

Evolutionary Modification of Pereopods in Phronimid Amphipods (Crustacea: Amphipoda: Hyperiidea: Phronimidae) Reflects Host Differences

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Abstract. Phronimid amphipods are oceanic crustaceans associated with gelatinous zooplankters. Their host organisms belong mainly to two taxonomic groups: tunicates (salps or pyrosomes; subphylum Tunicata) and siphonophores (Cnidaria). After these amphipods devour the inner tissues of their hosts, they display the unique behavior of modifying their hosts into hollow barrel-shaped shelters, which are then utilized as neonatal nurseries by the females. Although previous studies have revealed the host specificity of these amphipods, it has not been inferred which types of hosts ancestral phronimids could have originally used. Moreover, morphological changes associated with host switching have not yet been studied. To deduce the evolutionary patterns of host switching, we investigated the phylogenetic relationships of phronimid species by using two genes: (1) cytochrome *c* oxidase subunit I (*COI*) and (2) 18S ribosomal RNA (*18S*). In addition, a morphometric analysis was conducted in order to better understand the morphological relationships between phronimids and their host organisms. Our phylogenetic analysis suggests that the ancestral host ani-

mals of phronimids could have been tunicates and that the host organisms have independently switched from tunicates to siphonophores at least twice in the family Phronimidae. Our morphometric analysis revealed that phronimids using siphonophores as hosts have a relatively shorter pereopod 5 compared to those using tunicates. The shortening of pereopod 5 seems to be an adaptation to the narrower internal space of siphonophore barrels compared to those of tunicates.

Introduction

Host switching is suggested to play an important role in speciation and diversification of parasites and symbionts (Favret and Voegelin, 2004; Hayakawa *et al.*, 2008; Sato *et al.*, 2017). For example, palaemonid shrimps that are known to be symbiotic with a variety of invertebrates have shown inter-phylum host switching many times in different shrimp lineages. These features contribute to the extraordinary diversification in this group (Horká *et al.*, 2016). Speciation by host switching is also known to have occurred in monogeneans (Ziętara and Lumme, 2002), isopods (Boyko *et al.*, 2013), copepods (Huys *et al.*, 2007), and nudibranchs (Faucci *et al.*, 2007). In contrast to the symbionts on nektonic and benthic animals, host switching is rarely studied in the marine plankton community.

Hyperiid amphipods are associated with a wide variety of gelatinous zooplankters, including scyphozoans, siphonophores, ctenophores, salps, and even rhizarians (Harbison *et al.*, 1977; Laval, 1980; Gasca and Haddock, 2004; Nakamura *et al.*, 2019). Some species are considered parasitoids rather than parasites because they always kill their hosts (Laval, 1980). They utilize their host animals as food sources, nurseries for juveniles, and vehicles for transportation (Brusca *et al.*, 2016).

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Abbreviations: AK, Akuseki-jima Island; BC, Bungo Channel; BI, Bayesian inference; BS, bootstrap; CW/CL, width/length ratio of pereopod 5 carpus; HB, Hibiki-nada Sea; KC, Kuchinoerabu-jima Island; LH, length of head; ML, maximum likelihood; MW/ML, width/length ratio of pereopod 5 merus; OG, Ogasawara Islands; OM, Oumi-jima; OR/IR, length ratio of outer ramus to inner ramus in uropod 2; OS, Osezaki; P3, pereopod 3; P3/P5, length ratio of pereopod 3 to pereopod 5; P3/Body, length ratio of pereopod 3 to body; P5/Body, length ratio of pereopod 5 to body; PCR, polymerase chain reaction; Ple1/Per7, length ratio of pleonite 1 to pereonite 7; PP, posterior probability; PL/CW, ratio of propodus length to carpus width; TN, Tanegashima Island.

Online enhancements: supplements.

Hyperiids exhibit a range of body forms, swimming abilities, and reproductive strategies (Bowman and Gruner, 1973; Laval, 1980). There is no single morphological synapomorphy known in this group (Vinogradov *et al.*, 1982; Browne *et al.*, 2007). Recent molecular phylogenetic analyses have revealed that hyperiids may be polyphyletic (Browne *et al.*, 2007) and may consist of four separate lineages of amphipods (Hurt *et al.*, 2013). The presence of morphological homoplasy attributed to their association with gelatinous zooplankton hosts has been suggested by Bowman and Gruner (1973) and Vinogradov *et al.* (1982). Thus, a better knowledge of the parasite-host relationships between hyperiid amphipods and gelatinous zooplankton would assist in the understanding of evolution in this group.

The hyperiid family Phronimidae contains 11 known species—all of which are parasitoids (Bush *et al.*, 2001; Zeidler, 2004). Phronimid females consume the inner tissues of gelatinous zooplankters to modify the bodies into hollow barrels for brooding their offspring (Diebel, 1988). The barrels originate from two taxonomic groups: tunicates (salps, pyrosomes, doliolids) and siphonophores (Laval, 1978). Salps are most commonly used as barrels, whereas siphonophores are utilized by only three species of phronimids (Laval, 1968, 1978, 1980; Daniel, 1973; Hirose *et al.*, 2005; Table 1) and may be an example of host switching if tunicates are the ancestral hosts for phronimids. Both oozooid and blastozooid phases are used in salps (Laval, 1978). Because no studies of the phylogenetic relationships of the Phronimidae seem to be available, it is difficult to deduce how phronimids switched host organisms. Therefore, we aimed to explore the evolutionary relationships and host association of phronimids by using the molecular phylogenetic analyses in two genes—cytochrome *c* oxidase subunit I (*COI*) and 18S ribosomal RNA (*18S*). A morphometric analysis was also conducted to assess the evolutionary trends associated with host types.

Materials and Methods

Specimen collections

Phronimid specimens were collected from eight stations in Japan from 2017 to 2019: off Oumi-jima (OM), Hibiki-nada Sea (HB), off Osezaki (OS), Bungo Channel (BC), off Ogasawara Islands (OG), northeast off Tanega-shima Island (TN), south off Kuchinoerabu-jima Island (KC), and southeast off Akuseki-jima Island (AK) (Fig. 1). The specimens were sorted from plankton samples collected by a Motoda (MTD) horizontal net (Motoda, 1971) (diameter: 56 cm, mesh size: 0.35 mm) and an Ocean Research Institute (ORI) net (Omori *et al.*, 1965) (diameter: 160 cm, mesh size: 0.33 mm) operated by the crew of the TRV *Toyoshio Maru*, Hiroshima University. Phronimid amphipods collected by a towing net carried no barrels. In addition, specimens were collected by entrapping them in plastic bottles while scuba diving or in hand nets from an embankment. These phronimid specimens were gently collected together with their barrels and were used for the identification

Table 1

Records of host organisms of phronimid species

Species, host organisms	Reference(s)
<i>Phronima sedentaria</i>	
Tunicata, Thaliacea, Salpida <i>Salpa fusiformis</i> Cuvier, 1804	Laval, 1978
<i>Salpa maxima</i> (Forskal, 1775)	Diebel, 1988
<i>Ihlea punctata</i> (Forskal, 1775)	Laval, 1978
<i>Thalia democratica</i> (Forskal, 1775)	Laval, 1978
Unidentified species of Salpidae	This study
Tunicata, Thaliacea, Pyrosomatida <i>Pyrosoma atlanticum</i> Peron, 1804	Laval, 1978
<i>Pyrosoma</i> sp.	Aoki <i>et al.</i> , 2013; this study
Unidentified species of Pyrosomatida	Risso, 1816
Tunicata, Thaliacea, Doliolida <i>Doliolum</i> sp.	Daniel and Surya Rao, 1968
Cnidaria, Hydrozoa, Leptothecata <i>Aequorea</i> sp. ^a	Risso, 1816
Cnidaria, unidentifiable	
<i>Medusa</i> sp. ^{a,b}	Risso, 1816
Ctenophora <i>Beroe</i> sp.	Risso, 1816
<i>Phronima atlantica</i>	
Tunicata, Thaliacea, Salpida <i>Traustedtia multitentaculata</i> (Quoy & Gaimard, 1834)	Aoki <i>et al.</i> , 2013
Unidentified species of Salpidae	This study
<i>Phronima solitaria</i>	
Tunicata, Thaliacea, Salpida Unidentified species of Salpidae	This study
<i>Phronima stebbingii</i>	
Tunicata, Thaliacea, Salpida Unidentified species of Salpidae	This study
Unidentified barrel	Laval, 1968
<i>Phronima bucephala</i>	
Tunicata, Thaliacea, Salpida <i>Traustedtia multitentaculata</i> (Quoy & Gaimard, 1834)	Aoki <i>et al.</i> , 2013
<i>Phronima curvipes</i>	
Cnidaria, Hydrozoa, Siphonophorae <i>Abylopsis tetragona</i> (Otto, 1823)	Laval, 1968
<i>Phronima pacifica</i>	
Tunicata, Thaliacea, Salpida <i>Salpa aspera</i> Chamisso, 1819	Harbison <i>et al.</i> , 1977
Cnidaria, Hydrozoa, Siphonophorae <i>Abylopsis tetragona</i> (Otto, 1823) <i>Lensia fowleri</i> (Bigelow, 1911)	Harbison <i>et al.</i> , 1977
Unidentified species of Abylidiae	Daniel, 1973
<i>Phronima colletti</i>	
Tunicata, Thaliacea, Salpida <i>Salpa aspera</i> Chamisso, 1819	Harbison <i>et al.</i> , 1977
Cnidaria, Hydrozoa, Siphonophorae <i>Abyla trigona</i> (Quoy & Gaimard, 1827) <i>Agalma okenii</i> (Eschscholtz, 1825)	Chun, 1889, 1895
<i>Agalma elegans</i> (Sars, 1846)	Daniel, 1973
<i>Chelophyes appendiculata</i> (Eschscholtz, 1829)	Mańko <i>et al.</i> , 2017
<i>Diphyes</i> sp.	Laval, 1968
<i>Phronimella elongata</i>	
Unidentified barrel	Vosseler, 1901
	Mayer, 1879; this study

^a Reported as the host organisms of *Phronima custos*, which is recognized now as a junior synonym of *P. sedentaria*.

^b Formerly used genus name for some species of cnidarians.

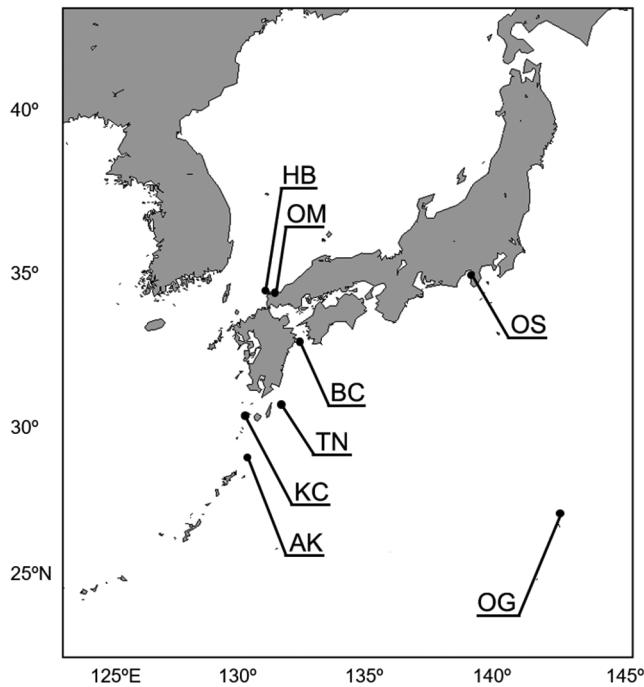


Figure 1. Sampling localities of phronimid species around Japan. AK, southeast off Akuseki-jima Island; BC, Bungo Channel; HB, Hibiki-nada Sea; KC, south off Kuchinoerabu-jima Island; OG, Ogasawara Islands; OM, off Oumi-jima; OS, Osezaki; TN, northeast off Tanega-shima Island.

of host types. Scuba diving and hand net sampling were mainly carried out at night, using a light trap.

All specimens were preserved in 70% or 99% ethanol for subsequent morphological and molecular analyses. Specific determinations were made following the key to phronimid species by Shih (1991). The presence of embryos within female marsupia and juveniles within barrels was recorded. Host types were identified on the basis of the morphological features of barrels: salp-origin barrels were identified by shape and ridges on the surface structure, pyrosome-origin barrels were judged from the presence of zooids, and siphonophore-origin barrels were identified if the posterior processes of the swimming bell remained. Species and reproductive phases of both salps and siphonophores are unknown because of the difficulties in identifying the diagnostic morphological characters of the highly modified barrels. Molecular work was not conducted for barrel species identification in this study.

Morphometrics of phronimids

Adult females were used for morphometric analysis because only a small number of male specimens were obtained. *Phronimella elongata* (Claus, 1862) and six species of *Phronima* (*Phronima sedentaria* (Forskål, 1775), *Phronima atlantica* Guérin-Méneville, 1836, *Phronima solitaria* Guérin-Méneville, 1844, *Phronima stebbingii* Vosseler, 1901, *Phronima curvipes* Vosseler, 1901, and *Phronima pacifica* Streets, 1877) were

newly collected in this study. Specimens loaned from the Department of Invertebrate Zoology at the Smithsonian Institution were also examined: *P. solitaria* (catalog nos. USNM137867, 137868, and 137871), *P. curvipes* (USNM137818 and 250099), *P. pacifica* (USNM137822), and *Phronima colletti* Bovallius, 1887 (USNM137809–137812 and 137814). The sexual maturity of the specimens was determined by the development of oostegites, following Shih (1969). The seven morphological parameters used by Shih (1969, 1991) for species identification were employed in the morphometric analysis (Fig. 2): (1) length of head (LH), expressed by a distance from the anterior end of pereonite 1 to the posterior-most pereonite part that is equivalent to the head length; (2) the length ratio of pereonite 1 to pereonite 7 (Per1/Per7); (3) the length ratio of pereopod 3 (P3) to pereopod 5 (P5) (P3/P5); (4) the width/length ratio of P5 merus (MW/ML); (5) the width/length ratio of the P5 carpus (CW/CL); (6) the ratio of propodus length to the carpus width (PL/CW); and (7) the length ratio of the outer ramus to the inner ramus in uropod 2 (OR/IR). The uropod 2 of *Phronimella elongata* is reduced, and, thus, OR/IR was not measured for this species. In addition to these seven parameters, the length ratios of P3 to the body (P3/Body) and of P5 to the body (P5/Body) were used. Body length was measured along the axis from the front of the head to the tip of the telson, according to Aoki *et al.* (2013). Pereopod 3 and pereopod 5 lengths were expressed as the sum of distances between proximal and distal ends of each segment. Tukey honest significant

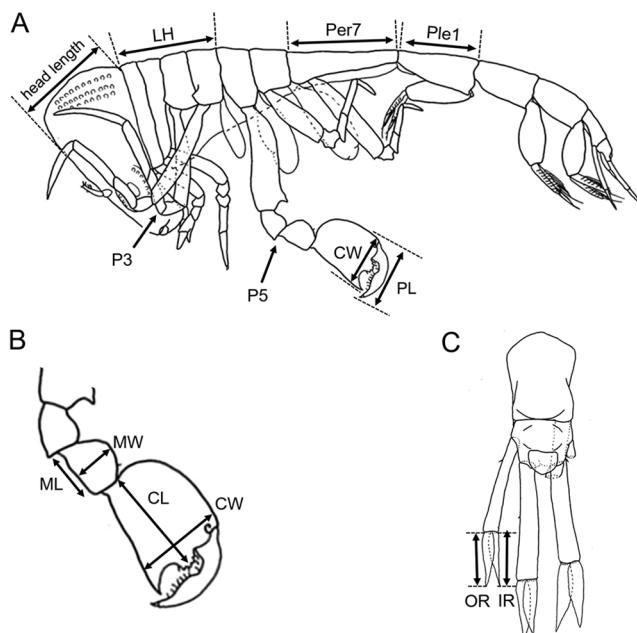


Figure 2. Diagrams of phronimid amphipod (based on *Phronima curvipes* NSMT-Cr 26924) showing body parts used for morphometric analysis. (A) Left side of body. (B) Distal part of left pereopod 5 (P5), lateral view. (C) Urosome, dorsal view. CL, carpus length; CW, carpus width; IR, inner ramus; LH, length of head; ML, merus length; MW, merus width; OR, outer ramus; P3, pereopod 3; P5, pereopod 5; Per7, pereonite 7; PL, propodus length; Ple1, pereonite 1.

difference (HSD) test was performed to find morphological parameters correlated with the host types. The width/length ratios of the internal spaces were compared between the tunicate and siphonophore barrels by Welch's *t* test. The correlation between body length of the phronimid amphipods and the length of the barrels was evaluated using Pearson's correlation coefficient. Statistical analyses in this observation used R version 3.5.2 (R Core Team, 2018).

DNA extraction, polymerase chain reaction, and cycle sequencing

Genomic DNA was extracted from the body tissue (pleopods, P5, or posterior half of body) of seven phronimid species as well as *Phrosina semilunata* Risso, 1822 as an outgroup (Table 2), using NucleoSpin Tissue XS (Macherey-Nagel, Duren, Germany) under the manufacturer's protocol. The concentration and purity of genomic DNA were measured by NanoDrop 2000 UV visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Polymerase chain reaction (PCR) was performed using TaKaRa Ex Taq (Mg^{2+} free buffer) (Takara Bio, Shiga, Japan). The primer pair of LCO1490/HCO2198 (Folmer *et al.*, 1994) was used to amplify the partial *COI*, and the primers E4/E1628 (van Hannen *et al.*, 1999) with a modification (18SPhF 5'-TGG TTG ATC CTG CCA GTG TC-3'/18SPhR 5'-CCC GTG TTG AGT CAA ATT G-3') were used to amplify the partial *18S*.

The PCR solution contained 10× Ex Taq buffer, 2.5 mmol L⁻¹ dNTP (deoxynucleoside triphosphate) mix, 25 mmol L⁻¹ MgCl₂, 10 pmol of each forward and reverse primer, 5 U μ L⁻¹ of Ex Taq polymerase, 5–20 ng μ L⁻¹ of template DNA, and sterilized water. The final volume of PCR mix was 20–50 μ L. PCR was conducted by Thermal Cycler Dice mini (Takara Bio) under the following temperature regimes. For *COI*, initial denaturation was carried out at 94 °C for 1 min, followed by 35 cycles of denaturation for 40 s at 94 °C, annealing for 40 s at 50 °C, extension for 60 s at 72 °C, and a 10-min final extension at 72 °C. For *18S*, initial denaturation was at 94 °C for 3 min, followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 54 °C, extension for 90 s at 72 °C, and a final extension at 72 °C for 10 min. The PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Cycle sequencing used BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) under the following temperature regimes: initial denaturation at 95 °C for 1 min followed by 25 cycles of denaturation for 10 s at 95 °C, annealing for 5 s at 50 °C, and extension for 4 min at 60 °C. Nucleotide sequences were determined with an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) at the Natural Science Center for Basic Research and Development, Hiroshima University. All sequences were checked on BioEdit version 5.0.9 (Hall, 1999). Bases expressed with "N" were visually checked with the original waveforms.

Phylogenetic analysis

Multiple sequence alignment was performed using MUSCLE (Edger, 2004) with default parameters (penalty for gap open: -400; maximum number of iterations: 8; clustering method: UPGMB) on MEGA7 (Kumar *et al.*, 2016). In addition to the seven phronimid amphipods, three more hyperiid species were chosen as an outgroup: *Phrosina semilunata* is a member of the family Phrosinidae, which is closely related to the Phronimidae (Hurt *et al.*, 2013); *Hyperia macrocephala* (Dana, 1853) and *Themisto abyssorum* (Boeck, 1871) were chosen as representative members of the family Hyperiidae and are classified in the same superfamily as Phronimidae and Phrosinidae (Hurt *et al.*, 2013; Table 2).

Poorly aligned and non-informative sites on *18S* were removed using Gblocks server version 0.91b (Castresana, 2002) with no options for less stringent selection. The final lengths of aligned genes of *COI* and *18S* were 608 bp (Supplement S1, available online) and 1180 bp (Supplement S2, available online), respectively. Models of nucleotide evolution were determined for each sequence; and for *COI*, each codon position was determined by the Akaike Information Criterion (AIC) score in MEGA7 under default parameters (gaps/missing data treatment: complete deletion, branch swap filter: none), using a neighbor-joining tree. For *COI*, a general time-reversible model with invariant sites (GTR+I), Hasegawa-Kishino-Yano model with gamma distribution (HKY+G), and Hasegawa-Kishino-Yano model with gamma distribution having invariant sites (HKY+G+I) were determined to be the best-fit models for first, second, and third codon positions, respectively.

For *18S*, Kimura two-parameter model with gamma distribution (K2P+G) was the best-fit model. Phylogenetic trees were reconstructed using two methods after concatenating the two gene fragments into a single sequence (1788 bp): maximum likelihood (ML) estimation and Bayesian inference (BI). The ML analysis was performed using IQ-TREE version 1.6.7 (Nguyen *et al.*, 2015). Branch support was evaluated by bootstrap (BS) resampling 1000 times. Other parameters were used in default conditions: tree search: nearest-neighbor interchange [NNI]; initial tree: 100 parsimony trees + BioNJ tree. The BI analysis was conducted using MrBayes 3.2.6 (Ronquist *et al.*, 2012). The tree search was run for 1,000,000 generations with trees every 1000 generations, and the first 250,000 trees were discarded as "burn-in." Support was given as posterior probabilities (PP) calculated under default parameters. Both ML and BI reconstructed trees were visualized in Seaview version 4.7 (Gouy *et al.*, 2010).

Ancestral state reconstruction was performed in Mesquite 3.6 (Maddison and Maddison, 2018), using a parsimony model, and the tree from the ML analysis inferred the ancestral host type. One of the three types of hosts (salp, salp and pyrosome, and siphonophore) was applied to each terminal species according to the host records (Table 1). *Phronimella*

Table 2

List of specimens used for phylogenetic analysis in this study

Species, locality	Sex	Museum voucher ID	GenBank accession nos.		Reference			
			COI	18S				
Phronimidae								
<i>Phronima sedentaria</i>								
OM	F	NSMT Cr-26895	MT062491	MT062587	This study			
OG	F	NSMT Cr-26897	MT062493	MT062589	This study			
TN	F	NSMT Cr-26898	MT062492	MT062588	This study			
KC	M	NSMT Cr-26899	MT062494	MT062590	This study			
<i>Phronima atlantica</i>								
OM	F	NSMT Cr-26905	MT062495	MT062591	This study			
HB	F	NSMT Cr-26906	MT062497	MT062593	This study			
HB	M	NSMT Cr-26907	MT062496	MT062592	This study			
<i>Phronima solitaria</i>								
OM	F	NSMT Cr-26914	MT062500	MT062596	This study			
OS	F	NSMT Cr-26911	MT062499	MT062595	This study			
OG	F	NSMT Cr-26915	MT062498	MT062594	This study			
<i>Phronima stebbingii</i>								
OG	F	NSMT Cr-26918	MT062502	MT062598	This study			
BC	M	NSMT Cr-26923	MT062501	MT062597	This study			
<i>Phronima curvipes</i>								
KC	F	NSMT Cr-26924	MT062503	MT062599	This study			
KC	F	NSMT Cr-26925	MT062504	MT062600	This study			
<i>Phronima pacifica</i>								
OS	M	NSMT Cr-26932	MT062507	MT062603	This study			
OS	M	NSMT Cr-26933	MT062508	MT062604	This study			
OS	F	NSMT Cr-26930	MT062506	MT062602	This study			
OG	F	NSMT Cr-26934	MT062505	MT062601	This study			
<i>Phronimella elongata</i>								
OS	M	NSMT Cr-26947	MT062489	MT062585	This study			
OG	F	NSMT Cr-26942	MT062487	MT062583	This study			
HB	M	NSMT Cr-26946	MT062488	MT062584	This study			
AK	F	NSMT Cr-26938	MT062490	MT062586	This study			
Phrosinidae								
<i>Phrosina semilunata</i>								
OS	F	NSMT Cr-26948	MT062509	MT062605	This study			
Hyperiidae								
<i>Hyperia macrocephala</i>								
WS	NA	—	EF989666	NA	Browne <i>et al.</i> , 2007			
NA	NA	—	NA	DQ378047	U. Englisch, Ruhr Universität Bochum, unpubl. data			
<i>Themisto abyssorum</i>								
LS	NA	—	MH330790	NA	Tempestini <i>et al.</i> , 2017			
AB	NA	—	NA	KF609371	Olsen <i>et al.</i> , 2014			

18S, 18S ribosomal RNA gene; AB, Arctic basin; AK, southeast off Akuseki-jima Island; BC, Bungo Channel; COI, cytochrome c oxidase subunit I gene; HB, Hibiki-nada Sea; KC, south off Kuchinoerabu-jima Island; LS, Lancaster Sound; NA, not available; NSMT, National Museum of Nature and Science, Tsukuba, Japan; OG, off Ogasawara Islands; OM, off Oumi-jima; OS, off Osezaki; TN, northeast off Tanega-shima Island. WS, Weddell Sea. Dash indicates not applicable.

elongata was removed from the ancestral state reconstruction because its specific host type is still unknown.

Results

Phronimid species and their hosts

Phronima sedentaria (58 individuals) and *Phronimella elongata* (34 individuals) were collected from almost all lo-

calities: *P. sedentaria* from OM, OG, OS, TN, AK, and KC; and *P. elongata* from OM, OG, OS, TN, AK, and HB. Females of five species (*Phronima atlantica*, *Phronima solitaria*, *Phronima stebbingii*, *Phronima pacifica*, and *P. elongata*) carried embryos within the marsupium or juveniles within the barrels. The species of *Phronima* with a greater body length had a greater number of juveniles in their barrels (Table 3). Six phronimid species collected by scuba diving and hand net were

Table 3*Numbers of offspring carried by a single female phronimid individual*

Species	Marsupiated embryos	Median (25%–75% IQR)		N
		N	Demarsupiated juveniles	
<i>Phronima atlantica</i>	—	0	104.5 (79.3–130.5)	6
<i>Phronima solitaria</i>	107, 114 ^a	2	40, 158 ^a	2
<i>Phronima stebbingii</i>	20, 28 ^a	2	19.0 (12.5–22.0)	3
<i>Phronima pacifica</i>	43.0 (36.0–54.5)	7	38.0 (24.0–40.5)	3
<i>Phronimella elongata</i>	—	0	4.0 (2.0–7.5)	4

N indicates the number of adult females examined. IQR, interquartile range. Dash indicates no data.^a Exact counts.

found with barrels (Table 4). Four species (*P. sedentaria*, *P. atlantica*, *P. solitaria*, and *P. stebbingii*) had salp-origin barrels (Fig. 3A–C), and one specimen of *P. sedentaria* from OS had a pyrosome-origin barrel (Fig. 3D). Four barrels used by *P. pacifica* were all identified to be of siphonophore origin (Fig. 3E), and the origin of the barrel of *Phronimella elongata* is unknown, based on morphology (Fig. 3F).

Morphometrics of phronimids and barrel shape

Three species (*Phronima curvipes*, *P. pacifica*, and *P. colletti*) using siphonophores as hosts had significantly lower values in the P5/Body ratio than the other species using tunicates ($P < 0.05$; Table 5; Fig. 4A). In contrast, the P3/Body ratio showed no remarkable correlation with the host types (Fig. 4B). There

Table 4*Numbers of phronimid specimens and their host types collected in this study*

Phronimid amphipods	Host type							
	Scuba/hand net				MTD/ORI			
	Sa	Py	Si	Un	No	No	Subtile	Total
<i>Phronima sedentaria</i>								
Immature	2	0	0	0	2	39	43	
Female	0	1	0	0	3	7	11	58
Male	0	0	0	0	0	4		
<i>Phronima atlantica</i>								
Immature	0	0	0	0	3	81	84	
Female	8	0	0	0	5	0	13	100
Male	0	0	0	0	1	2	3	
<i>Phronima solitaria</i>								
Immature	3	0	0	0	18	0	21	
Female	8	0	0	0	7	2	15	37
Male	0	0	0	0	1	0	1	
<i>Phronima stebbingii</i>								
Immature	1	0	0	0	0	0	1	
Female	5	0	0	0	1	1	7	11
Male	0	0	0	0	2	1	3	
<i>Phronima curvipes</i>								
Immature	0	0	0	0	0	0	0	
Female	0	0	0	0	0	3	3	3
Male	0	0	0	0	0	0	0	
<i>Phronima pacifica</i>								
Immature	0	0	1	0	3	0	4	
Female	0	0	6	0	8	0	14	21
Male	0	0	0	0	3	0	3	
<i>Phronimella elongata</i>								
Immature	0	0	0	0	1	11	12	
Female	0	0	0	5	6	0	11	34
Male	0	0	0	1	8	2	11	

MTD, Motoda horizontal net; No, no host; ORI, Ocean Research Institute net; Py, pyrosome; Sa, salp; Si, siphonophore; Un, unidentified.

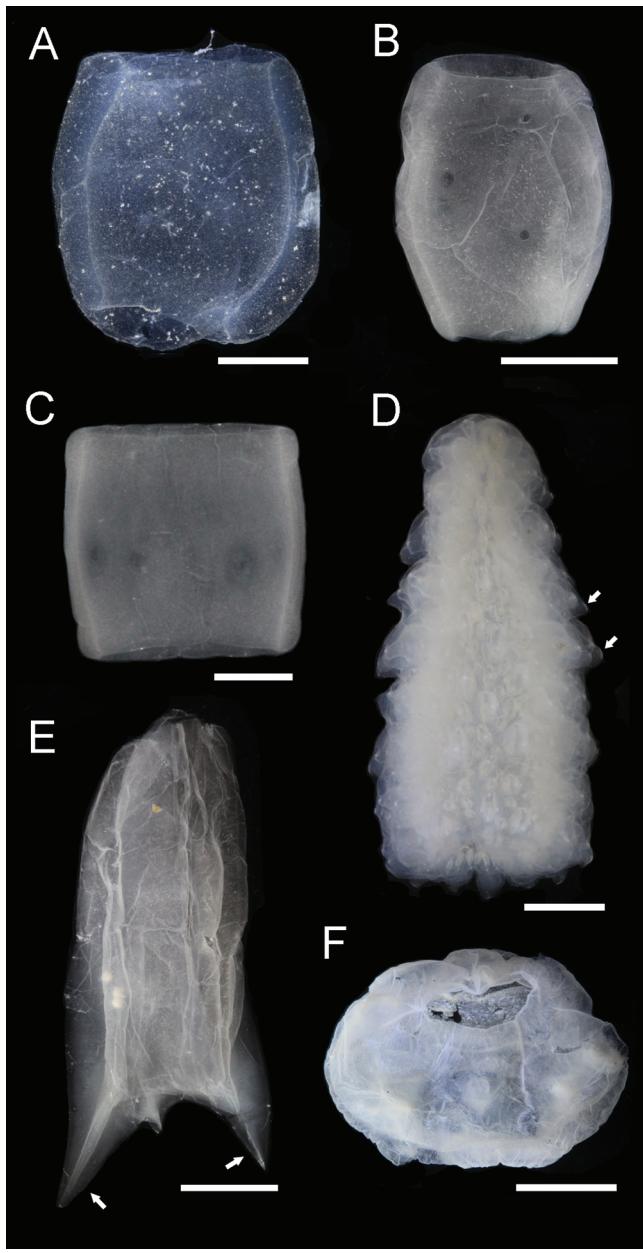


Figure 3. Barrels of phronimid amphipods. (A–C) Salp-origin barrels carried by *Phronima atlantica* (A), *Phronima solitaria* (B), and *Phronima stebbingii* (C). (D) Pyrosome-origin barrel carried by *Phronima sedentaria*; arrows indicate zooids. (E) Siphonophore-origin barrel carried by *Phronima pacifica*; arrows show the posterior processes of swimming bell. (F) Unidentified barrel carried by *Phronimella elongata*. All specimens were preserved in 99% ethanol. Scale bars = 5 mm (A, B, D, F); 1.5 mm (C); 3 mm (E).

were three clear groups of phronimid species when analyzed by the P3/P5 ratio (Table 5; Fig. 4C); however, they were not correlated with the host types.

Phronima stebbingii showed significantly higher ratios in Ple1/Per7 and uropod 2 OR/IR than the other six species of *Phronima* ($P < 0.01$; Table 5). Uropod 2 OR/IR of *P. curvipes* was significantly lower than *P. stebbingii* and signifi-

cantly higher than *P. solitaria*, *P. pacifica*, and *P. colletti* ($P < 0.05$; Table 5). Except for these parameters, no significant differences were detected in any one species compared to other species of *Phronima* (Table 5).

The larger phronimid amphipods such as *P. sedentaria* and *P. atlantica* carried the longer barrels; the smaller phronimids such as *P. stebbingii* formed the shorter barrels ($r = 0.778$, $P < 0.01$; Fig. 5). Two size groups of *P. solitaria* were found, and both consisted of individuals collected from OS and OG (Fig. 5). Barrels made of siphonophores had a width/length ratio of 0.37, which is significantly lower than the ratio of 0.82 found in barrels made of salps ($P < 0.01$; Fig. 6).

Phylogeny of phronimid species

Trees from the ML and BI analyses had an identical topology (Fig. 7A, B). Phronimid species formed a monophyletic group with *Phronimella elongata* placed within the genus *Phronima*, suggesting that Phronimidae is a polyphyletic group. Each of the conspecific specimens formed a monophyletic group. Two well-resolved subclades were present for *P. sedentaria*, *P. solitaria*, and *Phronimella elongata*. The *Phronima sedentaria* from TN was genetically distant from the subclade formed by the other specimens collected from three different localities. *Phronimella elongata* specimens from OS and AK formed a subclade, and specimens from OG and HB formed another subclade. *Phronima solitaria* collected from OG was genetically distant from the OM and OS specimens.

Interspecific relationships were resolved with a moderate to high reliability on each node ($BS \geq 66$, $PP \geq 0.68$; Fig. 7). A sister relationship was recovered between *P. pacifica* and *P. stebbingii* ($BS/PP = 66/0.68$), which collectively formed the sister group to *P. solitaria* ($BS/PP = 70/1.00$). *Phronima curvipes* was found to be sister to *P. atlantica* ($BS/PP = 99/1.00$), and these collectively formed the sister group to the clade containing *P. pacifica*, *P. stebbingii*, and *P. solitaria* ($BS/PP = 94/1.00$). *Phronimella elongata* was found to be sister to the clade comprising the five *Phronima* species ($BS/PP = 89/1.00$), and *Phronima sedentaria* was placed in the most outside position, being sister to the clade including all other phronimid species analyzed in this study ($BS/PP = 100/1.00$).

Discussion

Phylogenetic relationships among phronimid species

The morphology of all eight species agreed well with those described by Shih (1969, 1991), except for the P5 merus width/length ratio of *Phronima colletti* (longer than wide in Shih 1991 vs. wider than long in this study; Table 5). Each species clearly formed a single clade in our molecular phylogenetic analyses, demonstrating that the morphologically based classification proposed by Shih (1991) is genetically supported. However, we also found that three species, *Phronima sedentaria*, *Phronima solitaria*, and *Phronimella elongata*,

Table 5

Morphometrics of female phronimid species

Morphological parameters	Species							
	<i>Phronima sedentaria</i> (n = 5)	<i>Phronima atlantica</i> (n = 5)	<i>Phronima solitaria</i> (n = 6)	<i>Phronima stebbingii</i> (n = 6)	<i>Phronima curvipes</i> (n = 5)	<i>Phronima pacifica</i> (n = 7)	<i>Phronima colletti</i> (n = 6)	<i>Phronimella elongata</i> (n = 9)
Body length (mm)	24.7 ± 5.0	18.6 ± 1.9	17.6 ± 3.4	5.9 ± 0.6	11.0 ± 3.0	9.5 ± 0.9	7.9 ± 1.5	13.8 ± 2.1
P3 length (mm)	11.3 ± 3.0	8.4 ± 1.1	9.2 ± 1.7	3.5 ± 0.4	5.6 ± 2.2	5.3 ± 0.4	4.9 ± 1.2	11.1 ± 1.7
P5 length (mm)	14.5 ± 3.2	11.2 ± 1.4	10.7 ± 2.4	3.3 ± 0.3	5.4 ± 2.0	4.7 ± 0.3	3.8 ± 0.8	8.9 ± 1.4
LH	3.6 ± 0.3	2.4 ± 0.2	2.8 ± 0.2	3.5 ± 0.3	4.0 ± 0.2	2.8 ± 0.2	4.2 ± 0.4	2.7 ± 0.2
Ple1/Per7	0.8 ± 0.1 ^a	0.7 ± 0.1 ^{bc}	0.7 ± 0.1 ^c	1.3 ± 0.2^d	0.7 ± 0.1 ^{abc}	0.7 ± 0.1 ^{bc}	0.8 ± 0.1 ^{ab}	1.0 ± 0.1^e
P3/P5	0.8 ± 0.1 ^a	0.8 ± 0.1 ^a	0.9 ± 0.2 ^a	1.1 ± 0.1 ^b	1.0 ± 0.1 ^b	1.1 ± 0.1 ^b	1.3 ± 0.2 ^c	1.2 ± 0.1 ^c
P5 MW/ML	0.6 ± 0.2 ^a	0.5 ± 0.1 ^a	0.7 ± 0.1 ^b	0.7 ± 0.1 ^b	0.8 ± 0.1 ^b	1.1 ± 0.1 ^c	1.1 ± 0.2 ^c	0.2 ± 0.1^d
P5 CW/CL	0.5 ± 0.1 ^a	0.5 ± 0.1 ^a	0.5 ± 0.0 ^a	0.8 ± 0.1 ^b	0.8 ± 0.1 ^{bc}	0.9 ± 0.1 ^d	0.9 ± 0.1 ^{cd}	0.2 ± 0.0^e
P5 PL/CW	1.5 ± 0.3 ^{ab}	1.6 ± 0.7 ^a	1.3 ± 0.2 ^{bc}	1.3 ± 0.1 ^{bc}	1.0 ± 0.1 ^{cd}	1.0 ± 0.1 ^{cd}	0.9 ± 0.1 ^d	2.1 ± 0.6^e
OR/IR	1.3 ± 0.1 ^{ac}	1.2 ± 0.1 ^{ac}	1.0 ± 0.1 ^a	2.3 ± 0.4^b	1.5 ± 0.2 ^c	1.0 ± 0.1 ^a	1.0 ± 0.1 ^a	NA
P3/Body	0.5 ± 0.1 ^a	0.5 ± 0.0 ^a	0.5 ± 0.1 ^{ab}	0.6 ± 0.0 ^b	0.5 ± 0.1 ^{ac}	0.6 ± 0.1 ^{bc}	0.6 ± 0.0 ^b	0.8 ± 0.1^d
P5/Body	0.6 ± 0.0 ^{ac}	0.6 ± 0.0 ^{ac}	0.6 ± 0.1 ^{ac}	0.6 ± 0.0 ^a	0.5 ± 0.1 ^b	0.5 ± 0.0 ^b	0.5 ± 0.1 ^b	0.6 ± 0.1 ^c

Ratios and lengths are shown as median ± IQR (interquartile range) and average ± SD (standard deviation), respectively. Numbers in bold indicate that significant difference was shown in one species against all other species. The same lowercase letters in each ratio indicate no significant differences under Tukey honest significant difference test ($P < 0.05$). OR/IR is not applicable (NA) for *Phronimella elongata* because of its reduced condition of uropod 2. P3, pereopod 3; P5, pereopod 5; LH, length of head; Ple1/Per7, length ratio of pereonite 1 to pereonite 7; P3/P5, length ratio of pereopod 3 to pereopod 5; MW/ML, width/length ratio of P5 merus; CW/CL, width/length ratio of P5 carpus; PL/CW, ratio of propodus length to carpus width; OR/IR, length ratio of outer ramus to inner ramus in uropod 2; P3/Body, length ratio of P3 to body; P5/Body, length ratio of P5 to body.

showed subclades within each clade. This may suggest the existence of cryptic species or several separate populations within each species. Moreover, our phylogenetic trees showed strong support for polyphyly of the genus *Phronima* with *P. elongata* placed within *Phronima*. Hence, a taxonomic review of the family Phronimidae may be required. The remaining four species of *Phronima* that are missing in this study should

also be added to obtain a more reliable tree. Further sampling of specimens from different populations as well as additional genetic information for all species would be helpful. Recent molecular phylogenetic analyses also showed polyphyletic relationships in another hyperiid genus, *Hyperoche* (Hurt *et al.*, 2013), and even among the families in the suborder Hyperiidea (Browne *et al.*, 2007).

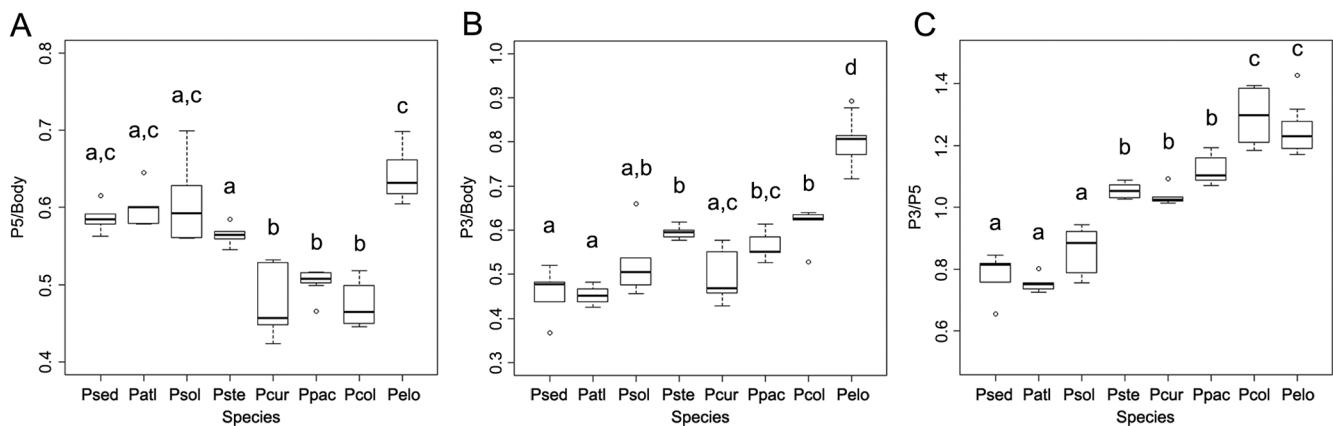


Figure 4. Median ratios of morphological parameters of eight female phronimid species. (A) Length ratio of pereopod 5 to body (P5/Body). (B) Length ratio of pereopod 3 to body (P3/Body). (C) Length ratio of pereopod 3 to pereopod 5 (P3/P5). Psed, *Phronima sedentaria*; Patl, *Phronima atlantica*; Psol, *Phronima solitaria*; Pste, *Phronima stebbingii*; Pcur, *Phronima curvipes*; Ppac, *Phronima pacifica*; Pcol, *Phronima colletti*; Pelo, *Phronimella elongata*. Whiskers show third (upper) and first (lower) quartiles. The same lowercase letters in each species indicate no significant differences under Tukey honest significant difference test ($P < 0.05$).

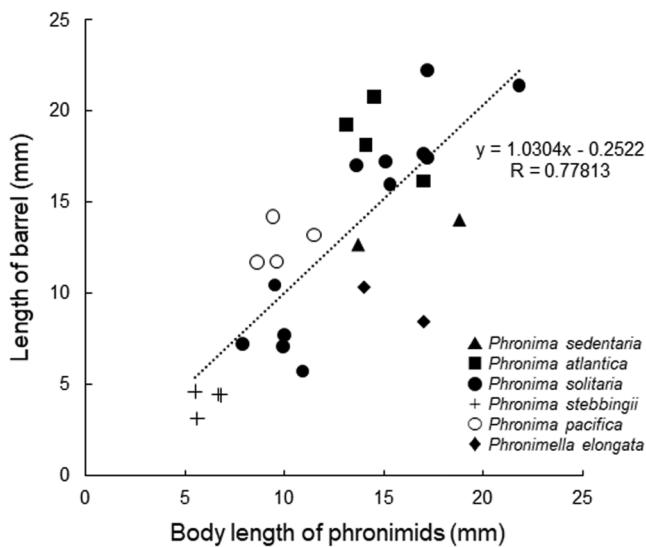


Figure 5. Positive linear correlation between body length of phronimid amphipod and barrel length. Pearson's correlation coefficient $r = 0.77813$ ($P < 0.01$).

Host selection and switching patterns in phronimid amphipods

Our ancestral state reconstruction suggests that tunicates are the ancestral host group and that some species of phronimids switched their host organisms from salps to siphonophores. The host-switching events seem to have occurred independently at least twice during the evolution of phronimid amphipods (Fig. 8).

Morphological observations (Laval, 1978; Diebel, 1988; Aoki *et al.*, 2013) and immunochemical analysis (Nishikawa *et al.*, 2005) of barrels showed that the main host organisms of *P. sedentaria* are tunicates (salps or pyrosomes). To date, this is the only species known to utilize a pyrosome as a host, which is also confirmed in our study (Table 4). However, there are reports of *P. sedentaria* having barrels made of *Aequorea* (Cnidaria) (Risso, 1816), *Beroe* (Ctenophora) (Risso, 1816), and *Doliolum* (Chordata) (Daniel and Surya Rao, 1968). Although tunicates are mainly used, *P. sedentaria* may utilize diverse gelatinous zooplankters as hosts. Among the other species of the genus *Phronima*, four species (*P. atlantica*, *P. solitaria*, *P. stebbingii*, and *P. bucephala*) are known to exclusively use salps, while three species (*P. pacifica*, *P. colletti*, and *P. curvipes*) mainly use siphonophores; two of them (*P. pacifica* and *P. colletti*) were once observed to use the salp *Salpa aspera* Chamisso, 1819 (Tables 1, 4). Thus, on rare occasions, the siphonophore parasitoids exhibit a plasticity and use salps rather than siphonophores. The ability to use different host types might be advantageous when there is a low abundance of the main host organisms—studies have shown that the abundance of gelatinous zooplankton varies among

oceans and across seasons (Lavanegos and Ohman, 2003; Primo *et al.*, 2009).

The host of *Phronimella elongata* is still unknown. However, it seems unlikely that tunicate-origin barrels are used by this species because tunicates are well known as the only animals producing cellulose (Kimura and Itoh, 2007), and Mayer (1879) used chemical testing to show that cellulose was not detected from the barrels of this species. Clarification of the barrel origin for *P. elongata* may also assist in understanding the host-switching pattern in phronimid amphipods. We tried to obtain a molecular identification of the barrel of *P. elongata* by using a direct sequencing method, but this approach failed as a result of DNA contamination from the amphipod and other foreign matter. The method of Nishikawa *et al.* (2005), using immunochemical analysis, may be useful to identify barrel organisms of *P. elongata*.

Doliolids are also an abundant gelatinous zooplankton but are rarely used as barrels by phronimid amphipods. This may be because they are too small (generally less than 10 mm in tunic length of a gonozooid) for most adult species.

Modification of pereopod length with respect to host switching

Barrels made of salps have a greater interior width than those made of siphonophores, which are more elongated (Figs. 3, 5). Phronimids utilizing a siphonophore-origin barrel may need to adapt to the narrow interior space in the barrel. On the basis of the morphometric analysis, the only significant difference between tunicate and siphonophore parasitoids was the P5/Body ratio (Table 5; Supplement S3, available online). Three siphonophore parasitoids, *Phronima curvipes*,

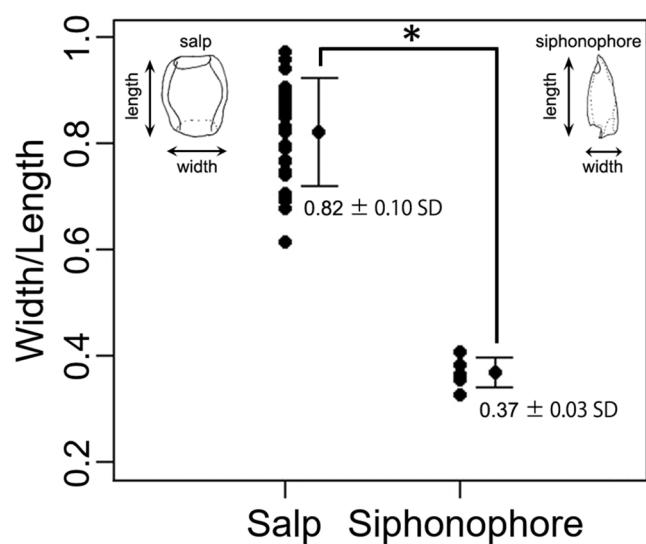


Figure 6. Width/length ratio of barrels from salps ($n = 29$) and siphonophores ($n = 6$). Error bars indicate standard deviation. Asterisk indicates significant difference under Welch's t test ($P < 0.01$).

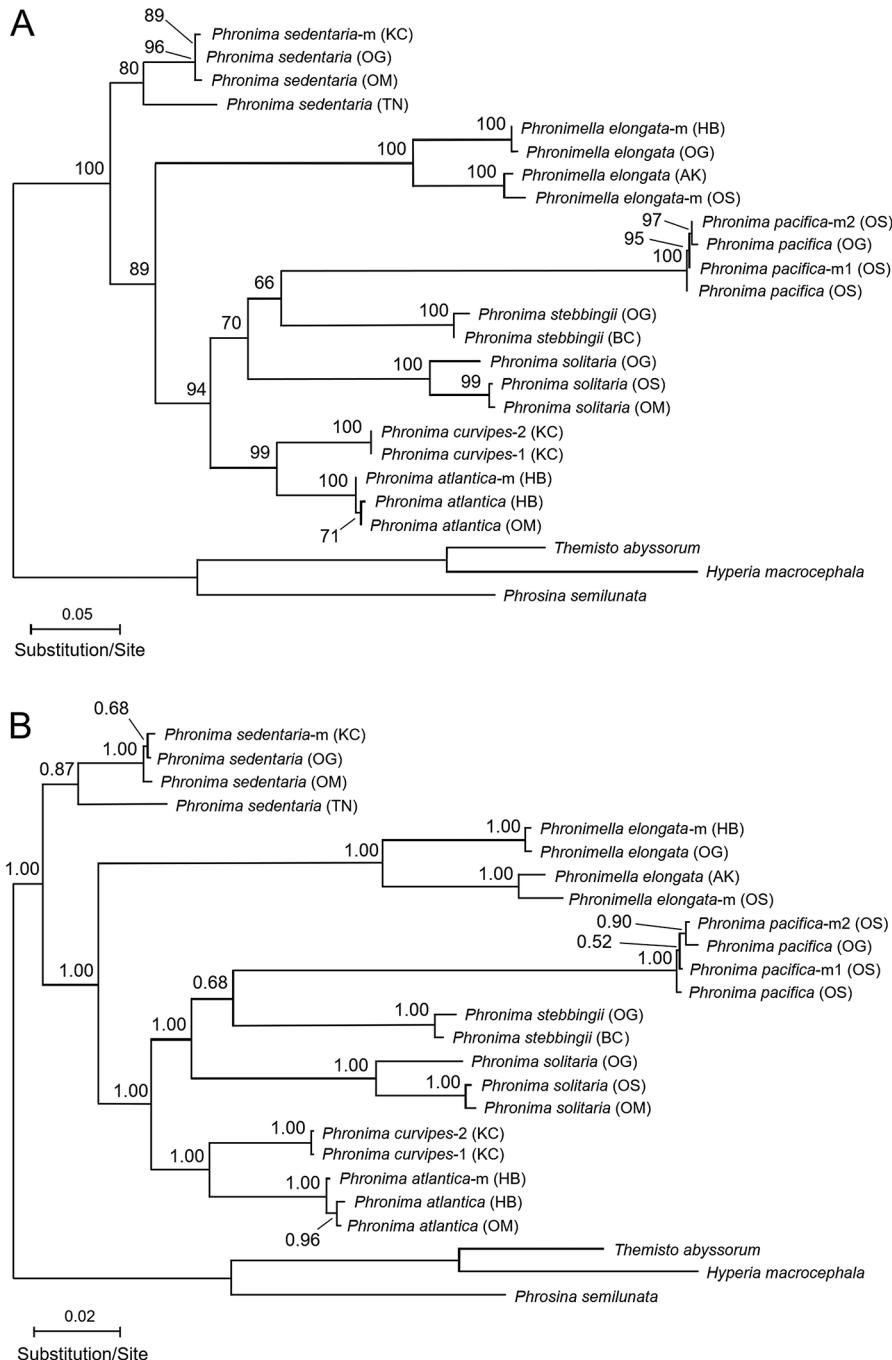


Figure 7. Phylogenetic trees using cytochrome *c* oxidase subunit I (*COI*) and 18S ribosomal RNA (*18S*) genes. (A) Maximum likelihood phylogenetic tree. (B) Bayesian inference phylogenetic tree. Numbers on each branch show bootstrap values resampling 1000 times and posterior probabilities, respectively. “-m” indicates male specimens. OM, off Oumi-jima; OG, off Ogasawara Islands; OS, off Osezaki; HB, Hibiki-nada Sea; TN, northeast off Tanega-shima Island; KC, south off Kuchinoerabu-jima Island; AK, southeast off Akuseki-jima Island; BC, Bungo Channel.

P. pacifica, and *P. colletti*, have lower ratios of P5/Body than other species, indicating that these phronimids have a relatively shorter P5 than the other four species. Our molecular phylogenetic trees demonstrate that *P. curvipes* and *P. pacifica* are not sister species (Fig. 7), suggesting that the length of P5 may

have been reduced in each lineage independently during the process of host switching from tunicates to siphonophores. While the function of P5 is poorly understood, pereopods 3, 4, 6, and 7 are used to attach to the barrel, and the gnathopods are used for feeding (Minkiewicz, 1909; Diebel, 1988).

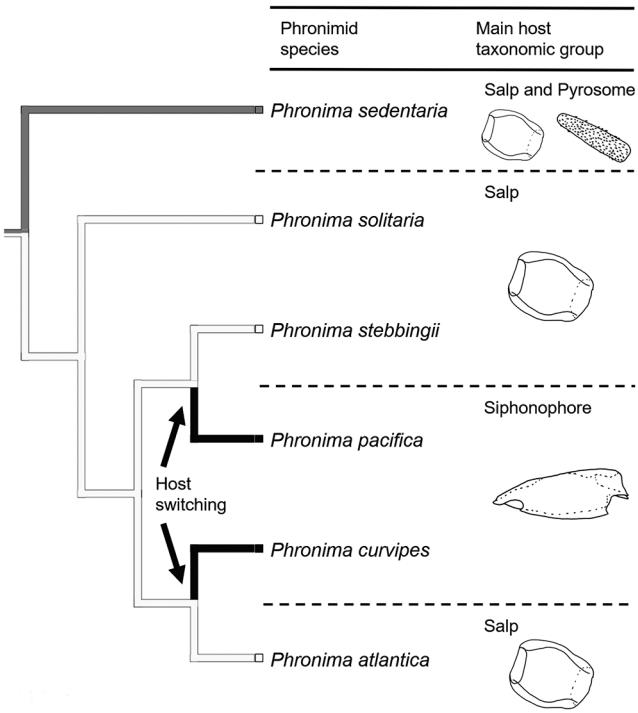


Figure 8. Ancestral state reconstruction based on best maximum likelihood (ML) tree. Host types of terminal taxa mapped onto ML tree using Mesquite parsimony model (Maddison and Maddison, 2018). Branch colors represent different host types (black, siphonophores; white, salps; gray, salps and pyrosomes).

Minkiewicz (1909) also showed a schematic drawing of *P. sedentaria* extending its P5 outside the barrel, but no detailed information of its purpose was given. The distal end of P5 is subchelate and is formed by the carpus and propodus; it is probably used as a pincer not only for modifying the host but also for processing prey in the barrel. If this is the case, then the P5 of siphonophore parasitoids may have been shortened to adapt to the interior width of the barrel for their movement within the barrel. *In situ* observations from scuba and/or remotely operated vehicles are needed to understand the exact functions of P5.

The barrel is used by phronimids for various purposes (Hirose *et al.*, 2005; Bishop and Geiger, 2006), but one of the most important functions of the barrel is as a nursery (Laval, 1980). Mother phronimids demarsupiate the offspring at an immature stage onto the interior wall of the barrel and then cohabit with them until the juveniles leave the barrel (Shih, 1969; Richter, 1978; Laval, 1980). They are capable of steering the barrels with juveniles inside by flapping their pleopods (Laval, 1980). Phronimid species with greater body lengths use a longer barrel regardless of the host type (Fig. 5). Hence, the smaller adult females might modify the host organisms into a shorter length, which is convenient for them to manage in the natural environments. The species of *Phronima* with a greater body length (*i.e.*, species inhabiting a larger bar-

rel) tend to produce a greater number of juveniles than do the smaller species (*i.e.*, species using a smaller barrel) (Table 3). The volume or surface area of the interior space, rather than the shape of the barrel, may limit the number of offspring that can be accommodated.

Presumption of environmental and physiological factors driving host switching

Salps are exposed to parasitism not only by phronimid amphipods but also by many other hyperiid amphipods, such as members of the families Vibiliidae, Hyperiidae, and Brachyscelidae (Harbison *et al.*, 1977; Laval, 1980; Gasca *et al.*, 2007) as well as parasitic copepods (*e.g.*, Heron, 1973). In subtropical waters the occurrence frequency and abundance of salps occasionally become lower than those of hyperiid amphipods, whereas the abundance of siphonophores is generally almost equal to or higher than that of hyperiids (Andersen *et al.*, 1997; Steinberg *et al.*, 2008). The low availability of host organisms and the presence of competitors can be a driver to switch host species (Cornell and Pimentel, 1978); thus, host switching might have occurred in phronimids in order to find suitable hosts. Previous studies have shown that both salp- and siphonophore-origin barrels have an antibacterial system (Hirose *et al.*, 2005). This benefit may be related to their choice of siphonophore as another option to make a barrel when phronimid amphipods seek a new host. Phronimids must overcome nematocyst stings when using siphonophores as hosts and may possess a resistance to nematocysts independent of host type, because they have been reported to prey on a variety of jellyfish (see Richter 1978; Laval 1980).

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Data Accessibility

Alignment files for molecular phylogenetic analysis used in this study can be found in Supplements S1 and S2 (available online) for *COI* and *18S* sequences, respectively. Morphometric data used in this study are available in Supplement S3 (available online).

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