ABSTRACT

Title of thesis: MULTI-LOCUS PHYLOGENETIC
ANALYSIS OF AMPHIPODA INDICATES
A SINGLE ORIGIN OF THE PELAGIC
SUBORDER HYPERIIDEA

Leann M. Biancani
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Thesis directed by: Dr. Michael P. Cummings, Professor
Department of Biology

Dr. Karen J. Osborn, Adjunct Professor
Department of Biology

Hyperiidea is an exclusively pelagic suborder of amphipod crustaceans, exhibiting a wide array of unique adaptations to life in the dark, open expanse of the oceanic midwater. No common morphological synapomorphy unites approximately 350 described species. Instead, hyperiid amphipods are defined only by their pelagic existence. Hyperiidea exhibits many of the characteristics of an adaptive radiation and could represent a midwater example of this phenomenon. Previous morphological and molecular analyses have led to uncertainty in the shared ancestry of Hyperiidea. The evolutionary history of their diverse adaptations, as well as their relationship to other amphipods, remains unknown. Here we present results of a multi-locus phylogenetic analysis of publicly available amphipod sequences for three nuclear loci (18S, 28S, and H3) and two mitochondrial loci (COI and 16S) from over 300 amphipod genera, 40 of which are hyperiids. We recover strong support for a monophyletic Hyperiidea as well as reciprocally monophyletic hyperiid infraorders Physocephalata and Physosomata (with enigmatic genera *Cystisoma* and *Paraphronima* more closely related to Physosomata). We also identify several benthic, commensal amphipods representing potential sister groups for Hyperiidea. These taxa have not previously been considered close hyperiid relatives and include the genera *Amphilochus*, *Colomastix*, *Anamixis*, *Paranamixis*, and *Leucothoe*. Our results support the current definition of Hyperiidea and inform the phylogenetic placement of the suborder within Amphipoda.

MULTI-LOCUS PHYLOGENETIC ANALYSIS OF AMPHIPODA INDICATES A SINGLE ORIGIN OF THE PELAGIC SUBORDER HYPERIIDEA

by

Leann M. Biancani

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Advisory Committee:
Professor Michael P. Cummings, Chair/Co-Advisor
Dr. Karen J. Osborn, Co-Advisor
Professor Alexandra Bely
Dr. Allen Collins
Professor Charles Delwiche

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Chapter 1: Introduction

Adaptive radiation is the divergence of a single lineage into an array of species exhibiting high phenotypic diversity [Osborn, 1902]. This widespread evolutionary process is believed to have generated much of the Earth's biodiversity [Schluter, 2000] and has profound implications for investigating speciation, convergence, trait evolution, and the complex links between genotype, phenotype and environment. There are numerous examples of well-studied adaptive radiations in a variety of organisms and ecosystems, the most familiar of which include African rift-lake cichlids, Caribbean anoles, Hawaiian *Drosophila*, and Darwin's Galápagos finches. Few examples, however, have been described from the planet's largest ecosystem, the oceanic midwater.

Several features are used to identify a group of species as an adaptive radiation: relatively high morphological diversity, ecological specialization, common ancestry, and rapid speciation [Schluter, 2000]. Hyperiidea, a suborder of amphipod crustaceans, exhibits characteristics that may be consistent with features of an adaptive radiation. However, uncertainty in their evolutionary history currently precludes a formal examination of these features.

Hyperiid morphology is highly diverse among the 350 described species. The group is defined by their exclusively pelagic existence as no common morphological synapomorphy unites Hyperiidea. Body shapes are highly varied (Figure 1.1) and include compact, nearly spherical forms (Platyscelidae, Parascelidae), globular bodies with a thin cuticle (Lanceolidae, Cystisomatidae), slender, elongated shapes (Oxycephalidae), and compact bodies with strong swimming muscles (Pronoidae, Lycaeidae) [Bowman and Gruner, 1973, Vinogradov et al., 1982]. Hyperiids also exhibit a truly exceptional variety of eye types (Figure 1.1). Their visual systems range from a complete absence of eyes to numerous retinas within each eye and even replicated eye pairs [Vinogradov et al., 1982, Land, 1981a]. Additional characteristics include cylindrical eyes with a 360° field of vision [Meyer-Rochow, 1978], fiberoptic-like crystalline structures [Land and Nilsson, 2012], and eyes with mirrored parabolic pigment cups [Marshall et al., 2003].

There are only a small number of morphological traits common to hyperiids and none are exclusive to the group (Figure 1.2). Such characteristics include the absence of an accessory flagellum on the first antennae, a pereon which is never fused with the head, a double-urosomite (fused second and third urosomites), an entire telson (never cleft or incised) with no setae, and uropods with single-segmented rami and no marginal setae [Bowman and Gruner, 1973]. Such wide morphological diversity and few common characters suggests Hyperiidea could satisfy the high diversity feature of an adaptive radiation, if it can be shown that hyperiids possess greater morphological diversity than their sister group. Although the variations among hyperiids is described as greater than that of typical amphipods [Dana, 1853], there are a number of morphologically divergent amphipod groups (Stock, 1981;

Laubitz, 1970; Leung, 1967) as well a known example of an adaptive radiations within Amphipoda [Naumenko et al., 2017]. Knowing which of 10,000 species of amphipods are most closely related to Hyperiidea is necessary to assess relative morphological diversity in the group.

Hyperiids also appear to exhibit ecological specialization. This is particularly apparent in the diversity of their limb structures. Although a number of hyperiids spend the majority of their time free-swimming in the water column, many form strong associations with gelatinous zooplankton, living a near-benthic lifestyle as parasites or commensals [Laval, 1980, Gasca and Haddock, 2004, Gasca et al., 2015]. Phenotypic diversity in hyperiids may to be linked to these varied lifestyles. In most amphipods the first and second pair of pereopod appendages form subchelate gnathopods used in feeding and copulation [Schram, 1986]. However, a diversity of additional limb specializations are found within Hyperiidea. In species of *Phronima*, pereopods III and IV are simple and elongated and pereopod V is enlarged and robustly sub-chelate. Pereopods III and IV are used in prey capture and manipulation as well as to grip the inside of the transparent barrels they fashion from salps, pyrosomes, or siphonophores [Laval, 1968, Diebel, 1988]. The enlarged pereopod V hangs loosely during these behaviors, but is employed (along with III and IV) in a defensive response when the barrel is disturbed [Laval, 1968]. In species of *Vibilia*, the digitform dactyls on Pereopod VII of females are used to transfer newly-hatched larvae to a salp host where they can feed and mature [Laval, 1963]. It has also been suggested that variations in body shape may be associated with environmental conditions [Bowman and Gruner, 1973, Vinogradov et al., 1982] and that the limitations of the midwater light environment constitute a selective pressure driving the evolution of pelagic eyes [Warrant and Johnsen, 2013]. Understanding the evolution of hyperiid diversity requires an accurate phylogenetic hypothesis onto which patterns of ecology and morphology can be mapped. Only then can we test whether this diversity is truly correlated with hyperiid habitat and ecology.

Hyperiid diversity and lack of common characters unique to the group has led to uncertainty in the monophyly of the suborder. One early and extreme view suggested Hyperiidea consisted of eight to ten phylogenetically disparate lineages, which arose from distinct benthic amphipod ancestors [Pirlot, 1932]. Vinogradov et al. [1982] argued that although nearly all the morphological features characteristic of hyperiids have analogies in other amphipod groups, no single group possesses a majority of these features. This more moderate view suggested possible polyphyletic origins of hyperiid infraorders (Physosomata and Physocephalata), but monophyletic relationships within those groups. Based on a morphological phylogenetic analysis of Amphipoda, Lowry and Myers [2017] conclude Hyperiidea is a monophyletic group, sister to all amphipods except Ingolfiellidea (now split into the newly-constructed Pseudoingolfiellidea and Ingolfiellida).

The first molecular study of hyperiid phylogenetic relationships was a single gene analysis of mitochondrial COI [Browne et al., 2007], which aimed to address the question of hyperiid monophyly and included sequences from 54 hyperiid species, 94 non-hyperiid amphipods and two isopod outgroups. The study recovered three hyperiid clades but was unable to resolve the relationships among them. A multilocus phylogenetic analysis of 467 species of the amphipod family Gammaridae included three hyperiid species among their outgroup taxa [Hou and Sket, 2015]. This study recovered a monophyletic hyperiid clade as sister to all other taxa in the analysis. Neither of the aforementioned studies were able to identify close hyperiid relatives. A multi-locus phylogenetic analysis of 51 hyperiid species used three nuclear loci in addition to COI [Hurt et al., 2013]. Based on their analyses, the authors suggested alternative taxonomic relationships within Hyperiidea including the movement of *Cystisoma* and *Paraphronima*, two genera with large, unique eyes, out of the large-eyed Physocephalata and into Physosomata. This remains the most robust phylogenetic hypothesis available for Hyperiidea. The study did not, however, address the question of hyperiid monophyly as no non-hyperiid amphipods were included in the analysis.

Exploring Hyperiidea as an adaptive radiation requires (i) understanding whether the lineage represents a single or multiple invasions of the midwater environment and (ii) identifying potential sister-groups (necessary for comparing levels of morphological diversity and speciation rates). To investigate the monophyly of hyperiids and their relationship to other amphipods, we performed a multi-locus phylogenetic analysis of publicly available sequences from across Amphipoda. This analysis includes representatives from over 300 genera, 40 of which are hyperiids.

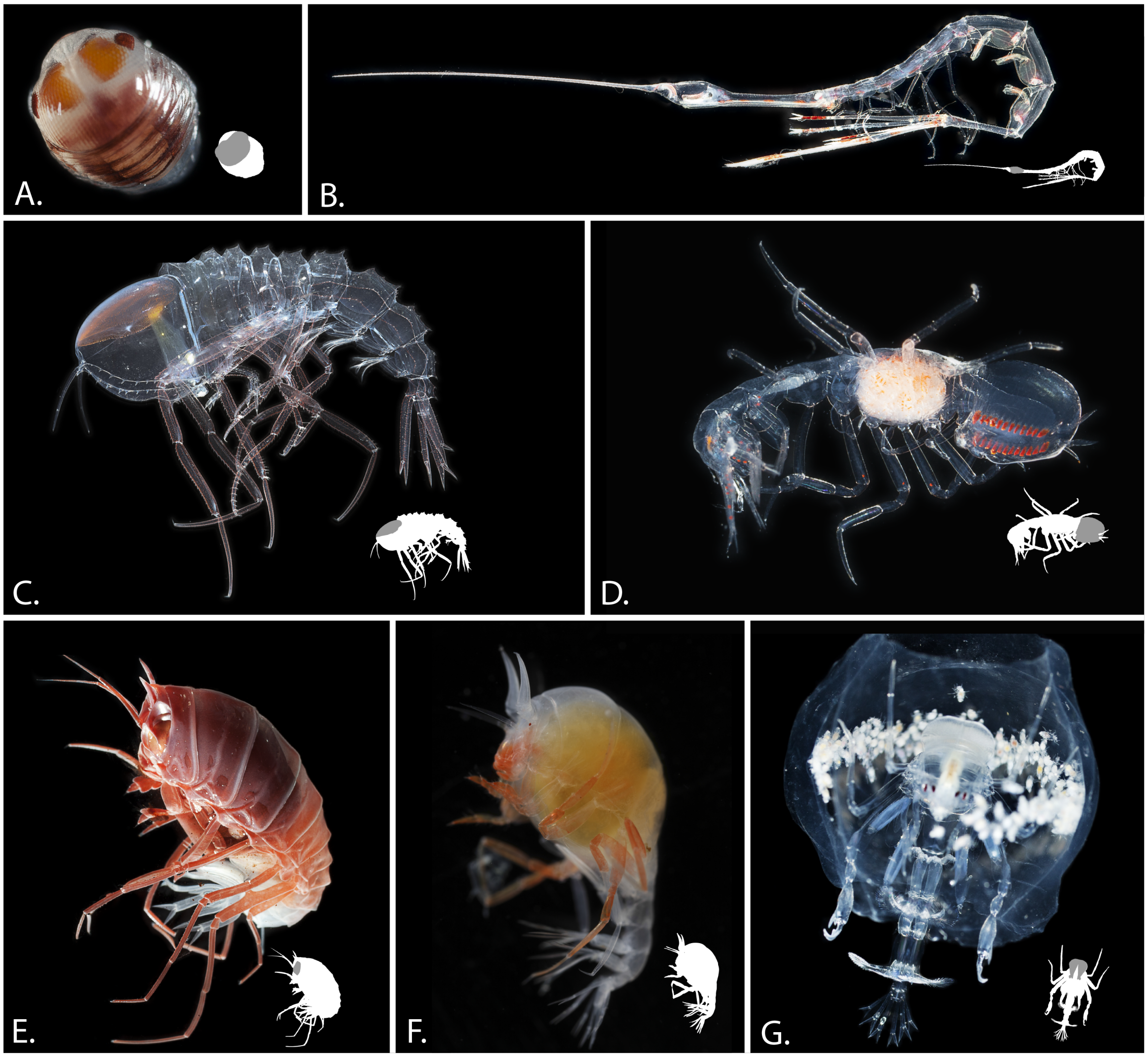

Figure 1.1: Examples of Hyperiid Morphological Diversity. Eye size and location indicated by gray shading on white silhouettes. A. *Platyscelus*, spherical bodies, 2 pairs of eyes. B. *Rhabdosoma*, elongated bodies, cylindrical eyes. C. *Cystisoma*, thin cuticle, eyes with diffuse retinal sheet. D. *Paraphronima*, thin cuticle, eyes with numerous retinas. E. *Scypholanceola*, globular bodies, eyes with mirrored pigment cups. F. *Mimonectes*, globular bodies, small or absent eyes. G. *Phronima* (with juveniles inside a salp barrel), 2 pair of eyes with fiberoptic-like crystalline structures.

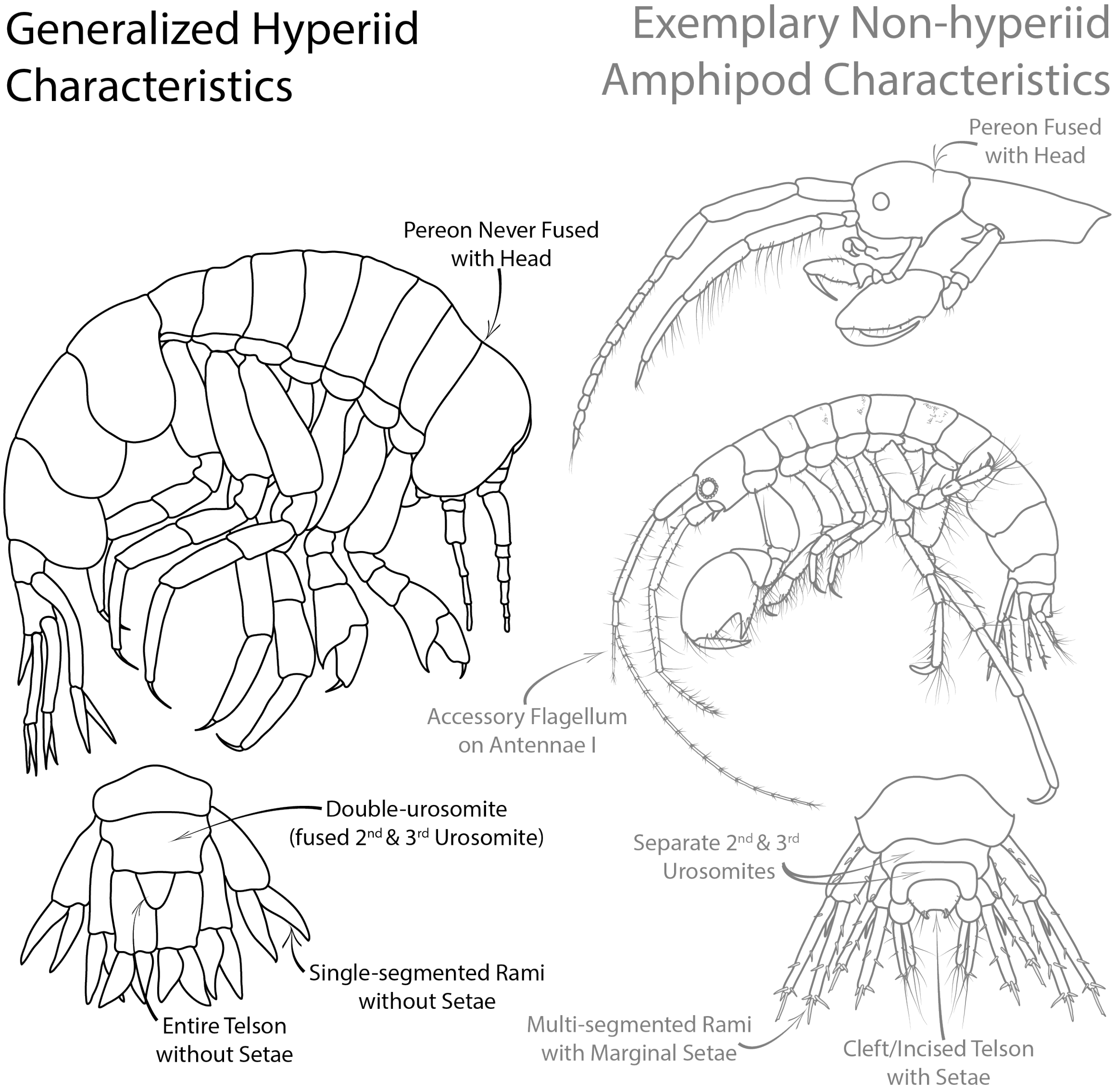

Figure 1.2: Morphological Characteristics common to hyperiid amphipods illustrated (in black) on a generalized hyperiid form and characteristics usually absent in hyperiids illustrated (in grey) on exemplary non-hyperiid amphipods.

Chapter 2: Methods

Data Selection

Gene selection across Amphipoda was based on the availability of hyperiid sequences and includes the nuclear genes encoding the 18S ribosomal RNA subunit (18S), the 28S ribosomal RNA subunit (28S), and histone H3 (H3), as well as the mitochondrial genes encoding cytochrome c oxidase subunit I (COI) and the 16S ribosomal RNA subunit (16S). Nucleotide sequences and associated metadata were obtained from GenBank, release number 224 [Benson et al., 2017], and parsed using a custom R script. The data were initially filtered to remove sequences from unclassified amphipod and environmental samples yielding a list of about 23,000 amphipod sequences. This list was used to design a taxon selection strategy for broad representation across Amphipoda and more detailed sampling among hyperiids and potential close relatives. All selected taxa and GenBank accession numbers are listed in Supplemental Section S4.

Selected taxa included every available species within Hyperiidea and within any promising closely-related genera uncovered during preliminary analyses. Multiple sequences were often available for a given genus or species. We chose to maximize gene representation and sequence length. Priority was given to taxa for which sequences from multiple genes of interest could be associated with the same specimen by a voucher or isolate number. In the absence of this information, sequences submitted by the same author were selected. If two groups of associated sequences covered different sets of genes for the same taxon, both groups were included. If a gene of interest was not available as an associated set, the longest unassociated sequence for the same taxon was included. Likewise, if an available unassociated sequence was much longer than the selected associated sequence, both were included. In preliminary analyses of concatenated gene sequences, the phylogenetic placement of taxa represented by only COI, H3, and/or 16S were highly variable and inconsistent. Hence, we chose to exclude all taxa not represented by either an 18S or 28S sequence. The outgroup for phylogenetic analyses comprised 12 isopod species for which at least four of the five genes of interest were available, and at least three of these genes could be linked by voucher or isolate number.

Multiple-Sequence Alignment

Multiple-sequence alignments were generated for each gene using MAFFT version 7 [Katoh and Standley, 2013]. The longest sequences were aligned first using the respective MAFFT algorithm listed in Table 2.1. The remaining sequences were grouped by length and added to the alignment using the `--addfragments` option in MAFFT, with groups of longer sequences added before shorter ones. The `--addfragments` option allows additional sequences to be added into an alignment independently, without considering the relationship among new sequences [Katoh and Frith, 2012]. Some sequences were occasionally, but not consistently, difficult to align. This issue was mitigated by separating out problematic sequences and adding them to the alignment after other sequences. For 18S and COI, the isopod outgroup sequences were particularly difficult to align and were therefore added to those alignments last. The protein-coding genes were aligned by amino acid sequence. The appropriate reading frame was determined from GenBank annotation information for most sequences, and for others by selecting the reading frame for which the translation appeared most fitting among all possible reading frames. Sequences were translated using the R package SeqinR [Charif and Lobry, 2007]. After multiple-sequence alignment, amino acid sequences were back-translated by supplying the alignment and original nucleotide sequences to RevTrans version 1.4 [Wernersson and Pedersen, 2003]. In each alignment, undetermined characters (`N`) were replaced by gap characters and sites containing data for fewer than four taxa (phylogenetically uninformative) were removed using a Perl script modified from Goodheart et al. [2015]. A summary of alignment characteristics is provided in Table 2.1 and additional details for the alignment of each gene are available in Appendix A.2.

Gene	Algorithm	Sequence Count	Alignment Length	Gaps
18S	Q-INS-i	353	5532 bp	70%
28S	E-INS-i	212	4590 bp	70%
COI	L-INS-i	369	1545 bp	58%
16S	E-INS-i	128	563 bp	34%
H3	E-INS-i	212	366 bp	9%

Table 2.1: Alignment Characteristics

Phylogenetic Analysis

Gene Trees Individual gene trees were inferred via maximum likelihood phylogenetic analysis with RAxML version 8.2.9 [Stamatakis, 2014] using a general time-reversible model of nucleotide substitution [Tavaré, 1986] with among site rate heterogeneity (GTR+Γ). The proportion of invariant sites is already accounted for when a model of rate heterogeneity is included in RAxML and was not specified separately. Alignments of protein-coding genes (COI and H3) were partitioned by codon position. For each alignment, a total of 42 independent optimization searches were used to find the topology with the highest likelihood. Twenty searches were started from randomized stepwise addition, maximum parsimony starting trees (`default`) and twenty searches were started from completely random starting trees (`-d`). The remaining two searches for the best-scoring tree were executed following 100 and 1000 rapid bootstrap replicates (`-f a`) where every fifth bootstrap tree was used as a starting tree [Stamatakis, 2014]. Support values for each node of the best-scoring topology were estimated with 1000 non-parametric bootstrap replicates (`-b`).

Analysis of Concatenated Genes To maximize representation of genes of interest, our sequence selection strategy very rarely led to the inclusion of more than one sequence per gene per taxon. This was the case when multiple sets of associated genes for a given taxon covered different, but overlapping, sets of genes or when a longer unassociated sequence was included in addition to a shorter associated sequence for the same gene. All sequences were included individually in the gene trees.

Provided duplicate sequences formed a clade in the gene tree, a consensus sequence was constructed with IUPAC nucleotide ambiguity codes. Consensus sequences were generated using the BioPerl `consensus_iupac()` method [Stajich et al., 2002] within scripts modified from Goodheart et al. [2015]. Consensus sequences were constructed for 12 COI sequences, 19 18S sequences, three 28S sequences, one 16S sequence, and no H3 sequences.

The five individual gene alignments were concatenated into a single matrix. The species tree was estimated via maximum likelihood phylogenetic analysis using the same methods described above for gene tree estimation. The concatenated alignment was partitioned by gene, with protein-coding genes (COI and H3) further partitioned by codon position.

Chapter 3: Results

Maximum likelihood analysis of the concatenated dataset recovers 100% bootstrap support (bss) for a monophyletic ingroup representing the peracarid order Amphipoda (Figure 3.1, Figure S5). This amphipod clade is also recovered with high bootstrap support in the single gene analyses of 18S (99%) (Figure S6) and 28S (100%) (Figure S7) and with moderate support in the analysis of 16S (81%) (Figure S10). The COI (Figure S8) and H3 (Figure S9) analyses lack resolution at deeper nodes.

Analysis of the concatenated dataset also recovers 100% bootstrap support for a monophyletic amphipod suborder, Hyperiidea (Figure 3.1). A hyperiid clade is recovered with high bootstrap support in the single gene analyses of 18S (97%) and 28S (100%). The COI, H3, and 16S analyses lack resolution at these deeper nodes.

Maximum likelihood phylogenetic analysis of the concatenated dataset recovers, with 100% bootstrap support, a clade that includes the monophyletic Hyperiidea as well as two clades of non-hyperiid amphipods representing potential Hyperiid sister groups. The relationships among these three clades could not be resolved and are presented as a polytomy. The first of these potential sister clades is recovered with 100% bootstrap support and comprises all 22 representatives of the genus *Leucothoe* which belong to the family Leucothoidae. Relationships among species of *Leucothoe* are largely in agreement with White and Reimer [2012a]. Disagreement exists only in the sister relationships within a well-supported clade comprising *L. akuma*, *L. amamiensis*, *L. vulagris*, and *L. akaisen* which are only moderately supported in both phylogenies. The second potential sister clade includes representative of three different amphipod groups. It includes a clade recovered with 100% bootstrap support and comprising the remaining representatives of the family Leucothoidae, which belong to two genera, *Anamixis* and *Paranamixis*. Recovered as sister to this clade (90% bss) is the only representative of the genus *Amphilochus* and recovered as sister to the resulting clade is the only representative of the genus *Colomastix*.

Analysis of the concatenated dataset recovers 100% bootstrap support for a clade representing the hyperiid infraorder, Physosomata, as it is classically defined (Figure 3.1). A clade comprising the enigmatic genera *Cystisoma* and *Paraphronima* and all representatives of Physosomata is recovered with 100% bootstrap support but we are unable to resolve the relationships among the three branches. Single-gene analyses of 28S and 18S recover highly supported clades that group *Cystisoma* and *Paraphronima* with the Physosomata. In the 28S analysis, the classically defined Physosomata is monophyletic with 100% bootstrap support (Figure S3). *Cystisoma* is recovered as its sister (96% bss) with *Paraphronima* as sister to both groups together (100% bss). In the 18S analysis, we do not recover the classically-defined Physosomata (Figure S2). Instead, a sister relationship is recovered between *Cystisoma* and *Paraphronima* (100% bss) and the clade they form falls within Physosomata. The COI and H3 analyses lack resolution and 16S data is only available for a single Physosomata representative.

Analysis of the concatenated dataset recovers 100% bootstrap support for a clade comprising all representatives of Physocephalata. This clade forms the sister group to the Physosomata (with *Cystisoma* and *Paraphronima* included in Physosomata). A monophyletic Physocephalata is also recovered with 100% bootstrap support in the analyses of 28S and 18S.

Relationships within Physocephalata are largely in agreement with Hurt et al. [2013]. We recover three clades representing the superfamilies, Vibilioidea, Phronimoidea, and Platysceloidea, each with 100% bootstrap support. We are unable to resolve the relationships among these superfamilies. Within Vibilioidea we recover a sister relationship between two monophyletic genera, *Cyllopus* (100% bss) and *Vibilia* (95% bss). Within Phronimoidea we recover strong support for the monophyletic families Phrosinidae (100% bss), Phronimidae (100% bss), Hyperiidae (99% bss), and Lestrigonidae (100% bss), with sister relationships between Phrosinidae and Phronimidae (99% bss) and between Hyperiidae and Lestrigonidae (100% bss). Within Hyperiidae, Hurt et al. [2013] recover a polyphyletic *Hyperoche* with a single representative of *Hyperia* nested within it. Our analysis added sequences for two additional representatives of *Hyperia* and, using morphological vouchers from Hurt et al. [2013], we re-identified a specimen of *Hyperoche medusarum* as *Hyperia sp.* (see Appendix A.1). Our analysis recovers monophyletic groupings for *Hyperia* and *Hyperoche*.

Within Platysceloidea we recover a monophyletic Parascelidae (100% bss) nested within a larger clade containing representatives of Amphithyridae and Platyscelidae. We also recover *Tryphana* (the only representative of Tryphanidae) as sister to all other representatives of Platysceloidea. Hurt et al. [2013] also recover this placement for *Tryphana* except with *Thyropus* (their only representative of Parascelidae) forming the sister group to *Tryphana*. Our analysis includes a second representative of Parascelidae, *Parascelus*, which we recover as sister to *Thyropus*, forming a monophyletic Parascelidae (100% bss) nested within a larger clade containing representatives of Amphithyridae and Platyscelidae. We also recover a clade containing all representatives of Oxycephalidae (95% bss) but nested within it is a representative of *Simorhynchotus* and two polyphyletic representatives of *Lycaea*. We recover a monophyletic Eupronoidae (77% bss) as sister to Oxycephalidae with 90% bootstrap support.

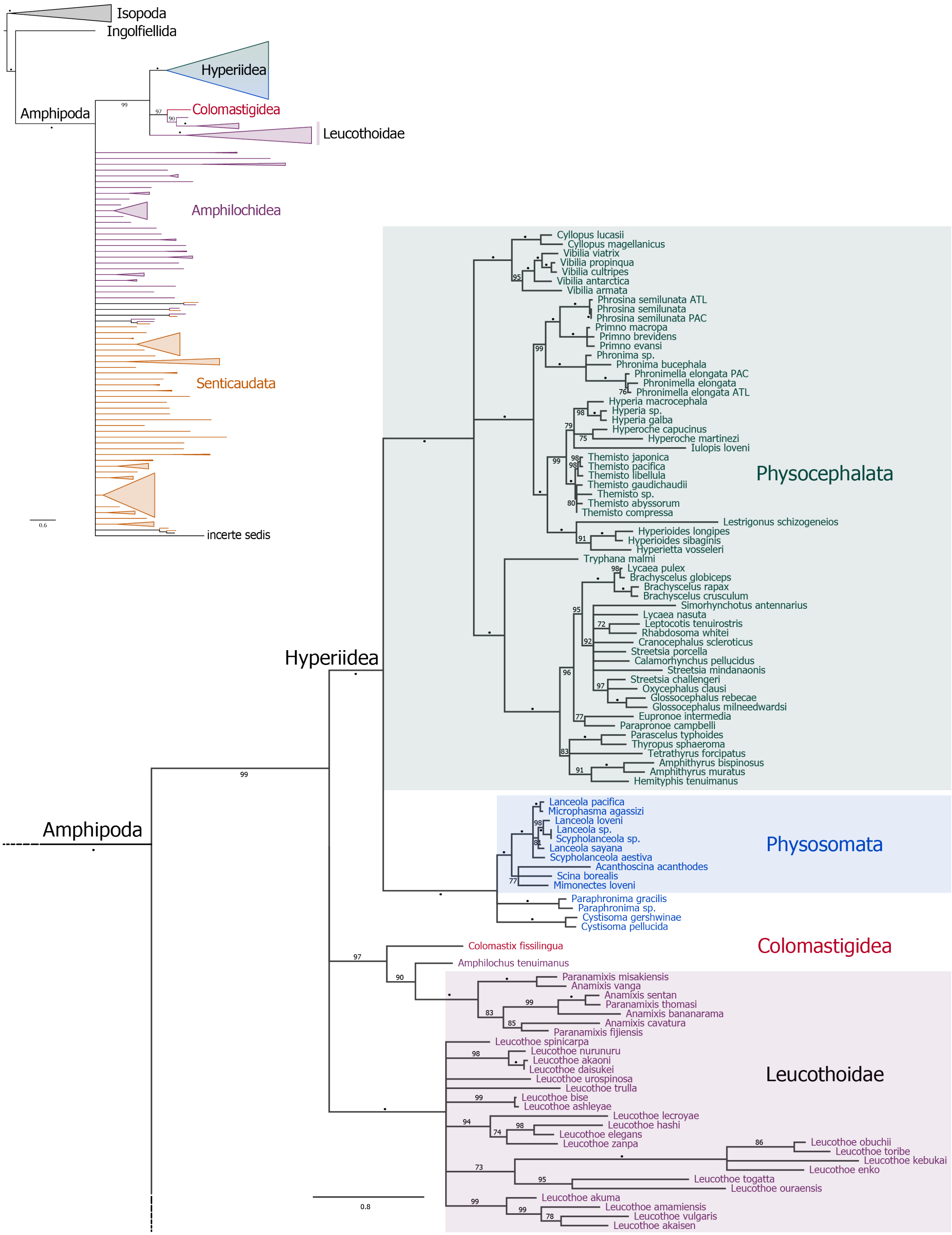

Figure 3.1: Phylogram of the maximum likelihood analysis of concatenated gene sequences. Branch support values reflect percent bootstrap values. 100% bootstrap support is indicated by a dot and all resolved nodes reflect >70% bootstrap support.

Chapter 4: Discussion

Our multi-locus phylogenetic analysis of Amphipoda strongly supports monophyly of the suborder Hyperiidea and therefore a single origin of the holopelagic lifestyle within hyperiids. This shared ancestry indicates their diverse morphological forms evolved either with or subsequent to their invasion of the midwater environment and may represent an evolutionary response to this ecological change. How hyperiid morphology and morphological diversity relates to ecology is still not well-understood. Many hypotheses have been suggested and continued advances in the ability to study pelagic animals *in vivo* have greatly facilitated ongoing research. Many studies have suggested that eye structure is correlated with depth [Land, 1981b, 1989], however, hyperiid species are predominantly collected in midwater trawls and most of their depth distributions are poorly characterized. Additional hypotheses link unique morphologies with the limitations imposed by a need see without being seen by predators in the open expanse of the oceanic midwater. Large, yet predominantly clear eyes with highly compact or thin, diffuse retina, allow for greater light-sensitivity while remaining nearly invisible [Land, 1981b, 1989, Ball, 1977, Johnsen, 2012] and clear bodies possessing and anti-reflective coating extend that invisibility to the rest of the animal [Bagge et al., 2016]. The function of duplicated eyes is not entirely clear but may be associated a the need to detect varying light sources including down-welling solar illumination and bioluminescence [Land and Nilsson, 2012, Land, 1981b, 2000]. Characteristics of hyperiid host and prey species may also be linked to morphologies necessary to locate, capture, consume, and/or inhabit such animals [Laval, 1980, Gasca and Haddock, 2004, Gasca et al., 2015, Baldwin-Fergus et al., 2015]. This may explain why many hyperiids are attracted to artificial light sources underwater. Land et al. [1995] show how *Brachyscelus*, *Phrosina*, and *Phronima* are attracted to and track a blue light source. They suggest this behavior functions to locate and pursue bioluminescent animals. The varied selective pressures imposed in the midwater environment by light sources, depth changes, predators, prey, and potential hosts are most likely driving hyperiid diversity and ecological specialization.

Hyperiid monophyly also indicates the few morphological traits shared among the group (see Figure 1.2) may represent true synapomorphies. Lowry and Myers [2017] identified several traits as significant hyperiid synapomorphies in their morphological analysis of the higher taxonomy of Amphipoda. These traits include the double-urosomite (fused second and third urosomites), uropods with lanceolate-shaped rami, a maxilliped (feeding appendage) lacking palps (segmented appendages), and minute serrations on the mandible incisor. Our identification of potential sister groups for Hyperiidea sheds light on the previously missing close-relatives and provides a rooting from which we can begin to explore the evolution of potential synapomorphies as well as their morphological diversity and the impact of ecological and environmental factors.

These potential hyperiid sister taxa are all small, benthic, commensal amphipods and have never before been considered close hyperiid relatives. These taxa are not particularly diverse, although some leucothoids possess strong sexual dimorphism and enlarged gnathopod claws thought to be a sexually selected trait [White, 2011]. The genera *Amphilochus*, *Leucothoe*, *Anamixis*, and *Paranamixis* are all representatives of the newly constructed amphipod suborder Amphilochidira. Species of *Amphilochus* are benthic amphipods commonly found as commensals of sea fans and hydroids as well as sponges, ascidians, and bivalves [Morales-Núñez and Chigbu, 2016]. Like most hyperiids, *Amphilochus* species have an entire telson (not cleaved/incised) without setae, the pereon is not fused with the head, and the first antennae often lack an accessory flagellum (if present it is minute, consisting of only a single article) [Morales-Núñez and Chigbu, 2016]. Unlike the hyperiids, however, all three urosomites are free (not fused) and there are palps on the maxilliped. The genera *Leucothoe*, *Anamixis*, and *Paranamixis* all belongs to Leucothoidae, a family of obligate endocommensal amphipods inhabiting sessile invertebrates including sponges, ascidians, and bivalves [White, 2011]. These are the only representatives of this family in our analyses. Although they all appear to be closely related to Hyperiidea, we do not see support for Leucothoidae as *Anamixis* and *Paranamixis* are recovered as more closely related to *Amphilochus* and *Colomastix* than to *Leucothoe*. *Anamixis* and *Paranamixis* together do form a highly-supported clade, however, our analyses do no support the separation of these genera. This is consistent with an 18S analysis of Leucothoidae [White and Reimer, 2012a]. We recover a sister relationship between representatives of *Anamixis sentan* and *Paranamixis thomasi*, the two species included in the 18S study, and the inclusion of an additional three *Anamixis* and two *Paranamixis* species results in the formation of three additional subclades with a represenative of each genera. This result strengthens the suggestion made by White and Reimer [2012a] that *Anamixis* and *Paranamixis* are in need of taxonomic review. A morphological analysis also found no support for the separation of these genera by the characters currently used to distinguish them [White, 2010] and they most likely need to be merged. *Leucothoe*, *Anamixis*, and *Paranamixis*, like hyperiids, have an entire telson (although sometimes possessing setae), a pereon free from the head, and no accessory flagellum on the first antennae (rarely present but with only one or two articles). However, like *Amphilochus*, these genera have three free urosomites and palps present on the maxilliped [White, 2011]. Lastly, *Colomastix* is a widespread genus typically occurring as commensals of marine sponges [LeCroy, 2009]. This genus belongs to one of two families that were recently elevated to form the amphipod suborder, Colomastigidea [Lowry and Myers, 2017]. No other sequence data is available for representatives of this suborder. *Colomastix* seems to share more traits with hyperiid amphipods. In addition to an entire telson without setae, a pereon free from the head, and a missing or minute accessory flagellum, *Colomastix* also possesses a double-urosomite (fused second and third urosomites) and a maxilliped with fused inner plates [Lowry and Myers, 2017, LeCroy, 2009].

The commensal lifestyle of these potential sister taxa is of particular interest because similar behaviors are frequently exhibited by hyperiids as well. Many hyperiid amphipods are parasites or commensals of gelatinous zooplankton [Laval, 1980, Gasca and Haddock, 2004, Gasca et al., 2015, Madin and Harbison, 1977, Harbison et al., 1977]. Although commensal lifestyles or life-stages are widely distributed across Amphipoda [Thiel, 1999], the presence of this behavior in all of the potential sister taxa leads us to speculate that commensal relationships may have played a role in the introduction of ancestral hyperiids into the midwater environment. Host organisms, whether benthic or pelagic, provide a stable microenvironment, shelter from predation, and often a source of nutrition. If ancestral hyperiids were adapted to live and thrive within the body of a benthic invertebrate host, it is possible they could have colonized closely-related bentho-pelagic or pelagic hosts before eventually solidifying a holopelagic lifestyle. If this is the case, it would appear the move from commensalism, where the inhabitants benefit from the host without inflicting harm, to parasitism or feeding on the host itself may have evolved within Hyperiidea. Although many amphipods form commensal relationships, parasitism is much less prevalent. The best known examples of parasitic amphipods are fish ectoparasites, whale lice (cyamid amphipods), and the hyperiids [Ruppert et al., 2004]. The potential sister taxa, however, feed on material available in or near their benthic hosts, and usually make use of host-generated feeding currents [White, 2011, Thiel, 1999]. The scarcity of food in the midwater environment many have pushed ancestral inhabitants to feed on their pelagic hosts. This hypothesis is supported by observations of hyperiids feeding on ctenophore hosts only when external food sources were not available [Laval, 1980].

Among the potential sister groups, the commensal lifestyles of *Leucothoe*, *Anamixis*, and *Paranamixis* have given rise to a number of social behaviors [White and Reimer, 2012b]. All of these genera exhibit extended parental care where juveniles remain under parental care or inhabit the same host after leaving the brood pouch [Thiel, 1999]. *Anamixis*, and *Paranamixis* have potentially developed a fully eusocial structure [White and Reimer, 1012]. These genera exhibit overlapping generations and an organized caste system with varying morphologies [White, 2010]. Social behaviors appear to be either absent or uncharacterized in *Amphilochus* and *Colomastix*. Social behavior is not extensive among hyperiids, but there are examples interactions among adults, extended parental care, and larval interattraction. Land (1992) described visually-mediated, potentially social (or predatory) interactions between adult hyperiids including tracking, pursuing, and ramming other hyperiids. Many hyperiids are known to exhibit swarming behaviors (Lobel, 1986). Some planktonic swarms are known to stem from social behaviors (Omari, 1982; Legendre, 1984), however, the forces driving hyperiid forms have not been characterized. Extended parental care is particularly apparent in species of *Phronima* which form a "maternal society" where the mother resides and cares for a brood of juveniles [Laval, 1980].

Our analyses are consistant with several characteristics of an adaptive radiation within Hyperiidea including shared ancestry and comparatively high morphological diversity.

Our analyses also indicates that representatives of the enigmatic genera *Cystisoma* and *Paraphronima*, traditionally grouped with infraorder Physocephalata (due to the presence of hypertrophied eyes), are more closely related to Physosomata. The movement of *Cystisoma* and *Paraphronima* from infraorder Physocephalata to Physosomata was first argued by Hurt et al. [2013] and our results indicate this finding is robust to the inclusion of additional hyperiid and non-hyperiid amphipods in the analyses.

Chapter 5: Conclusions

This work represents an important step in our understanding of the evolution of hyperiid amphipods as well as the diversification of a possible adaptive radiation within the largest habitat on the planet. We recover strong support for the monophyly of Hyperiidea and identify two clades comprising benthic commensal amphipods as potential hyperiid sister groups. Our results support the current definition of the amphipod suborder Hyperiidea and provide insight into its placement within Amphipoda. Like our analyses, most previous studies based on molecular data have exhibited at least some incongruence with morphological classifications of Amphipoda [Hou and Sket, 2015, Hurt et al., 2013, Ito et al., 2008, Verheye et al., 2016, Sotka et al., 2016]. Although confidence in a phylogenetic hypothesis of amphipod evolution is strengthened when morphological and molecular results agree [Lowry and Myers, 2017], incongruent analyses are vital to identifying potentially convergent characteristics and generating alternative hypotheses regarding the evolutionary history of the group. Broader genomic sampling will increase the number of molecular characters available for phylogenetic analysis and is a necessary next step towards a fully-resolved molecular phylogeny of Amphipoda. A well-resolved phylogeny would allow detailed morphological characterizations to be analyzed to test novel evolutionary hypotheses.

Appendix A: Supplemental Methods

Data Curation

Updated Identifications Due to their placement in preliminary gene trees, certain sequences from Browne et al. [2007] and Hurt et al. [2013] were re-identified by examining morphological vouchers provided by Dr. William Browne at the University of Miami and using the dichotomous key in Zeidler [2004]. The identifications of EF989686, KC428842, KC428893, and KC428944 were changed from *Hyperietta parviceps* to *Hyperioides sibaginis* and the identifications of EF989667, KC428897, KC428846, and KC428948 were changed from *Hyperoche medusarum* to *Hyperia sp.* Based on personal communication with Dr. William Browne, the identifications of KC428923, KC428872, KC428974, and EF989655 were changed from *Streetsia porcella* or *Glossocephalus sp. 19* to *Glossocephalus rebecae*, a new species described by Zeidler and Browne [2015].

Excluded Sequence A number of selected sequences were removed after preliminary analyses for reasons described below. Whenever possible, these excluded sequences were replaced by selecting another available sequence for the same taxon.

An 18S sequence identified as *Hyperietta sibaginis* (GU358617) was excluded because this is not an accepted genus and species combination and does not appear to have been one in the past. The sequence is similar to *Hyperioides* 18S sequences and therefore may be *Hyperioides sibaginis* but this identification is speculative so the sequence was excluded. An 18S sequence identified as *Hyperietta stephenseni* (DQ378051) may be miss-identified and was excluded. The sequences does not cluster with other *Hyperietta* or even hyperiid sequences and is not actually included in the doctoral dissertation cited for this sequence on GenBank [Englisch, 2001]. It is, however, very similar to a non-hyperiid amphipod sequence citing the same dissertation, which is identified as *Epimeriella walkeri* (DQ378005). Another 18S sequence, identified as *Eupronoe minuta* (DQ378052) and also citing Englisch [2001], was excluded because it is much more similar to *Hyperietta* sequences than to *Eupronoe* sequences and does not match the sequence length listed for this taxon and accession number in the cited dissertation [Englisch, 2001].

28S sequences mapping to the short D9-D10 region of the gene were excluded. There was little to no overlap with sequences from other regions of 28S.

A COI sequence identified as *Hyperia galba* (DQ889153), representing the only hyperiid in a crustacean-wide barcoding study [Costa et al., 2007], was excluded because a BLAST search indicated greater similarity to *Hyperoche* COI sequences. Three additional COI sequences were excluded because a BLAST search suggested they are not amphipod sequences: *Primno sp.* (GU145052), *Eupronoe intermedia* (HM053493), and *Oxycephalus clausi* (GU145053). Five COI sequences were removed after the sequences were trimmed according to the GenBank annotation of COI because only 186–190 bp of the 3′ end of COI remained. These sequences comprise AM749356, AM749332, AM749352, AM749345, and JQ319551.

Trimmed Sequences A COI sequence for *Platyscelus serratulus* (EF989662) was trimmed due to alignment issues. The sequence was not annotated with a reading frame or protein coding sequence. The reading frame was identified manually and 62 nucleotides were trimmed from the 5′ end to remove a region that would not align and included two stop codons. An H3 sequence for *Asellus aquaticus* (AJ238321) was trimmed according to the GenBank annotation, retaining positions 2600–3000. The 3′ half of a 16S sequence for *Lanceola sp.* (KP456062) was not alignable and was trimmed after position 119. 530 nucleotides at the 5′ end of a 16S sequence for *Niphargus kochianus* (KC315548) fell outside the gene region covered by any other sequence and were removed.

Concatenated Sequences Some sequences, linked by a voucher or isolate number, were found to be different regions of the same gene, sequenced from the same individual. After the order and direction was determined, such sequences were concatenated and subsequently aligned as a single sequence, labeled with both accession numbers. Concatenated sequences are indicated by a dash between accession numbers in the Taxon Selection Table (Supplemental Section S4).

Multiple-Sequence Alignment

Multiple-sequence alignments were generated using MAFFT version 7 [Katoh and Standley, 2013]. Alignment methods specific to individual genes are described here. The longest amphipod 18S sequences (>2000 bp) were aligned using the MAFFT Q-INS-i algorithm, a method recommended for the global alignment of divergent non-coding RNA sequences. This sequence-based, RNA alignment method incorporates structural information using an objective function derived from base-pairing probabilities [Katoh and Toh, 2008a]. The longest amphipod and isopod 28S sequences (>1225 bp) were aligned using the MAFFT E-INS-i algorithm [Katoh and Toh, 2008b]. The longest amphipod and isopod 16S sequences (>400 bp) were also aligned using the MAFFT E-INS-i algorithm. Before multiple sequence alignment, COI and H3 sequences were translated to amino acid sequences using the R package SeqinR [Charif and Lobry, 2007] with the invertebrate mitochondrial and standard genetic codes respectfully. For COI, the longest amphipod amino acid sequences (>220 aa) were aligned using the MAFFT L-INS-i algorithm [Katoh and Toh, 2008b]. All H3 sequences were aligned together using the E-INS-i algorithm in MAFFT [Katoh and Toh, 2008b]. The aligned COI and H3 sequences were then back-translated by supplying the original nucleotide sequences and amino acid alignments to RevTrans version 1.4 [Wernersson and Pedersen, 2003].

Appendix B: Supplemental Discussion

Introduction - Amphipod Systematics Amphipoda [Crustacea; Malacostraca; Peracarida] is a highly speciose order consisting of 223 families, over 1,600 genera, and nearly 10,000 described species [Lowry and Myers, 2017]. Amphipods are accepted as a monophyletic group defined by their unique arrangement of pereopods into two distinct forward and reverse-facing groups [Bousfield and Shih, 1994]. The order is further distinguished from other peracarids by the presence of sessile eyes, coxal gills, and a differentiated pleosome and urosome, each with three segments [Lowry and Myers, 2017]. The majority of amphipods are marine or estuarine; however, some species are found in freshwater, supralittoral, and terrestrial environments. The amphipod body is usually laterally compressed and always lacks a carapace. Until recently, Amphipoda was organized into four suborders: Hyperiidea, Caprellidea, Gammaridea and Ingolfiellidea. This classification was revised through a series of morphological phylogenetic analyses [Lowry and Myers, 2017, Myers and Lowry, 2003, Lowry and Myers, 2013]. The superfamily Corophoioidea was removed from Gammaridea and joined with Caprellidea forming suborder Corophiidea (characterized by a thickened telson and robust setae on uropods) [Myers and Lowry, 2003, Barnard and Karaman, 1984]. These robust setae were later deemed a synapomorphy of a much larger clade of amphipods including Corophoioidea as well as most freshwater and some benthic marine species of Gammaridea [Lowry and Myers, 2013]. These groups were combined to form the new suborder Senticaudata. Most recently, Lowry and Myers [2017] argued for splitting Gammaridea into three new suborders (Colomastigidea, Hyperiopsidea, Amphilochidea) and Ingolfiellidea into one new amphipod suborder (Pseudingolfiellidea) and a new peracarid order (Ingolfiellida). Amphipod suborders currently listed by the World Amphipod Database comprise Amphilochidea, Colomastigidea, Hyperiidea, Hyperiopsidea, Pseudingolfiellidea, and Senticaudata [Horton et al., 2018].

Ingolfiellida A single 18S sequence for *Ingolfiella tabularis* (the only available representative of Ingolfiellidea) is recovered as sister to all other amphipods in the concatenated analysis as well as the 18S gene tree. This result is consistent with the findings of a prior 18S analysis, which included the same *Ingolfiella tabularis* sequence [Verheye et al., 2016], as well as a recent morphological analysis, which raised Ingolfiellidea from an amphipod suborder to a new peracarid order, Ingolfiellida [Lowry and Myers, 2017]. Representatives of Ingolfiellida differ from those of Amphipoda in the presence of pedunculate eyes, a six-segmented pleosome, and reduced appendages (pleopods and uropods), but are considered sister to Amphipoda due to the shared presence of coxal gills and absence of a carapace [Lowry and Myers, 2017]. Assessing whether molecular data support an Ingolfiellida-Amphipoda sister relationship requires additional sequence data, particularly for representatives of the six other genera within Ingolfiellida as well as representatives of Pseudingolfiellidea.

Senticaudata and Amphilochidea Our results show no evidence of monophyly for either of the amphipod suborders Senticaudata and Amphilochidea. Although we are unable to resolve relationships along the backbone of Amphipoda, we recover four small clades that each include at least one representatives of each of Senticaudata and Amphilochidea (Figure 1). Furthermore, the single representative of the suborder Colomastigidea (*Colomastix fissilingua*), as well as all representatives of the suborder Hyperiidea, fall within a highly supported clade that also includes many representative of Amphilochidea (Figure 2). The suborder Amphilochidea is characterized by the presence of brush setae in adult males and was erected to unite two infraorders (Amphilochida and Lysianassida), comprising most of the families formally belonging to the now vacant suborder, Gammaridea [Lowry and Myers, 2017]. Both Amphilochida and Lysianassida are polyphyletic in all of our analyses. The suborder Senticaudata is defined by the presence of spines (robust apical setae) at the tips of the rami of the first and second uropods [Lowry and Myers, 2013], a characteristic which exhibits homoplasy when mapped onto molecular analyses [Verheye et al., 2016] and appears to be present at intermediate states in multiple dissimilar amphipods [d'Udekem d'Acoz and Verheye, 2017]. Myers and Lowry [2018] reassert these spines are a strong synapomorphy for Senticaudata, secondarily gained in only a few species outside the suborder. Our ability to use molecular data to resolve the deeper relationships within Amphipoda is currently limited by taxon sampling and and possible loss of phylogenetic signal due to substitution saturation in the five genes we analyzed.

Senticaudata Analysis of the concatenated dataset recovers, with at least 70% bootstrap support, 13 clades containing only representatives of the amphipod suborder Senticaudata. These clades range in size from two to 57 taxa. The placement of 19 representatives of Senticaudata could not be resolved and are shown as individual taxa branching from the Amphipoda polytomy (Figure S1). We recover monophyly of the superfamily Talitroidea (100% bss) with 30 taxa representing 29 genera and five families. A clade comprising 26 of the 27 taxa of infraorder Corophiida (representing 25 genera and 11 families) was recovered with 86% bss (placement of *Neohela monstrosa* was not resolved). A clade comprising all five taxa of the family Niphargidae as well as both taxa of the family Pseudoniphargidae was recovered with 86% bss. A clade comprising 57 of the 67 representatives of superfamily Gammaroidea is recovered with 70% bss. Five of the remaining taxa form a clade (86% bss) comprising the monopyletic family Anisogammaridae (99% bss) and the only representative of the family Bathyporeiidae. Placement of the remaining five representatives of Gammaroidea was not resolved.

Amphilochidea Analysis of the concatenated dataset recovers (with at least 70% bootstrap support) 12 clades containing only representatives of the amphipod suborder Amphilochidea. These clades range in size from two to 23 taxa. The placement of 15 representatives of Amphilochidea could not be resolved and are shown as individual taxa branching from the Amphipoda polytomy (Figure S1). We recover monophyly of the families Ampeliscidae (three taxa, 100% bss), Oedicerotoidea (four taxa, 100% bss), and Stegocephalidae (three taxa, 100% bss). A clade comprising five of the six taxa of the family Iphimediidae was recovered with 100% bss (placement of *Pariphimedia integricauda* was not resolved). The family Leucothoidea appears polyphyletic with representatives split among two clades. All representatives of the genus *Leucothoe* form one clade (100% bss). The other includes all representatives of the genera *Paranamixis* and *Anamixis* (100% bss) and is nested within a clade containing the single representatives of the family Amphilochidae and the suborder Colomastigidea (90% bss).

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