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A New Copepod With Transformed Body Plan and Unique Phylogenetic Position Parasitic in the Acorn Worm *Ptychodera flava*

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Abstract. Symbiotic copepods compose one-third of the known copepod species and are associated with a wide range of animal groups. Two parasitic copepods endoparasitic in acorn worms (Hemichordata), *Ive balanoglossi* and *Ubius hilli*, collected in the Mediterranean Sea and Australian waters, respectively, were described a century ago. Here we report a new parasitic copepod species, *Ive ptychoderae* sp. nov., found in *Ptychodera flava*, a widespread acorn worm in the Indo-Pacific Ocean and an emerging organism for developmental and evolutionary studies. The female of *I. ptychoderae* is characterized by having a reduced maxilliped and five pairs of annular swellings along the body that are morphologically similar but distinguishable from those in the two previously described parasitic copepods in acorn worms. Phylogenetic analysis based on the 18S rDNA sequence shows that *I. ptychoderae* may belong to Poecilostomatoida but represent a new family, which we name Iveidae fam. nov. *Ive ptychoderae* is commonly found in the acorn worm population with an average prevalence of 42% during the collecting period. The infection of the parasite induces the formation of cysts and causes localized lesions of the host tissues, suggesting that it may have negative effects on its host. Interestingly, most cysts contain a single female with one or multiple male copepods, suggesting that their sex determination may be controlled by environmental conditions. The relationships between the parasitic copepods and acorn worms thus provide a platform for understanding physiological and ecological influences and coevolution between parasites and hosts.

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Introduction

Copepods are one of the most diverse and abundant animal groups in marine ecosystems and also the most common and widespread crustaceans living in symbiotic relationship with other organisms (Humes, 1985a, 1994; Ho, 2001). They comprise 10 orders (Calanoida, Cyclopoida, Gelyelloida, Harpacticoida, Thaumatopsylloida, Mormonilloida, Platycopioida, Poecilostomatoida, and Siphonostomatoida) based on the morphological characters (Huys and Boxshall, 1991; Ho *et al.,* 2003; Huys *et al.*, 2007), with a total of approximately 14,617 valid species (Walter and Boxshall, 2013). Among them, symbiotic or parasitic copepods constitute about one-third of the known copepods (Ho, 2001), and the hosts include all major groups of marine animals, including Porifera (Bandera *et al.*, 2005), Cnidaria (Humes, 1985b), Mollusca (Huys, 2001), Crustacea (Humes and Ho, 1969), Echinodermata (Boxshall and Ohtsuka, 2001), Urochordata (Ooishi, 2001), Hemichordata (Mayer, 1879; Kesteven, 1913), and Chordata (Abaunza *et al.*, 2001). Although more than 4000 symbiotic copepod species have been named, it is apparent that many species are yet to be found (Ho, 2001). Recently, molecular studies based on DNA sequences have provided important insights into the phylogenetic relationships within copepods (Huys *et al.*, 2006, 2007, 2009, 2012). Parasitic copepods usually

exhibit transformed or reduced body plans with fewer morphological characters for taxonomic identification. Therefore, when new parasitic copepods are discovered from previously unexplored habitats or hosts, molecular data may provide useful information to determine the phylogenetic affiliation of the new species.

Acorn worms (Enteropneusta), a solitary class of hemichordates, are marine vermiform invertebrates that generally live concealed in burrows (Hyman, 1959). There are about 70 species of acorn worms, and most of them belong to the families Harrimaniidae and Ptychoderidae (Cannon *et al.*, 2009). Until now, only two species of copepods, *Ive balanoglossi* Mayer, 1879 and *Ubius hilli* Kesteven, 1913, have been described as parasites in acorn worms. *Ive balanoglossi* was collected from *Glossobalanus minutus* Kowalevsky, 1866 (= *Balanoglossus minutus*) in the Mediterranean Sea (Mayer, 1879); *Ubius hilli* was described as a parasite of *Balanoglossus australiensis* Hill, 1894 (= *Ptychodera australis or Ptychodera australiensis*) in Australian waters (Kesteven, 1913). Both parasitic copepods lack typical morphological features and cannot be allocated to any family of the Copepoda (Boxshall and Halsey, 2004). They were temporarily grouped together into the *Ive*-group due to the similarity in body transformation and number of appendages. Willey (1897) and Nishikawa (1977) also observed parasitic copepods from a widespread acorn worm, *Ptychodera flava* Eschscholtz, 1825, in the Marshall Islands and Kushimoto, Japan, respectively. These parasitic copepods were assumed to be *I. balanoglossi* even though no clear description was provided. The effects of the copepods on the acorn worm host are also not known.

Parasites can play important roles in all ecosystems and exert strong selection pressure on their hosts (Anderson, 1980; Price, 1980; Anderson and May, 1981; Michalakis and Hochberg, 1994). It has been inferred that parasitic copepods may feed on mucus, tissues, blood, and symbiotic algae as food sources from their hosts or associated animals (Humes, 1985a; Huys and Boxshall, 1991; Johnson *et al.*, 2004; Cheng and Dai, 2010). They may also cause variable levels of localized lesions to their hosts and elicit host tissue responses through the process of attachment and feeding activities (Bron *et al.*, 1991; Johnson and Albright, 1992; Roubal, 1994). Although many copepod species have been well documented for their potential influences upon their hosts, most studies have focused on the species causing significant mortality on economically important hosts, such as salmon and trout (Huys and Boxshall, 1991; Boxshall and Bravo, 2000; Johnson *et al.*, 2004).

In this study, we present a full description of a new copepod species, *Ive ptychoderae*, collected from the acorn worm *Ptychodera flava* in Taiwan. Our study combines morphological features and molecular approaches to determine the taxonomic identification and phylogenetic position of this copepod. We also report infestation parameters (prevalence and intensity) of *I. ptychoderae*, which are considered to be the primary information for understanding the possible roles of parasites on their host populations (Bush *et al.*, 1997; Rózsa *et al.*, 2000; Smallridge and Bull, 2000; Mihalca *et al.*, 2008). Moreover, we examined histological sections of the infected tissues of the acorn worms and the gut contents of their parasites to better understand the parasite-host relationships.

Materials and Methods

Sample collection and preparation

Acorn worms *Ptychodera flava* were collected monthly from September to December 2009, as well as in June 2010 and June 2011, with a shovel in the sandy beach at Chito, Penghu Islands, Taiwan (23°38′54.17″N, 119°36′14.40″E). To isolate the parasitic copepods, acorn worms were anesthetized with 0.2 mol l^{-1} magnesium chloride in seawater for 15 min, and the copepods were dissected from cysts of hosts by using insect needles. Location of cysts, number and sex of copepods, and sex of hosts were determined under a dissecting microscope (Olympus SZ61). Length and width of copepods were measured in 120 (60 ♀♀ and 60 ♂♂) individuals. All copepod specimens were preserved in 70% ethanol except for those used for scanning electron microscopic (SEM) observations and DNA extraction.

Morphological studies

Morphological observations of parasitic copepods were performed as described in Humes and Gooding (1964). Three copepods of each sex were cleared in 85% lactic acid for 1 h and dissected on a wooden slide under a dissecting microscope. The removed body parts and appendages were examined under a compound microscope (Zeiss AXIOSKOP-40) with a series of magnifications up to 1000×. All drawings were made with the aid of a drawing tube.

For SEM observation, six copepod individuals (3 ♀♀ and 3 ♂♂) were preserved in 2.5% glutaraldehyde in 0.1 mol l^{-1} MOPS buffer (0.1 mol l^{-1} MOPS, 2 mmol l^{-1} $MgSO_4$, 1 mmol l^{-1} EGTA, 0.5 mol l^{-1} NaCl, pH 7.5) at 4 °C overnight and then post-fixed with 2% osmid (WAKO) in phosphate buffered saline (pH 7.4) overnight. Fixed copepods were dehydrated through a graded series of ethanol concentrations followed by critical-point-drying in a critical point dryer (Pelco CPD2). Dry individuals were coated with gold using a Cressington sputter coater (TED Pella, USA) and observed in an FEI Quanta 200 scanning electron microscope.

For histological observations, 10 infected acorn worms were anesthetized with 0.2 mol l^{-1} magnesium chloride in seawater for 15 min, fixed with 4% paraformaldehyde in 0.1 mol l^{-1} MOPS buffer at 4 °C overnight, and then dehydrated through a graded series of ethanol concentrations. Samples

were embedded in paraffin and sectioned at 5 μm, or were embedded in low-viscosity Spurr's resin (EMS Cat. 14300) and sectioned at 1 μm using a Leica Ultracut UCT microtome. Hematoxylin and eosin and toluidine blue (1% in 2% sodium borate solution) stains were applied to the sections, and the images were documented using a Zeiss Axio Imager.A1 microscope.

Phylogenetic analysis

For molecular phylogenetic analysis, a DNeasy Blood and Tissue kit (Qiagen) was used to extract genomic DNA from a single adult female parasite. For amplifying the 18S rDNA gene, PCR was carried out using primers 18-e (5′-CTGGTTGATCCTGCCAGT-3′) (Hillis and Dixon, 1991) and 18-p-c (5′-TAATGATCCTTCCGCAGGTTCACCT-3′) (Winchell *et al.*, 2002) with the following conditions: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1.5 min. The PCR products were cloned and sequenced. The sequence was aligned with published copepod 18S sequences (Huys *et al.*, 2006, 2007, 2009, 2012) using MUSCLE ver. 3.8.31 (Edgar, 2004). Alignments were used to construct phylogenetic trees with two phylogenetic methods, maximum likelihood (ML) and Bayesian inference (BI). ML was performed using PHYML ver. 20120412 with default settings (Guindon *et al.*, 2010) and BI analysis with MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003). The level of bootstrap support for ML was calculated by 10,000 resamplings using SEQBOOT in the PHYLIP ver. 3.69 package. For BI analysis, the nucleotide substitution model was set to mixed with gamma-distributed rate variation across sites and a proportion of invariable sites. The Markov chain Monte Carlo analysis was set to run for 1,000,000 generations and sampled every 100 generations. The first 25% of the samples were discarded as the burn-in. Parameters not specified were set to the default. The phylogenetic trees were illustrated with the FigTree ver. 1.4.0 program. GenBank accession number for the 18S rDNA sequence of *I. ptychoderae* is JF417992 (under the name "Ive sp. YHS-2012").

Statistical procedures

Total numbers of parasites were determined directly by numerical count. Prevalence, mean intensity, and median intensity were recorded as described in Bush *et al.* (1997) and Rózsa *et al.* (2000). Monthly differences in parasite prevalence were determined using Fisher's exact test in the Quantitative Parasitology 1.0 software (Rózsa *et al.*, 2000). One-way ANOVA and Duncan's multiple comparison test in the SPSS 12 package programs were used for analyzing the differences of the mean intensities. Differences in median intensity were analyzed using Mood's median test in the Quantitative Parasitology 1.0 software.

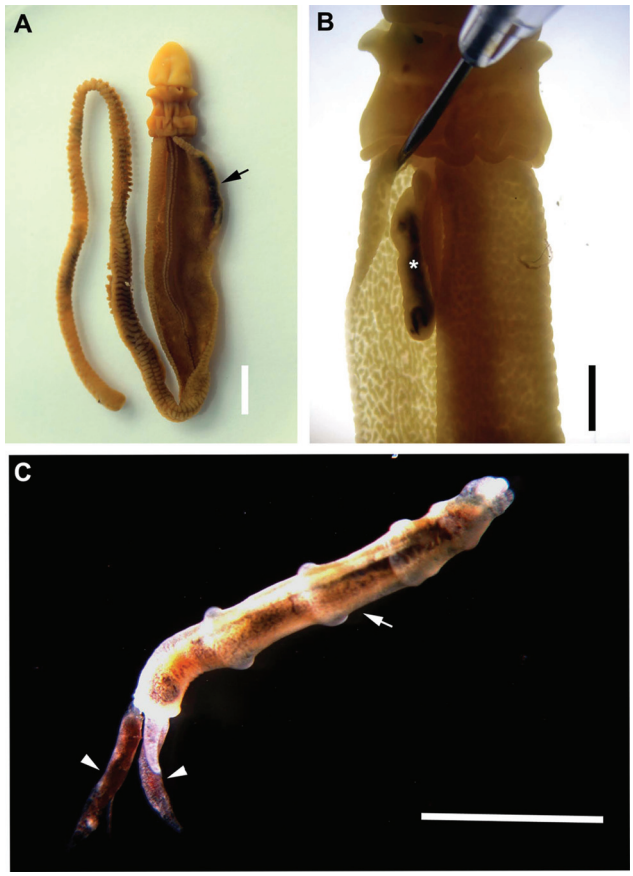

Figure 1. Host acorn worms (*Ptychodera flava*) infected by parasitic copepod *Ive ptychoderae*. (A) a parasitic copepod in the genital wing (indicated by the black arrow); (B) in the branchial region (white asterisk); (C) a female parasitic copepod (arrow) with two males (arrowheads) attached. *Scale bar* = 1 cm in A; 5 mm in B and C.

Results

Taxonomy
Genus *Ive* Mayer, 1879
***Ive ptychoderae,* n. sp.**

Type-host: *Ptychodera flava* Eschscholtz, 1825

Site: Parasitic copepods mostly appear in the cysts at the edges of the genital wings or the branchial region of the host (Fig. 1A, B). Male holds the female with clawlike maxilliped in the same cyst. Females were frequently accompanied by one or several males, which are smaller than the female (Fig. 1C).

Type-locality: Chito, Penghu Islands, Taiwan.

Etymology: The specific name, *ptychoderae*, is derived from the generic name of its host.

Type-material: Twenty-six ♀♀ and 31 ♂♂ collected on

Table 1

Summary of statistics for the parasite Ive ptychoderae *found in the acorn worm* Ptychodera flava

	Sep. 2009	Oct. 2009	Nov. 2009	Dec. 2009	Jun. 2010	Jun. 2011
No. of collected acorn worms	47	103	13	30	76	21
No. of infected acorn worms	16	46	9	16	25	11
Parasite abundance (male/female)	57 (31/26)	132 (72/60)	60 (40/20)	64 (40/24)	81 (50/31)	50 (27/23)
Sex ratio of parasites	1.19	1.20	2.00	1.67	1.61	1.17
No. of ovigerous females	7	33	6	9	11	5
Percentages of ovigerous females	26.92	55.00	30.00	37.50	35.48	21.74
Prevalence (%)	34.04	44.66	69.23	53.33	32.89	52.38
Mean (± SD) intensity	3.56 ± 1.50^{ab}	2.81 ± 1.80^{b}	6.67 ± 2.45^{a}	4.00 ± 1.83^{ab}	3.24 ± 1.42^{b}	4.55 ± 3.42^{ab}
Median intensity	4.0	2.0	7.0	4.0	3.0	4.0

During the sampling periods, 290 acorn worms were examined and 444 parasitic copepods (184 ♀♀ and 260 ♂♂) were found.
Various superscripts denote significant differences ($P < 0.05$) between groups.

24 September 2009; 60 ♀♀ and 72 ♂♂ collected on 26 October 2009, 20 ♀♀ and 40 ♂♂ collected on 17 November 2009, 24 ♀♀ and 40 ♂♂ collected on 2 December 2009, 31 ♀♀ and 50 ♂♂ collected on 30 June 2010, and 23 ♀♀ and 27 ♂♂ collected on June 2011 (Table 1). Holotype (SINICA-COPEPOD 001), allotype (SINICA-COPEPOD 002), and paratypes (SINICA-COPEPOD s001) were deposited at the Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan. Remaining specimens are retained in the first author's private collection.

Taxonomic description

Figures 2, 3 and 4

Female: Body (Figs. 2A and 4A) vermiform, lacking external segmentation. Average length 8.84 ± 0.51 mm (mean ± SD), ranging from 2.61 to 23.27 mm; average width 1.26 ± 0.04 mm, ranging from 0.45 to 1.94 mm, based on 60 specimens. Prosome (Figs. 2A and 4A) with 4 pairs of annular swellings. Urosome (Figs. 2B and 4A) much shorter than prosome, bearing only 1 pair of swellings. Caudal ramus (Fig. 2C) 1 and 4 lateral tubercles.

Rostral area (Fig. 2D) unarmed. Cephalic region swollen laterally. Antennule (Fig. 2E) short, unsegmented, with scattered spinules, bearing 3 (2 subterminal and 1 terminal) spines and 5 tubercles in distal region. Chelate antenna (Fig. 2F) robust, indistinctly 3-segmented; basal segment largest, bearing 4 patches of spinules; second segment with pointed process and basal spiniform seta; distal segment tipped with short claw in addition to carrying 2 spiniform setae. Oral aperture (Figs. 2D and 4C) distinct, with slightly raised rim (lip), which is divided into 2 halves (anterior and posterior) by a pair of tiny, bifid, lateral knobs (Fig. 4C, indicated with p).

Mandible, maxillule, and maxilla absent. Maxilliped (Fig. 2D) sexually dimorphic, reduced to tiny lobe located not far posterior to oral aperture.

Leg 1 (Fig. 2G) biramous; 2-segmented large protopod with strongly arched intercoxal sclerite, bearing bifid, unsegmented exopod and endopod. Leg 2 similar to leg 1. Legs 3–5 absent.

Male: Body (Figs. 3A and 4B) highly transformed, average length 3.40 ± 1.50 mm (mean ± SD) ranging from 1.79 to 7.54 mm; average width 0.53 ± 0.19 ranging from 0.32 to 1.13 mm, based on 60 specimens. Segmentation of body indistinct. Body without annular swellings. Caudal ramus (Fig. 3B) generally as in female but much smaller.

Antennule and antenna generally like those in female. Rim of oral aperture with more prominent lateral bifid knobs (Fig. 4D). Mandible, maxillule, and maxilla absent. Maxilliped (Fig. 3C) 2-segmented, first segment larger than the second, with scattered spinules; distal segment small, carrying 3 spines. Legs 1–2 as in female. Legs 3–5 absent.

Remarks: Inasmuch as the original species description of *I. balanoglossi*, the only existing congener of the present new species, lacked the morphological details of the appendages, it is impossible to make a close comparison with *I. ptychoderae*. Although *I. balanoglossi* has been sighted twice since Mayer's (1879) report, once by Willey (1897) found in *P. flava* in the Marshall Islands and another time by Nishikawa (1977) also found in *P. flava* but from Kushimoto, Japan, the parasite was only mentioned without addition of any morphological information. Nevertheless, the difference in the number of annular swellings, four in *I. balanoglossi* and five in *I. ptychoderae*, clearly indicates that the species of *Ive* from Taiwan is different from the species reported by Mayer (1879) from the Mediterranean.

Molecular phylogenetic analysis

In an attempt to determine the phylogenetic position of *I. ptychoderae* within the Copepoda, we performed molecular phylogenetic analyses using the 18S rDNA sequences (Fig

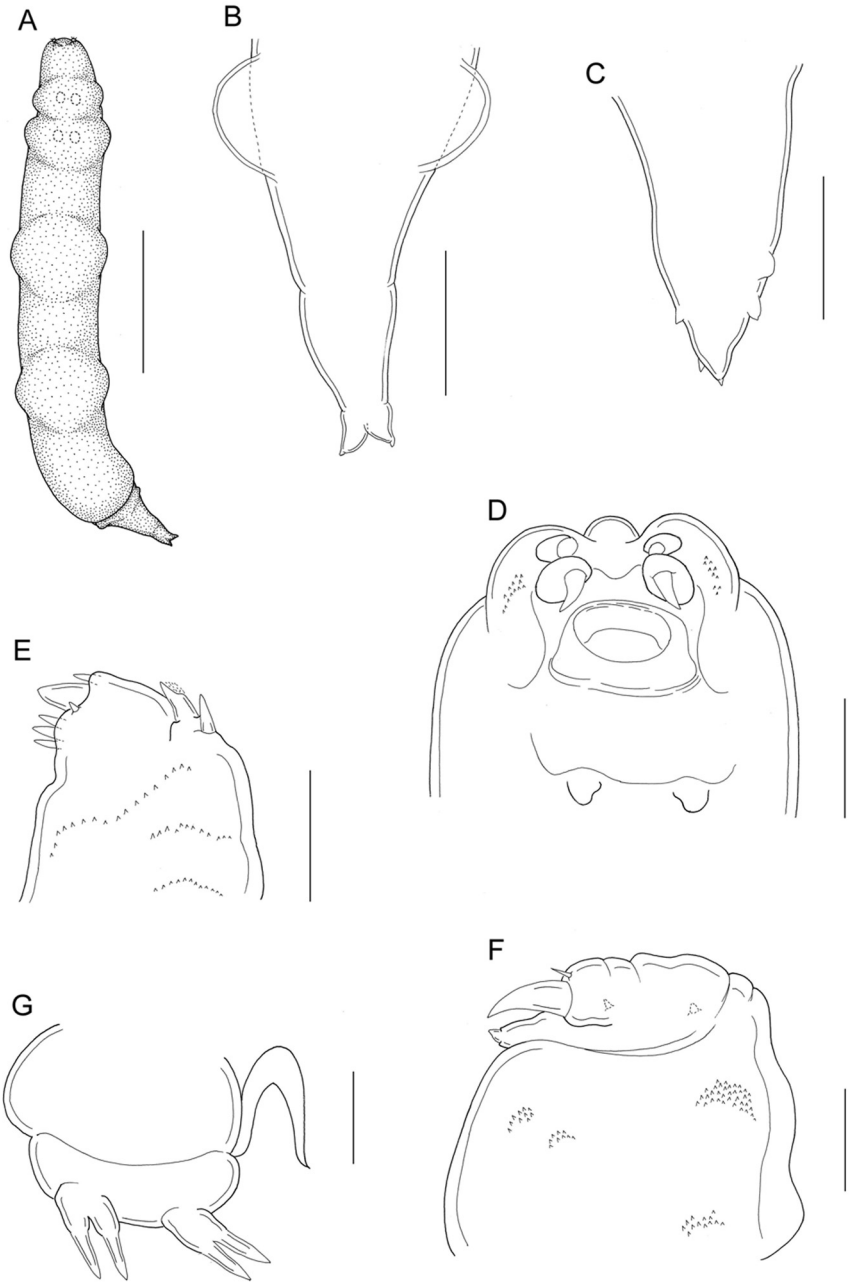

Figure 2. *Ive ptychoderae* n. sp., female. (A) habitus, dorsal; (B) urosome, dorsal; (C) caudal ramus, dorsal; (D) cephalic region, ventral; (E) antennule, anterior; (F) antenna, anterior; (G) leg 1 and intercoxal sclerite, anterior. *Scale bar* = 2 mm in A; 0.2 mm in D; 0.05 mm in B, C, and G; 0.025 mm in E and F.

5). Both the Bayesian and ML analyses showed that *I. ptychoderae* groups with Poecilostomatoida, although the supporting values are low (Bayesian posterior probability 0.84 and bootstrap value 32). In addition, *I. ptychoderae* is not closely related to any copepod family with known 18S sequences. We noted that the Clausidiiform complex of Poecilostomatoida does not form a monophyletic group with other Poecilostomatoida species and *I. ptychoderae*. This result is similar to a previous published study (Huys *et al.,* 2012). Therefore, we concluded that *I. ptychoderae* is possibly a member of Poecilostomatoida and may belong to a distinct clade within the order.

Biology of Ive ptychoderae *n. sp.*

According to our sampling records in Penghu Islands from September 2009 to June 2011 (Table 1), 444 individuals of *I. ptychoderae* were obtained from 290 acorn worms.

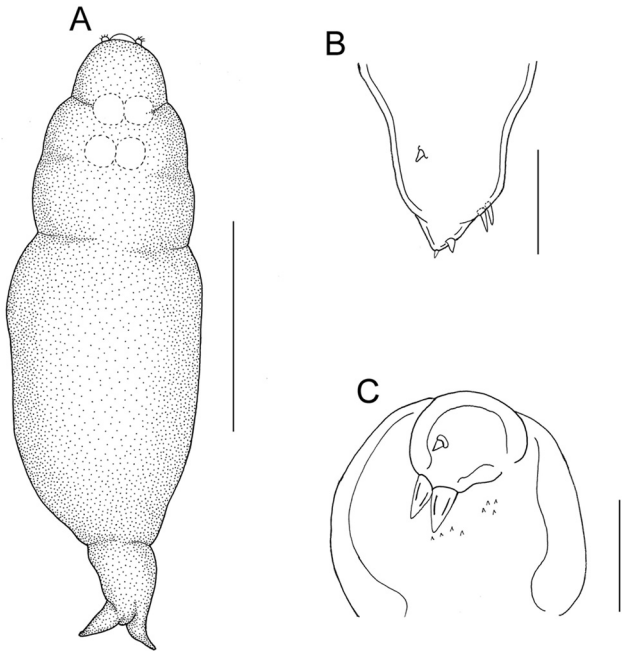

Figure 3. *Ive ptychoderae* n. sp., male. (A) habitus, dorsal; (B) caudal ramus, dorsal; (C) maxilliped, anterior. *Scale bar* = 0.5 mm in A; 0.025 mm in B and C.

The average prevalence of *I. ptychoderae* in the *P. flava* population during the collecting period was 42.41% (123/290), ranging from 32.89% (June 2010) to 69.23% (November 2009). The prevalence values were not significantly different between months during the collecting period (Fisher's exact test, $P = 0.0575$). The mean intensity of *I. ptychoderae* varied significantly between the months ($P < 0.05$), and a maximum mean intensity (6.67 ± 2.45) was recorded in November 2009. The median intensity, ranging from 2.0 to 7.0 parasites per infected acorn worm, also showed a similar trend with the mean intensities observed between each month (Table 1).

Individuals of *I. ptychoderae* were commonly found near the edges of the genital wings (86%, $n = 106$, Fig. 1A), in the branchial region (5%, $n = 6$, Fig. 1B), or in both regions (9%, $n = 11$) of the acorn worms. The number of cysts varied among acorn worm individuals. In most cases, the infected acorn worms contained one (60%, $n = 74$) or two (33%, $n = 41$) cysts. A few acorn worms were found to have three (4.88%, $n = 6$) or four (1.63%, $n = 2$) cysts. In general, a single cyst contains one female of *I. ptychoderae* accompanied by one or more (up to 7) males. The most common cases were 1 ♀/1 ♂ (37%, $n = 46$) followed by 1 ♀/2 ♂♂ (21.86%, $n = 27$). We also found that 15.85% ($n = 19$) of the cysts contained only a single female without any males, while cysts containing only male *I. ptychoderae* were rare.

We observed that 21.74% to 55.00% of *I. ptychoderae* females carried egg strings containing eggs or embryos (ovigerous females; Table 1), suggesting that they can produce offspring during our sampling period. Each egg string contained approximately 637.97 ± 307.90 eggs ($n = 30$) (Fig. 6A). When mature egg strings were dissected from hosts in seawater, nauplius lavae hatched rapidly (Fig. 6B). Swimming nauplius larvae maintained in seawater with mixed algae at room temperature moulted into copepodid larvae (Fig. 6C) in 3 to 4 days. *In situ* observations showed that *I. ptychoderae* went through embryonic development until the nauplius stage inside the egg strings within hosts (Fig. 6D), and the nauplius larvae were later released into seawater.

To observe the relationship between the parasitic copepod and acorn worm host *in situ*, we examined histological sections of the infected tissues of acorn worms and found that cysts in the genital wing of the infected acorn worm were enclosed by the hypertrophied epithelium and connective tissues of the host (Fig. 6E, F). We also observed significant degeneration of host muscular tissue in the cyst-containing regions (Fig. 6F). Inside the digestive tracts of *I. ptychoderae*, we observed chyme containing disintegrated host tissues (Fig. 6G, H) and host eggs covered with multilayered peritrophic membranes (Fig. 6I). These results suggest that *I. ptychoderae* feeds on somatic and gonadal tissues of its acorn worm host.

Discussion

Phylogenetic affiliation of Ive ptychoderae *n. sp.*

Since 1879, only two parasitic copepods, *Ive balanoglossi* and *Ubius hilli*, have been described from acorn worms. Boxshall and Halsey (2004) suggested that these two genera (*Ive* and *Ubius*) are clearly not related to Siphonostomatoida since they lacked an oral cone. With the absence of the labrum and concomitant modification of thoracopods into exopods and endopods, these two genera are also excluded from any of the existing poecilostome families. However, the presence of sexually dimorphic maxilliped in *I. ptychoderae* suggests that this new species (and the two genera) may belong to the Poecilostomatoida (Huys and Boxshall, 1991; Kim, 2004). Our molecular analyses placed *I. ptychoderae* with non-Clausidiiform Poecilostomatoida, although the supporting values are not confident enough (Fig. 5). Nevertheless, both morphological and molecular features suggest that *I. ptychoderae* may represent a member of a distinct copepod clade. Further molecular phylogenetic analysis using 18S rDNA sequences or other molecular markers from related species, such as *I. balanoglossi* and *U. hilli*, would be necessary to understand the exact phylogenetic position of these parasitic copepods. In addition, uncovering and comparing morphological features of postembryonic stages of *I. ptychoderae* may provide more insights into its phylogenetic position.

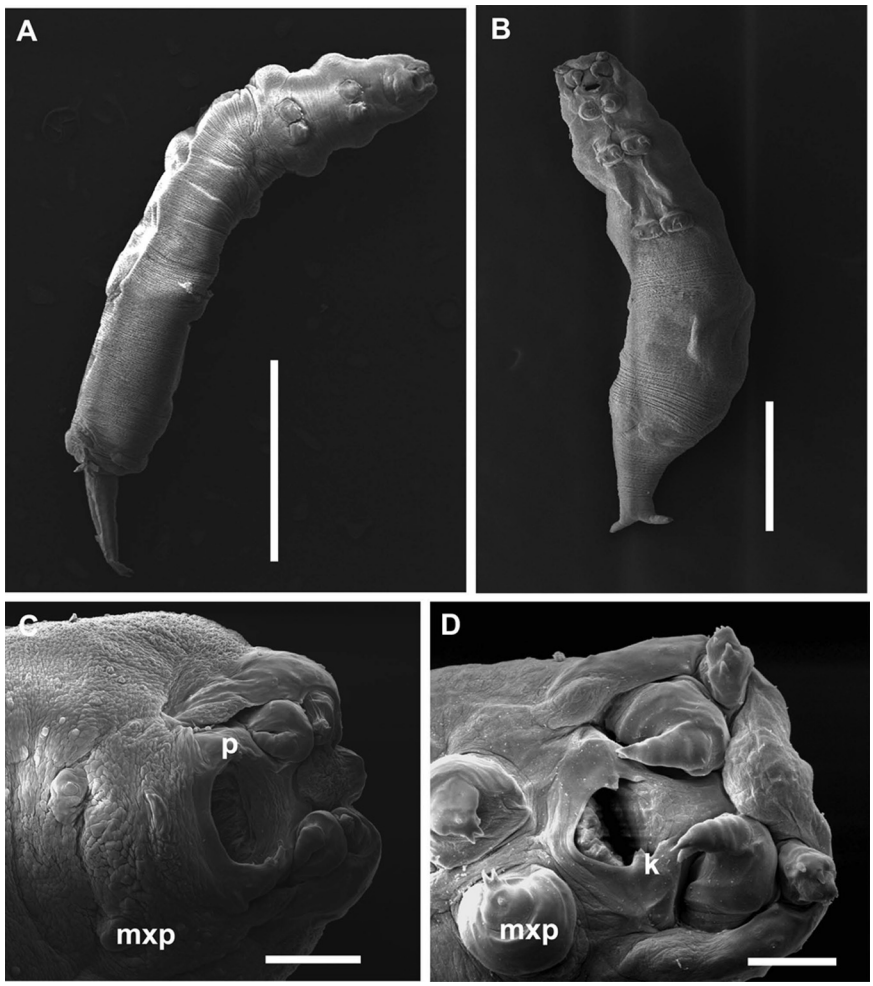

Figure 4. Scanning electron microscopic photos of *Ive ptychoderae* n. sp. (A) female, ventral; (B) male, ventral; (C) head region of female, showing paragnaths (p) and reduction of maxilliped (mxp); (D) head region of male, showing bifid lateral knobs (k) and maxilliped (mxp). *Scale bar* = 3 mm in A; 1 mm in B; 0.2 mm in C; 0.05 mm in D.

Because of the highly distinct morphological and molecular character of *I. ptychoderae*, we propose herein to establish a new family, Iveidae, to accommodate both *Ive* and *Ubius*. The diagnosis of this new family is as follows.

Female: Body highly transformed, vermiform, large (may be up to 23 mm), and lacking external segmentation; trunk cylindrical in both, but with annular swellings along body in *Ive*. Body tapering posteriorly in *Ubius*, but with paired caudal rami in *Ive*. First leg-bearing somite separates from cephalosome. Mouth aperture distinct, without labrum or labium.

Antennule stubby, unsegmented, and tipped with elements. Antenna uniramous, 3-segmented; forming chelate apparatus with pointed process of middle segment lying against claw of terminal segment. Mandible, maxillule, and maxilla absent. Maxilliped sexually dimorphic, reduced to tiny lobe in *Ive*, but retained as 2-segmented functional appendage in *Ubius*.

Protopod of first and second thoracopods large, carrying biramous rami, with each ramus transformed into movable chela. Legs 3 to 5 absent.

Male: Similar to female but with relatively much shorter body (up to 7.5 mm). Maxilliped 2-segmented, with large proximal segment and small distal segment tipped with 2 or 3 processes.

Prevalence and intensity of Ive ptychoderae *n. sp. within the hemichordate* Ptychodera flava

During our sampling period we found more than one-third of the acorn worms collected in Penghu Islands to be infected by *I. ptychoderae*. It suggests that *I. ptychoderae* is

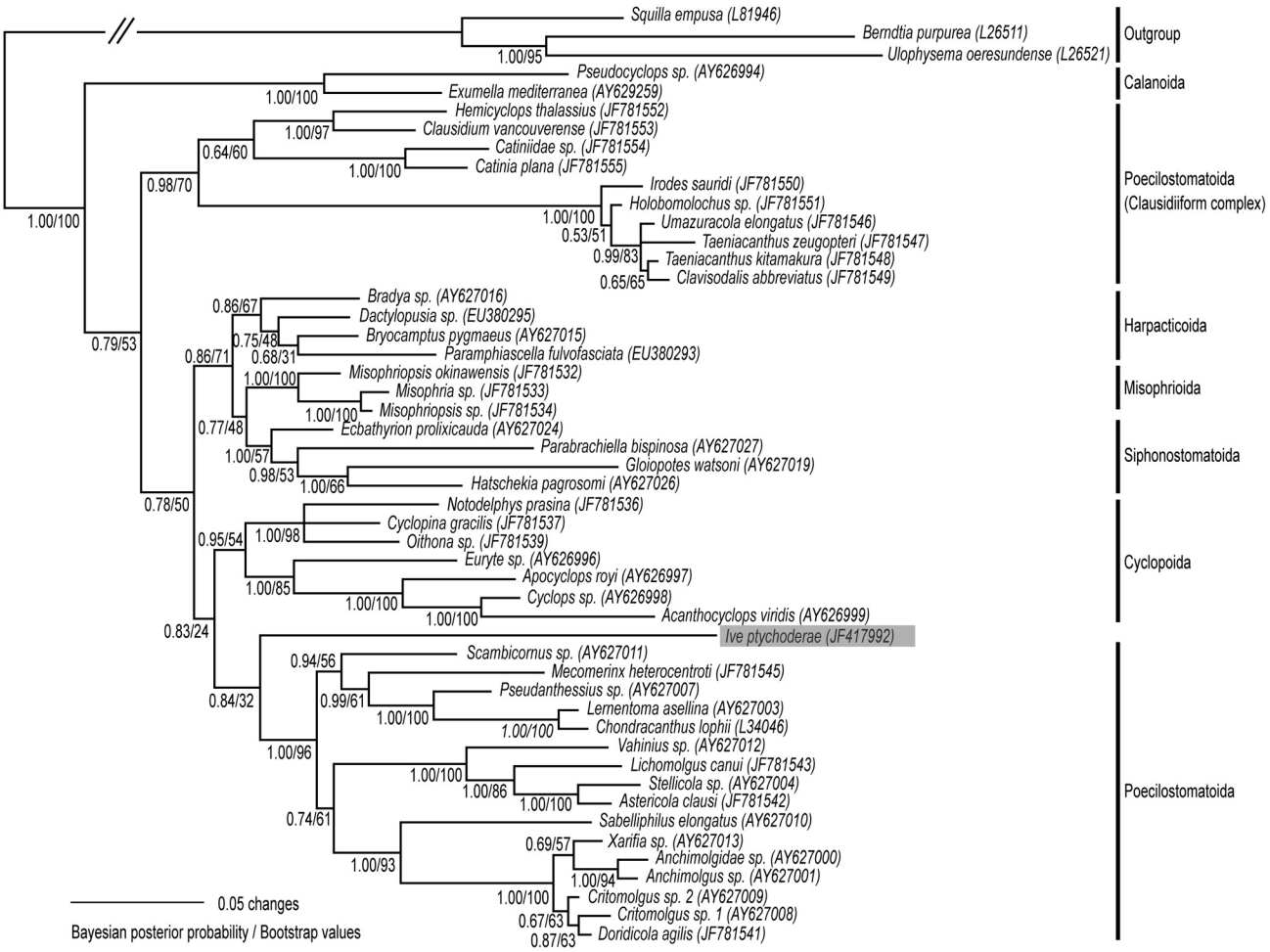

Figure 5. Phylogenetic analyses of *Ive ptychoderae* based on the 18S rDNA sequences of the copepod species using Bayesian inference analysis and maximum likelihood methods with three noncopepodan taxa as an outgroup. The phylogram is based on Bayesian inference and the nodal support based on posterior probabilities (Bayesian inference) and bootstrapping percentage (maximum likelihood). Scales indicate changes per site.

a common parasite within the acorn worm population. Our results also show that the highest infestation of *I. ptychoderae* occurred in November. Since sexual reproduction of *P. flava* is restricted primarily from October to December in Taiwan (unpubl. data), the highest infestation is possibly related to the weakening of the defense mechanisms of acorn worms during their reproductive season. These results support the hypothesis of a trade-off between reproductive effort and the ability to defend against parasitic infection (Festa-Bianchet, 1989; Norris *et al.*, 1994). The investment of acorn worms in reproduction may result in higher susceptibility to parasite infection. However, the ecological conditions may also play critical roles in altering the parasite community (Mouritsen and Poulin, 2002; Poulin and Mouritsen, 2005; Møller, 2010). Further studies with monthly sampling and long-term prevalence and intensity analyses in the wild are required to address these issues.

The same acorn worm species *P. flava* is commonly found in Hawaii and has been used for biological research, especially developmental studies, during the past 15 years (Lowe *et al.*, 2004; Röttinger and Lowe, 2012). However, the occurrence of parasitic copepods has as yet not been recorded from this population. If *I. ptychoderae* is indeed absent in Hawaiian *P. flava*, it would suggest that there may be some barriers preventing the long-distance dispersal of this copepod parasite. Alternatively, parasitic copepods are present within the *P. flava* population in Hawaii Islands but were not noticed by the researchers. It would require careful reexamination of the *P. flava* from Hawaii Islands to confirm this issue. This issue is particularly important since *P. flava* has recently become one of the major hemichordate model species for evolutionary developmental biology research (review in Röttinger and Lowe, 2012), and studies of its genome have provided new insights into the evolution of

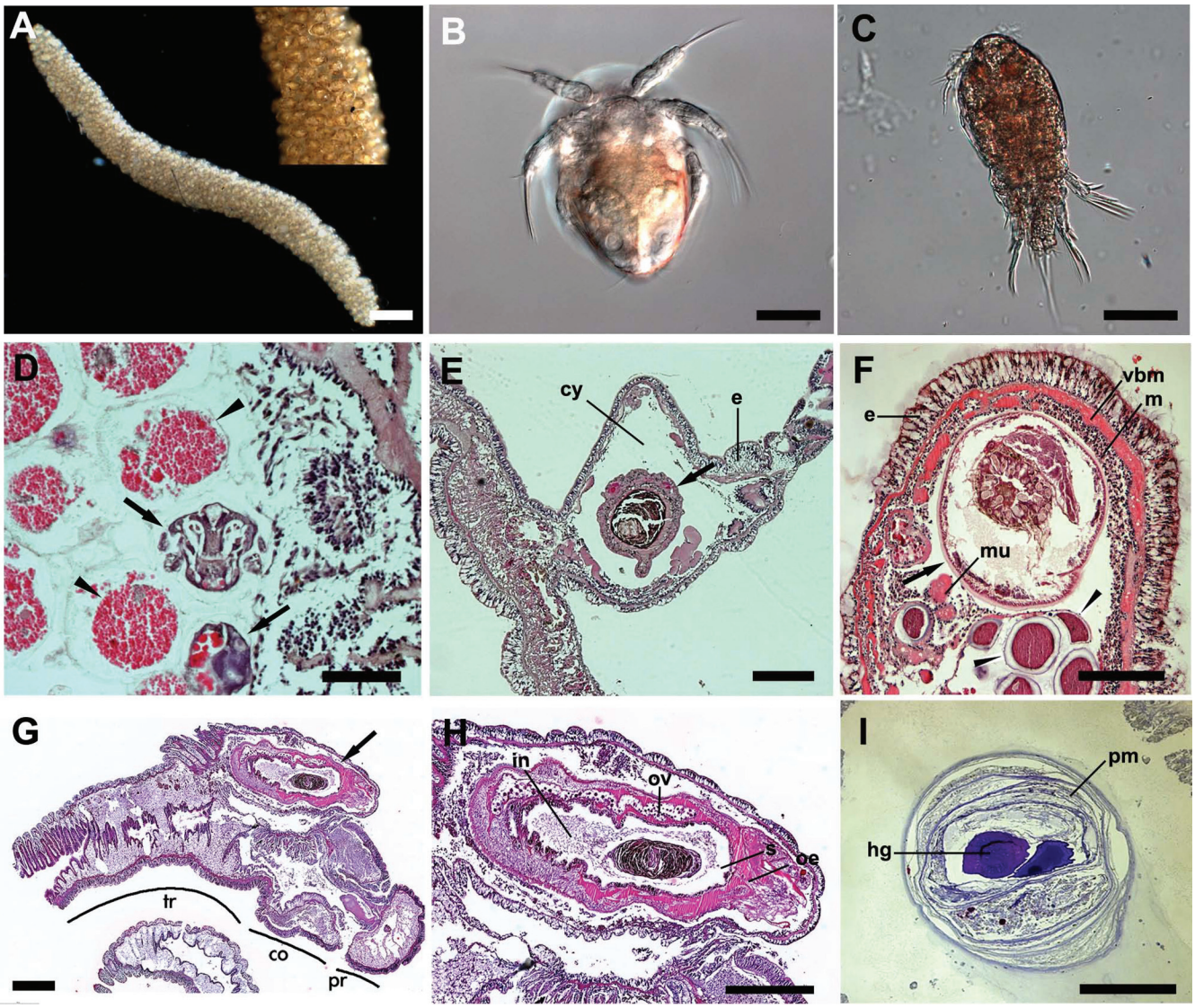

Figure 6. Postembryonic stages of *Ive ptychoderae* and histological examination of infected acorn worms (*Ptychodera flava*). (A) egg string with insert showing a higher magnification of enclosed embryos; (B) nauplius larva just hatched from the egg string; (C) copepodid larva; (D) histological section showing eggs (arrowheads) and enclosed nauplius larvae (arrows); (E) infection of *I. ptychoderae* (arrow) causing formation of cyst (cy) and expansion of epidermis (e) of host tissues; (F) cross section of *I. ptychoderae* (arrow) inside the genital wing of a female acorn worm showing thickening of vascularized basement membranes (vbm), expansion of epidermis (e), degeneration of host muscular tissue (mu), and aggregation of mesenchymal cells (m) surrounding the cyst; host oocytes are indicated by arrowheads; (G) longitudinal section of an acorn worm with *I. ptychoderae* (arrow) parasitic inside its genital wing; the tripartite body organization, proboscis (pr), collar (co), and trunk (tr) of the acorn worm is labeled; (H) higher magnification image of (G) showing the esophagus (oe), stomach (s), intestine (in), and ovary (ov) of *I. ptychoderae*; (I) host eggs (hg) covered with multi-layered peritrophic membranes (pm) inside the digestive tracts of the copepod. *Scale bar* = 0.5 mm in A, G, and H; 0.25 mm in F; 0.2 mm in E; 0.1 mm in D and I; 0.05 mm in B and C.

deuterostomes (Freeman *et al.*, 2012; Ikuta *et al.,* 2013). Therefore, to avoid the potential problem of contamination, it would be necessary to make sure that there is no parasitic copepod present in the biological materials of *P. flava* for future genomic studies. Similar care should be taken for other acorn worm species, such as *Saccoglossus kowalevskii*, which has also been used frequently for developmental and genomic studies in recent years (Freeman *et al.*, 2008; Cameron and Bishop, 2012), although no parasites have been reported yet from this species.

It should be noted that the differences in levels of parasite infection between sexes is a common phenomenon in host

species (Morand *et al.*, 2004; Christe *et al.*, 2007). In our studies, when the sex of the infested acorn worms can be identified during the gametogenesis period, we observed that the sex ratio (male/female) of infested acorn worms was similar to the sex ratio of acorn worms in the wild population at Chito, Penghu Islands (3:2, unpubl. data). Previous studies pointed out that the sex-specific parasitism may be mediated by gender differences in susceptibility to parasitism and that male hosts seem to be more susceptible than females (Zuk, 1990; Schalk and Forbes, 1997; Klein, 2000; Moore and Wilson, 2002). Our observations, to a certain extent, suggest that the infection of *P. flava* by *I. ptychoderae* in Penghu is not sex-biased and is a common phenomenon within this population.

Infection by Ive ptychoderae *causes damage in host tissues*

Ive ptychoderae may elicit defense reactions by its acorn worm host and trigger the formation of cystic dilatation in host tissues. We suspect that the process of cyst formation in acorn worms by *I. ptychoderae* may be similar to that of gall-forming copepods, whose appendages attach to soft tissues of their coral hosts and elicit defense reactions by depositing a calcareous barrier (gall) (Dojiri, 1988; Buhl-Mortensen and Mortensen, 2004). In addition, we observed that mature eggs hatched and directly developed into larval stages inside the cyst (Fig. 6D), and the larvae were subsequently released from their hosts, possibly through "birth" pores. The movements of the parasites may cause localized lesions in the tissues of their host. In our study a variety of such lesions were observed, including degeneration of muscular tissue, thickening of vascularized basement membranes, mesenchymal cell aggregation, and expansion of epidermis.

Sex ratio and sex determination in Ive ptychoderae

Although the sex ratio (male/female) of *I. ptychoderae* in the acorn worm population ranged from 1.17 to 2.00 (Table 1), suggesting a male-biased population, we rarely found cysts containing only male *I. ptychoderae* in our collection. In most cases, we observed one female, either with or without males. This observation raises the possibility that the sex determination in *I. ptychoderae* may be based on the environmental conditions experienced by different individuals after they infect the host. The environmental sex determination mechanism has been reported in the parasitic copepod *Pachypygus gibber* of the tunicate *Ciona intestinalis* (Becheikh *et al.*, 1998; Michaud *et al.*, 2004). In *P. gibber*, it has been demonstrated that in a rich environment (abundant food resource) the larvae tend to develop into females; in addition, it was reported that an existing sexual partner in a particular environment exerts a strong influence on sex determination of the newcomer (Becheikh *et al.*, 1998). It is possible that *I. ptychoderae* also employs a similar mechanism for sex determination. We hypothesize that *P. flava* represents a rich host environment, and the initial individuals of *I. ptychoderae* entering this environment tend to differentiate into females. On the other hand, if a female *I. ptychoderae* is already present inside an acorn worm, when the newcomers encounter this female they may differentiate into males and attach to the female with a maxilliped.

Parasite-host coevolution

Ptychodera flava is considered a widespread species across the Indo-Pacific Ocean (Lowe *et al.*, 2004). Previously, Willey (1897) and Nishikawa (1977) reported the occurrence of parasitic copepods from *P. flava* in the Marshall Islands and Japan, respectively; however, detailed descriptions of the parasites were not provided, and the name "*Ive balanoglossi"* was given in the literature. In the present study, we show that the parasitic copepod found within *P. flava* in Penghu Islands is a new species, *I. ptychoderae*. Further re-sampling would be required to confirm whether the previously reported parasitic copepods from *P. flava* in the Marshall Islands and Japan are different species or indeed belong to *I. ptychoderae*. In addition, *I. ptychoderae* found in *P. flava* in the Pacific Ocean represents an additional parasite-host pair to the two previously described cases, *I. balanoglossi* of *Glossobalanus minutus* in the Mediterranean Sea and *U. hilli* of *Balanoglossus australiensis* in Australian waters. Although the morphology of *I. ptychoderae* is distinguishable from *I. balanoglossi* and *U. hilli*, they are similar in their highly transformed body plans. Further studies on the phylogenetic relationships and interactions between parasitic copepods and acorn worms among the three pairs will be needed to investigate whether the parasites coevolve with their hosts in different geographic areas.

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