunas. Future analyses should include multiple genetic loci from both mitochondrial and nuclear markers to moderate the effects of differential rates of gene evolution. Increased taxon sampling is also urgently needed to better represent the enormous diversity of extant Amphipoda (currently more than 8000 described species). Hypotheses generated from these additional molecular analyses can, and should be, used to independently evaluate morphological attributes that appear to play important roles in patterns of amphipod diversity but that have proven recalcitrant to traditional morphological based evolutionary analyses due to homoplasy. Given the high branch support observed within hyperiid clades in our analysis, independent contrast methodologies could prove particularly useful in exploring morphological character convergence within and between these lineages (Felsenstein 1985).

For a number of reasons, the expansion of sampled genetic loci and the inclusion of additional taxa should be undertaken to further improve lineage relationships within the hyperiid clades identified here. Pelagic hyperiid amphipods have very sharp differences in the organization of their heads and anterior nervous systems relative to most other benthic amphipods, likely due to constraints imposed by disparate life histories (W.E.B., personal observation). Clearly, associations of hyperiids with gelatinous zooplankton are sufficiently old to have played an important role in the evolution of various hyperiid morphologies, as evidenced by the modifications of appendages used for feeding on/with and for attachment to their preferred hosts (Bowman and Gruner 1973; Vinogradov et al. 1996). Many ecdysozoans (e.g., crustaceans, insects, chelicerates, nematodes, and nematomorphs) have representatives that possess a parasitic life history (Price 1977; Poulin and Morand 2000). Coevolution between hosts and their parasites is well characterized as leading both to strong stabilizing and strong destabilizing selection (Frank 1993). As details of associations between hyperiids and gelatinous zooplankton continue to emerge, it will be important to consider the role that hosts have played during the evolution of various hyperiid lineages. For example, in contrast to the majority of benthic amphipods, the hyperiids exhibit a range of posthatching larval stages that in most cases reflect specific interactions with preferred hosts (Laval 1980). We suggest that the hyperiid amphipods offer a unique intersection of attributes that further our understanding of biological evolution; they are members of an extremely

successful clade of metazoans, display a range of parasitic associations, and are well adapted to the largest habitat on the planet.

Supplementary data

Supplementary data are available at *ICB* online.

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