

A molecular phylogeny of marine amphipods in the herbivorous family Ampithoidae

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Ampithoid amphipods dominate invertebrate assemblages associated with shallow-water macroalgae and seagrasses worldwide and represent the most species-rich family of herbivorous amphipod known. To generate the first molecular phylogeny of this family, we sequenced 35 species from 10 genera at two mitochondrial genes [the cytochrome *c* oxidase subunit I (COI) and the large subunit of 16 s (LSU)] and two nuclear loci [sodium-potassium ATPase (NAK) and elongation factor 1-alpha (EF1)], for a total of 1453 base pairs. All 10 genera are embedded within an apparently monophyletic Ampithoidae (*Ampitholina*, *Ampitboe*, *Biancolina*, *Cymadusa*, *Exampitboe*, *Paragrubia*, *Perampitboe*, *Pleonexes*, *Plumitboe*, *Pseudoampithoides* and *Sunampitboe*). *Biancolina* was previously placed within its own superfamily in another suborder. Within the family, single-locus trees were generally poor at resolving relationships among genera. Combined-locus trees were better at resolving deeper nodes, but complete resolution will require greater taxon sampling of amphipods and closely related outgroup species, and more molecular characters. Despite these difficulties, our data generally support the monophyly of Ampithoidae, novel evolutionary relationships among genera, several currently accepted genera that will require revisions via alpha taxonomy and the presence of cryptic species.

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Introduction

Amphipod crustaceans in the family Ampithoidae Boeck 1871 dominate invertebrate assemblages associated with shallow-water macroalgae and seagrasses worldwide (Conlan 1982; Poore *et al.* 2008) and represent the most species-rich family of herbivorous amphipod known (209 species described in 15 genera, Horton & De Broyer 2014). These amphipods are ‘insect-like’ herbivores (Hay *et al.* 1987) in that they are locally abundant, small relative to the hosts that they use for both habitat and food, and can sometimes have strongly negative impacts on macrophyte dynamics in the field (Chess 1993; Graham 2002;

Mukai & Iijima 1995; Reynolds *et al.* 2012, but see Carpenter 1986; Poore *et al.* 2009). Despite featuring in hundreds of studies on the ecology of nearshore habitats, and proposed hypotheses about the influence of phylogeny on their feeding and behaviour (Poore *et al.* 2008), there are no molecular-based phylogenies that compare relationships within and among the genera of Ampithoidae. Molecular studies have been used to describe relationships among lineages for a small subset of species within amphipod genera (McCarty & Sotka 2013; Sotka *et al.* 2003; Pilgrim & Darling 2010; Kim *et al.* 2012). Based on morphological traits, the family Ampithoidae is a robust monophyletic group

defined by the outer ramus of uropod 3 having 1–2 recurved, robust apical setae (Barnard & Karaman 1991; Myers & Lowry 2003).

Within the family, only one major evolutionary hypothesis has been offered, with Conlan (1982) generating a phylogeny for 12 genera based on 27 morphological characters (Fig. 1). In that work, *Amphitholina* Ruffo, 1953 was ‘strongly separated’ from the other genera within the Ampithoidae, supporting previous work by Barnard (1972) and Myers (1974) which recognized Amphitholinae as a distinct subfamily. The remaining ampithoid genera were grouped into three clades: the genus *Pleonexes*; genera which have their first gnathopod with oblique palms (e.g. *Ampitboe* Leach, 1814); and genera with transverse palms on gnathopod 1 (e.g. *Exampitboe* K.H. Barnard, 1926). The genus *Pseudoamphithoides* Ortiz, 1976 was an exception, as it shares several characters with the transverse-palmed genera, but has oblique palms on gnathopod 1. The phylogeny proposed by Conlan (1982) was based on phenetic methods, and there have been no family-level phylogenies produced using either cladistic or molecular methodology. Furthermore, most of the genera with the Ampithoidae are currently characterized by few morphological characters, and in at least one case, by continuous traits that overlap

between genera. For example, the presence of a mandibular palp separates the genera *Peramphithoe* Conlan and Bousfield, 1982 and *Sunamphithoe* Bate, 1857, but is both present and absent within the genus *Exampitboe* (Myers & Lowry 2003). These uncertainties, and the likelihood that many morphological traits in the amphipods are homoplasious (Hurt *et al.* 2013; Lowry & Myers 2013), suggest that a phylogeny based on molecular data could lead to new insights into evolution in this group.

In this study, we used sequences from two mitochondrial genes [the cytochrome *c* oxidase subunit I (COI) and the large subunit of 16 s (LSU)] and two nuclear loci [sodium–potassium ATPase (NAK) and elongation factor 1-alpha (EF1)] to construct the first molecular phylogeny of 10 ampithoid genera, including nine of the 12 genera from Conlan (1982).

Methods

Taxon sampling

Amphipods from the family Ampithoidae were collected by hand from shallow-water habitats worldwide (Table S1) and placed into 95% denatured ethanol. Our collections included 35 species from 10 genera from Europe, North America, South America, Asia and Australasia. For two

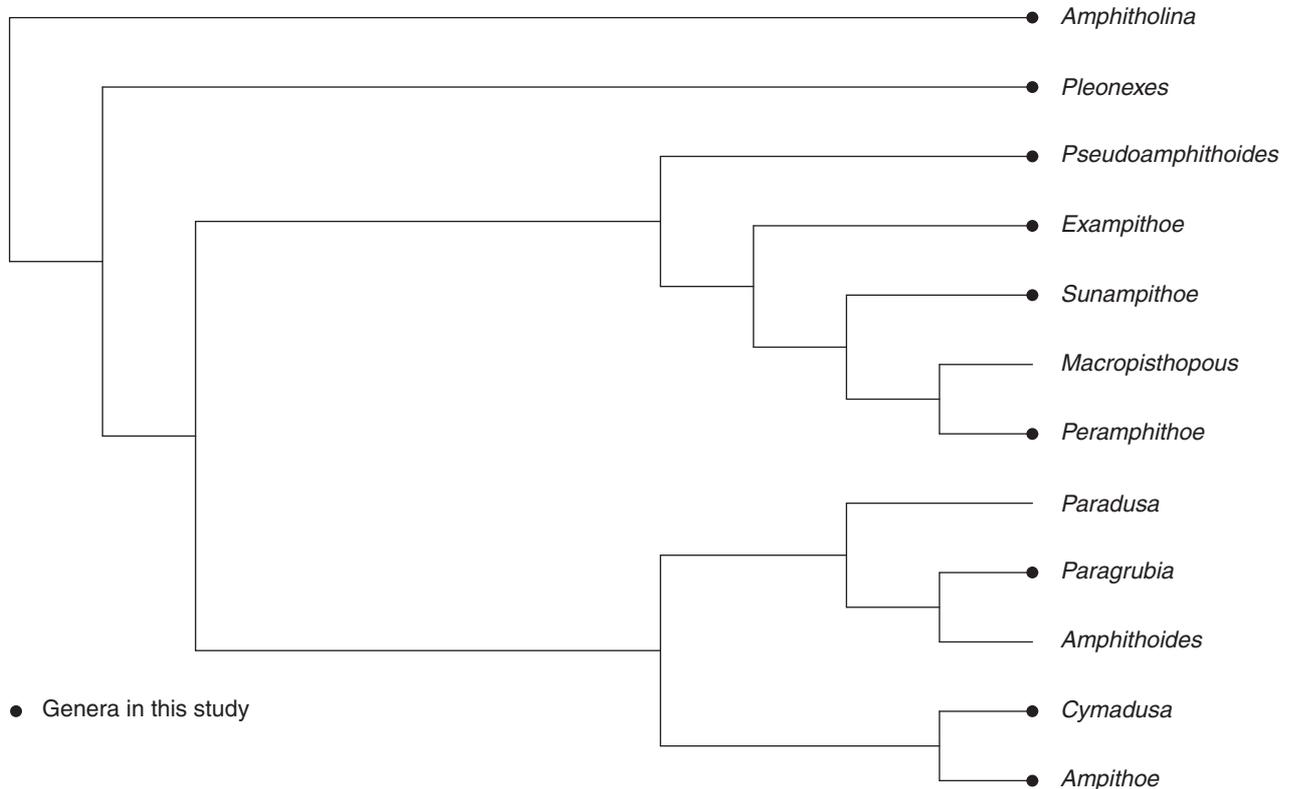


Fig. 1 The phylogeny of Ampithoidae proposed by Conlan (1982) based on phenetic analysis of 27 morphological characters. Genera with a black dot were sequenced in this study.

species with wide geographic distributions, we separately analysed samples from more than one region (*Ampithoe ramondi* from Brazil and Hawaii, *A. valida* from east coast USA, west coast USA and Japan). For *A. tarasovi*, we separately analysed two colour morphs recognized by researchers in Japan (Hiroyuki Ariyama, personal communication). We also collected *Biancolina japonica* from the family Biancolinidae J.L. Barnard, 1972 as this genus has been closely associated with the Ampithoidae in past taxonomic treatments (Stebbing 1906; Ruffo 1953).

Amphipods were identified to genus and species before extraction of DNA. To be confident of species identifications, male specimens were typically used as the characters involved in species-level identification of amphipods are frequently those associated with secondary sexual characters, in particular the morphology of the enlarged second gnathopod. All samples were identifiable to described taxa except for three novel species; one from *Sunamphitoe* and two from *Exampithoe*. In most cases, we had to destructively sample entire amphipods during extraction. Other individuals from the same samples were held as voucher specimens and have been deposited in the Australian Museum. Table S1 includes GenBank accession numbers and voucher identification for all species sampled. Three other species from the suborder Senticaudata were used as outgroups: *Gammarus mucronatus* (infraorder Gammarida; family Gammaridae), *Parhyale hawaiiensis* (infraorder Talitrida; family Hyalidae) and *Melita plumulosa* (infraorder Hadziida; family Melitidae).

Generating DNA sequences

Amphipod DNA was extracted using Qiagen (Limburg, the Netherlands) DNeasy Blood and Tissue kit. We amplified loci using the primers listed in Table S2. Gene regions were amplified using the following 20 μL recipe: 4 μL Promega GoTaq 5X Buffer (Promega, Madison, WI, USA), 0.75 mM total dNTP, 3.75 mM MgCl_2 , 0.25 μM (5 pmol) of forward and reverse primers, 1 $\mu\text{g}/\mu\text{L}$ Bovine Serum Albumin (NEB, Ipswich, MA, USA), 1 unit of GoTaq polymerase and approximately 10 to 50 ng of DNA. Standard PCR conditions were used for 16 s and COI with the following cycles and temperatures: 95 °C for 60 s, 90 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min, for 30–35 cycles. For EF1, the above PCR conditions were used except for 52 °C annealing temperature instead of 45 °C. For NAK, the above conditions were used or a touchdown PCR protocol to increase the success of PCR amplification.

Unincorporated primers and dNTPs were removed using Antarctic phosphatase and exonuclease I (NEB) using the following protocol: in a 12–17 μL reaction, 10–15 μL of PCR product was combined with 1 μL of exonuclease I

(20 units/ μL), 1 μL of Antarctic phosphatase (5 units/ μL). This reaction mixture was then incubated at 37 °C for 15 min and 80 °C for 15 min. Each of these ‘clean’ reactions was then quantified using a NanoDrop spectrophotometer (Thermo-Scientific, Waltham, MA) and diluted to 40 ng/ μL . These reactions were then sent to Macrogen USA (Rockville, MD, USA) for sequencing. Forward and reverse sequences were assembled and edited using Geneious 4.8 (<http://www.geneious.com>, Kearse *et al.* 2012) Any sequences with lower than 80% quality scores within Geneious were discarded and resequenced. Heterozygotes were scored as ambiguous with codes of R or Y depending on purine or pyrimidine changes.

Analyses

Alignment. For COI, EF1 and NAK, all sequences were aligned using CLUSTAL-X (Larkin *et al.* 2007) and visually inspected. In cases in which more than one individual of a terminal node was sequenced, we generated a majority consensus sequence using CONSENSUS in the package SEQNR (v. 3.0-7c) (Charif & Lobry 2007). For LSU (or the mitochondrial 16 s) sequences, we made the consensus sequence first and then generated alignments with multiple gap and extension penalties (10/5, 10/1, 5/5, 5/1, 2/2, 2/1) following MacDonald *et al.* (2005). A visual inspection of the resulting trees indicated that the topology was broadly similar, and we pursued analysis using the 10/5 alignment because of its consistency with other loci across most nodes. We inferred phylogenies using three or four loci for each taxa. Sequences were concatenated using APE (Paradis *et al.* 2004) and manipulated using SEAVIEW (Gouy *et al.* 2010) and MESQUITE (Maddison & Maddison 2015).

Substitution saturation. We assessed substitution saturation of codon positions in our protein-coding loci (COI, EF1 and NAK) as implemented in DAMBE (Xia 2013). At codon positions 1 and 2 of COI, I_{SS} (a measure of substitution saturation) was significantly lower than $I_{SS,C}$ (a critical value determined from computational simulation) while I_{SS} and $I_{SS,C}$ were indistinguishable at codon position 3. This pattern indicates substantial saturation at codon position 3, and we removed this locus for subsequent analyses. By these same criteria, all codon positions at EF1 and NAK were not saturated.

Phylogenetic analysis – single-locus. We randomly subsetted 10 taxa and used jMODELTEST2 (Darriba *et al.* 2012) to evaluate the best model of evolution (as determined by AIC criteria) for each locus (Table S3). We analysed all individuals for which we had data at a given locus, and thus, the number of individuals differed across loci. Single-locus data sets were analysed with a Bayesian search in

MRBAYES (Ronquist *et al.* 2012) with 10 million generations, sampling every 1000 generations. After removal of 2500 of 10 000 trees, log likelihood (LnL) was consistent across two independent runs at each locus and effective sample sizes (ESS) exceeded 1000 (as summarized by TRACER 1.4 (Rambaut *et al.* 2014) or LOGANALYZER (Drummond *et al.* 2012). The *comparetrees* command within MrBayes showed strong convergence of topologies across independent runs. We then generated a majority-rule consensus tree using the *sumt* command within MrBayes and visualized using FIGTREE (Rambaut 2014) and APE. See Figs S1–S4 for individual trees.

Phylogenetic analysis – multilocus. Multilocus analyses included all species ($n = 40$ amphitoid + 3 outgroup taxa) for which at least three or more loci were sequenced (1453 bp total). Because incorporating a partitioning strategy may fundamentally alter phylogenetic inference, we inferred phylogenies using no partitions (P_1), a partition by genome (P_{2A}), by locus type (P_{2B}) and by locus (P_4 ; Table S3). Bayesian searches were implemented in BEAST (Drummond *et al.* 2012) on the CIPRES server (Miller *et al.* 2010) using 50 million generations, sampling every 10 000 generations. After removal of 10 000 of 50 000 trees, log likelihood (LnL) was consistent across two independent runs at each locus and ESS values exceeded 1000. A Bayes-factor analysis (or an absolute difference in the harmonic means of LnL; (Kass & Raftery 1995) indicated that P_{2A} was the most appropriate model (Table S3).

We also pursued bootstrap ML analyses with RAXML (Stamatakis 2014) using RAXML (Silvestro & Michalak 2011) on all (un)partitioned data sets using GTRGAMMA for each partition, two independent runs and 10 000 bootstrap replicates. Support values were summarized using SUNTTREES (Sukumaran & Holder 2010), and trees were visualized using PHYLOCH (Heibl 2013) and APE.

Results

Phylogeny of the Ampithoidae

Single-locus phylogenies (Figs S1–S4) showed strong support for the monophyly of the Ampithoidae at three of four loci. The exception was at LSU where a polytomy included all amphitoid species plus an outgroup of *Gammarus* and *Parbyale* species. When all data were combined (Fig. 2; Fig. S5), there was high posterior probability (100%) and ML-bootstrap support (100%) of a monophyletic Ampithoidae.

Within the Ampithoidae, the monophyly of *Cymadusa* Savigny 1816 and *Exampithoe* was well supported in both multilocus (Fig. 2) and some single-locus phylogenies. In contrast, we found no statistical support for a distinction between *Perampithoe* and *Sunampithoe*. The genus

Biancolina Della-Valle, 1893, most recently placed in the monotypic family Biancolinidae, is clearly nested within the Ampithoidae.

We detected two major clades within the most speciose genus in the family, the currently-accepted genus *Ampithoe*. One clade, termed here the *Ampithoe*-‘south’ clade, represents the most ancestral lineage of Ampithoidae. An analysis of occurrence data from the Global Biodiversity Information Facility indicates that the ‘south’ clade dominates temperate to tropical Pacific Oceans, as well as the Indian, Mediterranean and Atlantic Oceans. The *Ampithoe*-‘north Pacific’ clade is a more derived clade whose species occur within the northern, cold-temperate Pacific Ocean (Table 1; Fig. 3). The exception to this pattern is *Ampithoe valida* which occurs in both the north Pacific and Atlantic (see *Discussion*).

We also found several broadly dispersed species that represent a complex of multiple cryptic species or subspecies. Specimens identified as *Ampithoe ramondi* collected from Hawaii and Spain are likely different species, as the former clusters with *A. kava* (from Australia) and the latter with *A. plumulosa* (from Ecuador) and *A. longimana* (from the US east coast). Japanese specimens identified as *A. tarasovi* ‘light’, *A. tarasovi* ‘dark’ and *A. lacertosa* formed a single monophyletic clade.

Systematics

Order Amphipoda Latreille, 1816

Suborder Corophiidea Leach, 1814

Infraorder Corophiida Leach, 1814

Superfamily Corophioidea Leach, 1814

Family Ampithoidae Boeck, 1871

Diagnosis (unaltered from Myers & Lowry, 2003). Labium outer plate with or without distal notch or excavation. Uropod three outer ramus with two recurved robust setae, or with one small, straight or weakly curved robust seta. Telson cusps present or absent.

Subfamily Ampithoinae Boeck, 1871

Diagnosis (unaltered from Myers & Lowry, 2003). Mandible palp 3-articulate or absent. Labium outer plate with distal notch or excavation. Uropod three outer ramus with two recurved robust setae or with one small, straight or weakly curved robust seta.

Generic composition. *Ampithoe* Leach, 1814; *Ampithoides* Kossmann, 1880; *Amphitholina* Ruffo, 1953; *Austrothoe* Peart, 2014; *Biancolina* Della-Valle, 1893; *Cymadusa* Savigny, 1816; *Macropisthopus* K. H. Barnard, 1916; *Paradusa* Ruffo, 1969; *Paragrubia* Chevreux, 1901; *Paranexes* Peart, 2014; *Perampithoe* Conlan and Bousfield, 1982; *Plumithoe* Barnard and Karaman, 1991; *Pseudampithoides* Ortiz, 1976;

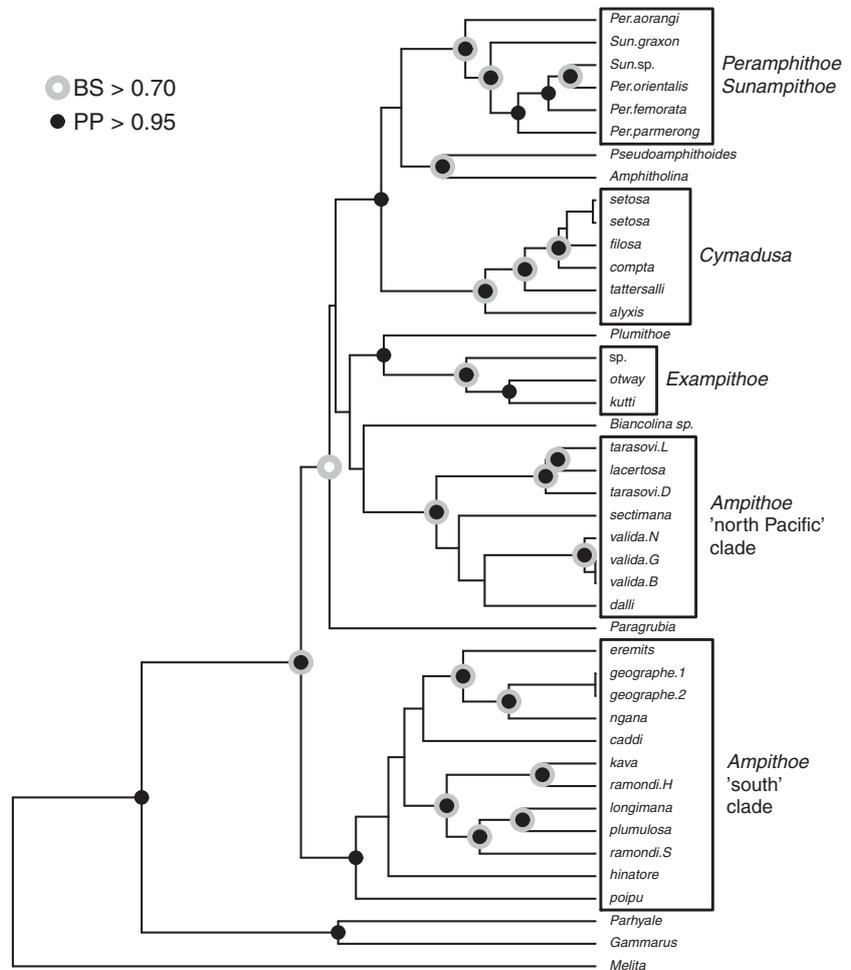


Fig. 2 A consensus Bayesian phylogeny of Ampithoidae using mitochondrial (COI, 16 s) and nuclear (NAK, EF1) loci and partition strategy P_{2A} (Table S3). Black dots indicate nodes with 95% posterior probability (PP), and grey circles indicate 70% bootstrap support (BS). Nodes of terminal sister taxa [e.g. *Ampithoe valida* (G) and *A. valida* (B)] had strong PP and BS support (Fig. S5).

Pseudopleonexes Conlan and Bousfield, 1982; *Sunamphithoe* Bate, 1857.

Remarks. Transferring *Biancolina* back to Ampithoidae subsumes its previous status as a monotypic superfamily. This is a substantial shift in taxonomic status and taxon relationships, transferring between suborders. Such extreme transitions can be expected for highly derived monotypic groups. Convergent morphology is evident in several genera of amphipods that are known to burrow inside algal tissues (Mejaes *et al.* 2015). Note that five genera were not sampled as part of the molecular analyses in this study (*Ampithoides*, *Macropisthopus*, *Pseudopleonexes* and the recently described *Austrothoe* and *Paranexes*).

Discussion

Ampithoid amphipods are numerically dominant members of shallow marine ecosystems worldwide and can have important ecological roles as herbivores. Here, we generated the first molecular treatment of phylogenetic

relationships within the Ampithoidae using 1453 base pairs across two nuclear and two mitochondrial loci for 35 amphipod species across 10 genera. Overall, single-locus trees were generally poor at resolving relationships among genera. Combined-locus trees were better at resolving deeper nodes, but complete resolution will require greater taxon sampling of both amphipods and closely related outgroup species and more molecular characters. Despite these issues, the existing data set suggests the monophyly of Ampithoidae, novel evolutionary relationships among genera, several currently accepted genera that will require revisions via alpha taxonomy and the presence of cryptic species.

Ampithoid genera

We sequenced eight of the currently accepted genera within Ampithoidae (*Amphitholina*, *Ampithoe*, *Cymadusa*, *Exampithoe*, *Paragrubia*, *Peramphithoe*, *Plumithoe*, *Pseudoamphithoides* and *Sunamphithoe*), and all are embedded within a monophyletic Ampithoidae (Fig. 4). We note, however, that our

Table 1 *Ampitboe* clade distributions. *Ampitboe* species were delineated by molecular phylogeny into north Pacific or south clades. Hypothesized clades for other *Ampitboe* species are based on reported geographic distributions. Citation: GBIF.org (4th June 2015) GBIF Occurrence Download <http://doi.org/10.15468/dl.1815go>

Species	Locations	Ocean region	Source
North Pacific clade			
<i>dalli</i>	Russia, Alaska, Canada, south to California	NW and NE Pacific	GBIF/this study/Conlan & Bousfield 1982;
<i>lacertosa</i>	Japan, Alaska, Canada, south to Baja California	NW and NE Pacific	GBIF/this study/Conlan & Bousfield 1982;
<i>sectimana</i>	Alaska to California	NW Pacific	GBIF/this study/Conlan & Bousfield 1982;
<i>tarasovi</i>	Japan	NE Pacific	This study
<i>valida</i>	N. America, Japan, Europe, S. America	Cosmopolitan	GBIF/this study/Conlan & Bousfield 1982
South clade			
<i>caddi</i>	Australia	SE Pacific	GBIF/this study/Peart 2007
<i>eremits</i>	Australia	SE Pacific	GBIF/this study/Peart 2007
<i>geographe</i>	Western Australia	SE Pacific/Indian Ocean	GBIF/this study
<i>hinatore</i>	New Zealand	SE Pacific	This study
<i>kava</i>	Fiji, Hawaii, Australia, Red Sea	SE Pacific/Indian Ocean	This study/Peart 2007
<i>longimana</i>	West and east US, Caribbean, west Mexico	Cosmopolitan	GBIF/this study
<i>ngana</i>	Australia and NZ	SE Pacific	GBIF/this study
<i>plumulosa</i>	BC to Ecuador	NW Pacific	GBIF/this study
<i>poipu</i>	Hawaii	C. Pacific	This study
<i>ramondi</i>	Atlantic, Pacific and Mediterranean	Cosmopolitan	GBIF/this study

Hypothesized North Pacific clade species: *kussakini*, *lindbergi*, *rubricatoides*, *sectimana*, *simulans*.

Hypothesized Basal clade species (Atlantic, Mediterranean, Indian or South Pacific distributions): *boiana*, *brevipes*, *cinera*, *cookana*, *ferox*, *gammaroides*, *guaspare*, *helleri*, *hiana*, *hinatore*, *hirsutimanus*, *hyalos*, *kaneohe*, *katae*, *kerqueleni*, *kuala*, *kulafi*, *marcuzzii*, *meganae*, *ningaloo*, *parakava*, *platycera*, *pollex*, *pseudongana*, *rachanoi*, *riedli*, *roly*, *rosema*, *rubricata*, *tea*, *ulladulla*, *virescens*, *Waiialua*.

Ambiguous distribution (Southern California; Baja): *humeralis*, *plumulosa*.

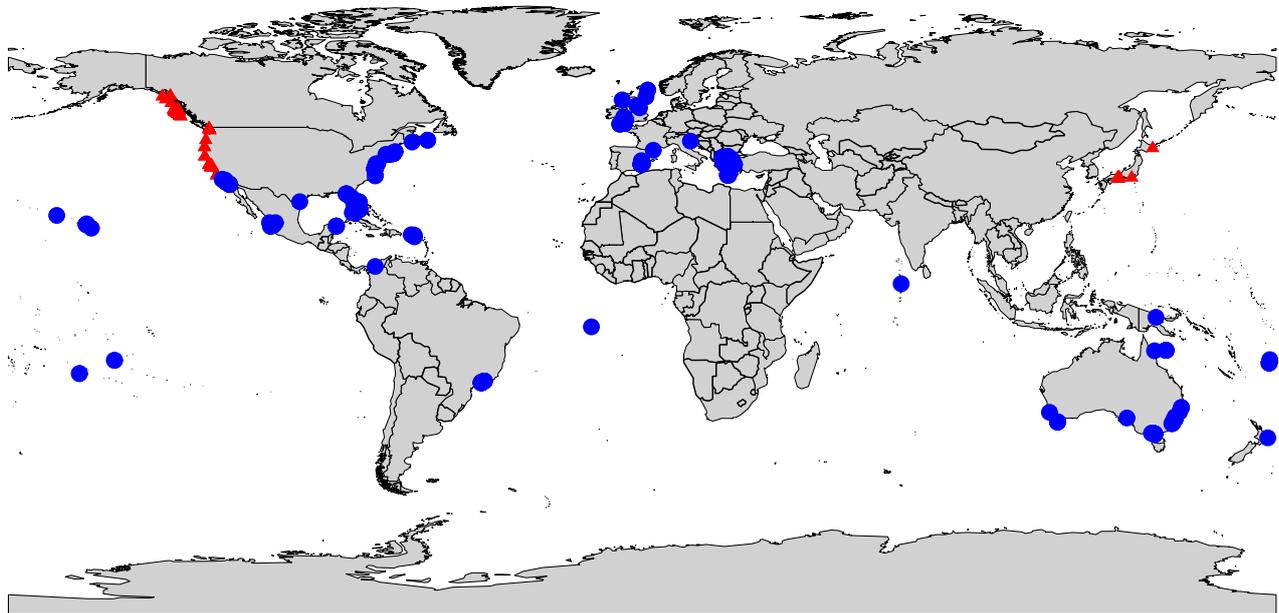


Fig. 3 The distribution of *Ampitboe*-‘north Pacific’ clade (red circles) and *Ampitboe*-‘south’ clade (blue dots). The cosmopolitan species *Ampitboe valida* is not included. See Table 1 for data sources.

phylogeny was rooted with outgroup species that are not Corophiida, the infraorder to which Ampithoidae belongs. We suggest that future work should attempt to confirm monophyly using outgroup species that are more closely related to Ampithoidae.

Our data also indicate that the genus *Biancolina* should be considered within the family Ampithoidae. Based on morphological characters only, *Biancolina* was positioned within the talitrids most recently by Lowry & Myers (2013). This taxonomic placement follows a highly mobile

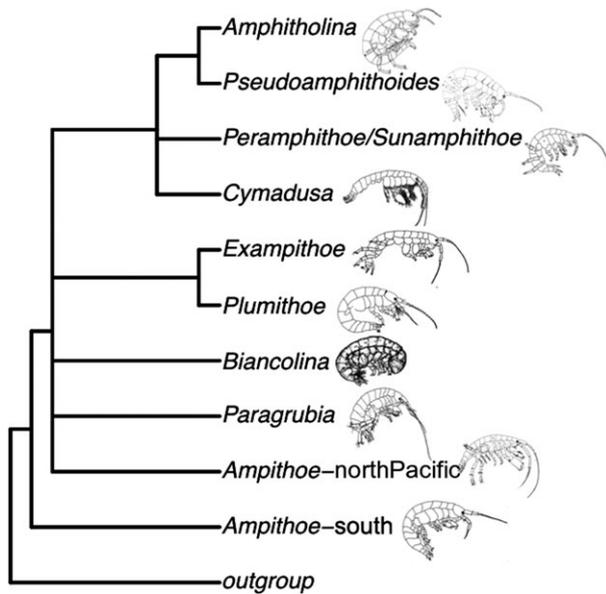


Fig. 4 Genus-level phylogeny of Ampithoidae. Nodes have posterior probability greater than 0.95, maximum likelihood bootstrap support greater than 0.70, or both. Image credits: Conlan and Bousfield 1982 (*Ampithoe lacertosa*); Hughes and Lowry 2009 (*Cymadusa tattersalli*, *Paragrubia edgari*); Just 1977 (*Pseudoampithoides incurvaria*); Lowry 1974 (*Biancolina brassicephala*); Myers 1974 (*Amphitholina cuculus*); Poore and Lowry 1997 (*Ampithoe caddi*, *Exampithoe kutti*, *Perampithoe parmerong*, *Plumithoe quadrimana*).

history of the higher classification of this genus, with previous workers each acknowledging the distinctness of the group. *Biancolina* was originally placed with the Dexaminidae (Della-Valle 1893). However, Stebbing (1906) synonymized the species with *Ampitholina cuculus*, which first placed *Biancolina* as a junior synonym within the family Ampithoidae. This placement of *Biancolina* within *Ampitholina* was subsequently rejected by all further workers.

With the description of a second species, *Biancolina* was included in the then-newly-established Prophiliantidae Nicholls, 1939. The genus was again placed in association with the Ampithoidae by Ruffo (1953) and further supported by Myers (1974), however, between this time, Barnard (1969) had proposed it as part of the Eophliantidae Sheard, 1936. *Biancolina* was then established within the monotypic family Biancolinidae by Barnard (1972), with remarks on its close relationships to *Ampitholina* (Ampithoidae) and *Wandelia* Chevreux 1906 (Eophliantidae), later expanded upon in Barnard & Karaman (1991). A phylogeny of the Talitroidea by Serejo (2004) included *Biancolina* based on its historic association with the Eophliantidae, a family more recognizably aligned with other talitrids. *Biancolina* was originally used as an outgroup by Serejo (2004), but eventuated as an ingroup within the Phliantidae

Stebbing 1899, at the family level. Most recently, Lowry & Myers (2013) further recognized the exceptional nature of *Biancolina* within the Talitrida and established Biancolinoidea as one of four distinct superfamilies within the Infraorder. In this article, we formally transfer the monotypic genus *Biancolina* back within the Ampithoidae based on our molecular findings (see Systematics section).

The name '*Pleonexes*' was proposed by Bate (1856), was considered a *nomen nudem* until 1857 when sufficient description of the group was provided. Conlan's (1982) cluster analysis (Fig. 2) recognized the group at the generic level. Barnard & Karaman (1991) considered *Pleonexes* as a subgenus within *Ampithoe* for species with prehensile pereopods and the presence of large recurved hooks on the telson. They included the species *A. poipu*, *A. aptos*, and *A. helleri* in this subgenus, as well as *A. auriculata*, where the telsonic hooks are considered intermediately developed. This study was able to include sequences from one of these taxa (*A. poipu*) and find it within the *Ampithoe*-'south' clade, which itself appears to be basal to Ampithoidae. This suggests that the *Ampithoe poipu* may represent a species from a group with telsonic hooks that is basal to the entire family. Testing this hypothesis will require more thorough sampling of the species in this group.

Relationships among genera

Our current understanding of generic relationships within the Ampithoidae is shown in Fig. 4 (nodes with >0.95 posterior probability). There is a large sister clade that includes *Cymadusa*, the sister taxa *Pseudoampithoides*-*Amphitholina* and *Sunampithoe/Perampithoe*. There is strong support for a *Plumithoe*-*Exampithoe* sister clade. The phylogenetic relationships between these two clades, *Biancolina*, *Paragrubia*, and *Ampithoe*-'north Pacific' remains uncertain. The basal status of *Ampithoe*-'south' has strong Bayesian support, and its separation from the remainder of the ampithoid taxa had 78% support from ML bootstrap (Fig. 2; Fig. S5).

Our molecular phylogeny contrasts in several ways with the morphological-based phylogeny of Conlan (1982) (Fig. 1). Conlan (1982) suggested *Cymadusa* and *Ampithoe* are sister taxa, but our molecular phylogeny clusters *Cymadusa* with *Amphitholina*, *Perampithoe*, *Sunampithoe* and *Pseudoampithoides*. *Exampithoe* clusters with these last three taxa by Conlan (1982), but our phylogeny suggests that *Exampithoe* and *Plumithoe* are sister taxa. Finally, Conlan (1982) and Myers & Lowry (2003) suggest that *Amphitholina* is a basal taxa, while our phylogeny suggests it is derived.

Among our most surprising findings, the cosmopolitan and most speciose genus, *Ampithoe*, is composed of two significantly supported clades that are split geographically between the north Pacific and other areas. We predict that

species we did not sample but with a distribution localized to the north Pacific will likely also fall into the *Ampithoe*-‘north Pacific’ clade (Table 1). This includes *A. kussakini*, *A. lindbergi*, *A. rubricatoides* and *A. simulans*. We also predict that any other species with Atlantic, Mediterranean, Indian or south Pacific distributions will be part of the *Ampithoe*-‘south’ clade (see Table 1 for species listing).

While it is possible that these clades reflect two different genera, this remains a preliminary hypothesis that will require greater resolution of the deeper nodes that delineate clades. Moreover, our preliminary investigation of existing morphological traits in the DELTA database (Hughes *et al.* 2008) to world amphithoid genera and species – including mouthpart and external morphological characters – identified only a single synapomorphic character that divides these two groups. The mandibular palp article 3 is clavate (medially or distally broad, proximally more narrow) in the *Ampithoe*-‘north Pacific’ while *Ampithoe*-‘south’ species have a palp that is slender (of uniform breadth). Identification of only a single morphological character is surprising given the significant genetic support for splitting these clades. We note that these two groups are not consistent with the split of *Ampithoe* species by Conlan & Bousfield (1982) into a cluster of three ‘subgroups’ based on their morphological survey of north-eastern Pacific species (1-*A. lacertosa*, *A. valida*, *A. plumulosa*; 2-*A. kussakini*, *A. volki*, *A. sectimana*; 3-*A. rubricatoides*, *A. dalli*, *A. simulans*). Instead, we find that *A. plumulosa* is embedded within the *Ampithoe*-‘south’ clade and *A. valida*, *A. sectimana* and *A. dalli* are *Ampithoe*-‘north Pacific’.

Molecular data also force a reevaluation of morphological traits that were used to define other genera. For example, *Peramphithoe* and *Sunamphitoe* were historically split by the presence and absence of a mandibular palp, respectively. Our molecular analyses indicate that this single trait is not a good generic character and that these genera should be synonymized. These genera remain united by the outer ramus of uropod one and the broad lower lip of inner lobes (as in Conlan 1982). The sister-taxa relationship of *Plumithoe* and *Examphithoe* is united by a feeble to poorly developed mandibular palp. These morphological traits are, however, tentative, and a more thorough sampling of species within and across these particular genera and examination of their morphological and molecular traits will be required to resolve these issues.

Cryptic species

Within species, there is clear evidence that cryptic, ancient lineages are present among some amphithoids. As an example, previous studies suggest that *Ampithoe valida* is composed of at least three deep genetic mitochondrial lineages (Pilgrim & Darling 2010). Consistent with their data, we find an Atlantic

specimen (*AmpVaN*) diverged from Pacific (*AmpVaG* and *AmpVaB*) using all molecular data (Fig. 2, Fig. S5) and with significant support at one of four single loci (COI; Fig. S2). Given that *Ampithoe valida* is embedded within the *Ampithoe*-‘north Pacific’ clade, it is likely that the *A. valida* complex originated with other *Ampithoe*-‘north Pacific’ species and then dispersed (via natural means) into the Atlantic where it then diverged at COI (Pilgrim & Darling 2010). There is also evidence that some Atlantic lineages have reinvaded the Pacific coastline (Pilgrim & Darling 2010), but this hypothesis will require future sampling. As with other amphipod taxa with cosmopolitan distributions, molecular data can be effective in identifying cryptic speciation (e.g. Pilar Cabezas *et al.* 2013).

Deep divergence among lineages at the population, subspecies or species level also occurs within *Ampithoe longimana* (McCarty & Sotka, 2013; Sotka *et al.* 2003), *Peramphithoe femorata* (E. Sotka and M. Thiel, unpublished), *Peramphithoe tea* (E. Sotka and J. Long, unpublished), *Ampithoe ramondi* and *Ampithoe tarasovi* (this study). Detailed taxonomic research on the morphology of species in this family has also demonstrated that species thought to be cosmopolitan are comprised of species complexes (e.g. *Cymadusa filosa*, Peart 2004). Their brooding lifestyle and close association with benthic macrophytes would suggest that localized distributions are likely for species in this family; however, several species are known to occur on drifting macroalgae (e.g. *Sunamphitoe pelagica*, Stoner & Greening 1984; *Peramphithoe femorata* Rothäusler *et al.* 2011) and further work is needed to understand likely patterns of dispersal in this group.

Conclusions

The molecular phylogeny of 10 genera of herbivorous amphipods in the family Ampithoidae revealed the inclusion of *Biancolina* as a monotypic genus, representing a significant transition from previous works which assess the taxa as a superfamily within a different suborder. Surprisingly, this study also revealed cryptic genus-level lineages in the currently accepted *Ampithoe* that will require more species sampling and morphological and molecular data.

The phylogeny represents a profoundly incongruent conclusion with previous morphological generic concepts within the Ampithoidae. Among the amphipods, this is not unique to Ampithoidae. Morphological and molecular traits are commonly incongruent, for example in the *Gammarus* species complex (Hou *et al.* 2007; Weiss *et al.* 2014), the Lake Baikal amphipod radiation (MacDonald *et al.* 2005), and among subterranean freshwater taxa (Finston *et al.* 2007). This is not surprising, as there are relatively few molecular studies of the higher levels of classification of the Amphipoda (Lowry & Myers 2013; Myers & Lowry 2003; Barnard & Karaman 1991) and amphipod

taxonomists have long bemoaned the macroevolutionary stasis within families of gross morphological traits (Myers & Lowry 2003).

For all groups of Crustaceans, regardless of size, the number of species described per year has not reached an asymptote (Martin & Davis, 2006), a trend repeated across most terrestrial and marine fauna (Costello 2015). For the Amphithoidae, the known fauna has recently and rapidly increased. For example, 40% of the known amphithoids occur in Australian waters, yet until as late as 1994 only six species were described from the region, which now has 54 recorded species and many more yet to be described (J. Lowry, R. Peart, L. Hughes, pers. comm.). This pool of new species offers further opportunities to test our understanding of relationships in the family.

Finally, the absence of a robust, family-level phylogeny of Amphithoidae has hindered our understanding of the evolution of morphology, and of feeding behaviour, in this abundant group of herbivorous amphipods. Amphithoid genera differ in their use of algal and seagrass species as hosts (Poore *et al.* 2008), but formal analyses of the influence of phylogeny on host use, as are commonplace among studies of the herbivorous insects (Futuyma & Agrawal 2009), require an understanding of the phylogenetic relationships among species in the family. As emerging evidence accrues on the strength of phylogenetic effects on feeding patterns of marine consumers (Craft *et al.* 2013; Best and Stachowicz 2013), amphithoid amphipods will be an ideal model system to understand these effects.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1–S5. Phylogenies using individual locus data sets (S1–S4) and a comparison of ML and Bayesian tree in Figure S5. Numbers indicate posterior support from MrBayes. Species abbreviations are noted in Table S1.

Table S1. List of species, collection locations, seaweed hosts, abbreviation and locus sequenced with GenBank Accession numbers.

Table S2. Primers for polymerase chain reactions. F1/R1 amplified NAK for all species except for *Peramphithoe* (F2/R2) and *Cymadusa* (F3/R3).

Table S3. Partitioning strategy during phylogenetic analyses.