

First report of freshwater atyid shrimp, *Caridina formosae* (Decapoda: Caridea) as a host of ectosymbiotic branchiobdellidan, *Holtodrilus truncatus* (Annelida, Citellata)

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Received: 5 May 2020 / Accepted: 1 July 2020

Abstract – In recent years, ornamental shrimps gained increasing popularity in the aquarium trade. Unfortunately, they are potential vectors of epibionts, which may be unintentionally introduced to aquaria with imported shrimps. This contribution presents the first report of the occurrence of *Holtodrilus truncatus* on aquarium freshwater shrimp *Caridina formosae*. A total of 120 shrimp imported from Taiwan as aquarium pets were examined for the presence of epibionts. *Holtodrilus truncatus* occurred in 23.3% of shrimps. A total of 29.6% of crustaceans showed signs of damages as a result of *H. truncatus* activity. The shrimp is not common in the ornamental trade and is not subject to selective breeding. Therefore *C. formosae* populations available on the market, if not wild-caught, are most likely very similar to those occurring in Taiwanese natural water bodies, where *H. truncatus* occurs in *Neocaridina* shrimp. *Neocaridina* spp. are a known host for this epibiont, and transmission between species might occur in nature as well as in the aquarium trade where densities of animals are often high. The ability of *H. truncatus* to infect also highly invasive crayfish *Procambarus clarkii*, might also pose concern for regions where this crustacean is widespread. The actual lack of preventive measures for shrimp epibionts as well as confirmed releases of ornamental crustaceans into new aquatic ecosystems may result in further spread of *H. truncatus*, a potential new threat to native crustaceans and other epibionts.

Keywords: aquarium / alien species / epibiont / pet trade / Atyidae

Résumé – Premier signalement de la crevette atyide d'eau douce, *Caridina formosae* (Decapoda: Caridea) en tant qu'hôte du branchiobdelle ectosymbiotique, *Holtodrilus truncatus* (Annélide, Citellate). Ces dernières années, les crevettes ornementales ont gagné en popularité dans le commerce aquariophile. Malheureusement, elles sont des vecteurs potentiels d'épibiontes, qui peuvent être introduits involontairement dans les aquariums avec des crevettes importées. Cette contribution présente le premier rapport sur la présence de *Holtodrilus truncatus* sur les crevettes d'eau douce d'aquarium *Caridina formosae*. Au total, 120 crevettes importées de Taïwan comme animaux d'aquarium ont été examinées pour détecter la présence d'épibiontes. *Holtodrilus truncatus* était présent chez 23,3% des crevettes. Au total, 29,6% des crustacés ont montré des signes de dommages résultant de l'activité de *H. truncatus*. La crevette n'est pas commune dans le commerce des plantes ornementales et n'est pas soumise à une reproduction sélective. Par conséquent, les populations de *C. formosae* disponibles sur le marché, si elles ne sont pas pêchées à l'état sauvage, sont très probablement très similaires à celles des plans d'eau naturels de Taïwan, où *H. truncatus* est présent dans les crevettes *Neocaridina*. Les *Neocaridina* spp. sont un hôte connu pour cet épibionte, et la transmission entre espèces pourrait se produire dans la nature ainsi que dans le commerce des aquariums où les densités d'animaux sont souvent élevées. La capacité de *H. truncatus* à infecter également l'écrevisse très envahissante *Procambarus clarkii*, pourrait également poser problème dans les régions où ce crustacé est très répandu. L'absence réelle de mesures préventives pour les épibiontes de crevettes ainsi que

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les lâchers confirmés de crustacés ornementaux dans de nouveaux écosystèmes aquatiques pourraient entraîner une nouvelle propagation de *H. truncatus*, une nouvelle menace potentielle pour les crustacés indigènes et autres épibiontes.

Mots clés : aquarium / espèces exotiques / épibionte / commerce d'animaux de compagnie / Atyidae

1 Introduction

Ornamental aquaculture has a long history with millions of enthusiasts globally (Novák *et al.*, 2020). Releases of aquarium crustaceans in novel aquatic environments by irresponsible owners and vendors have been reported worldwide (Chucholl, 2013; Patoka *et al.*, 2014, 2017). The vast majority of reported cases involve the release of crayfish in urban and conurban waters (Patoka *et al.*, 2016a). Freshwater shrimps, however, are also increasingly reported alien species in various new environments (Klotz *et al.*, 2013; Jabłońska *et al.*, 2018a; Weiperth *et al.*, 2019a; Levitt-Barmats *et al.*, 2019). Once released in a new environment, freshwater shrimps may compete with native species and possibly serve as potential vector of diseases (Klotz *et al.*, 2013; Maciaszek *et al.*, 2018; Levitt-Barmats *et al.*, 2019). Moreover, relatively low levels of pathogen prevention and control as well as ineffectiveness of some related laws, in the aquarium trade, may further contribute to the global spread of undesirable hitchhikers (Patoka *et al.*, 2016b; Patoka *et al.*, 2018).

Freshwater shrimps are among the most common ornamental crustaceans kept in aquaria. Their often intense colouration is valuable to both breeders and hobbyists, and play a key role in their diffusion in the global aquarium trade (Maciaszek *et al.*, 2018). The increasing popularity of these crustaceans has led to the development of many intensive breeding facilities, particularly in Taiwan. Shrimp farms often utilize artificial or seminatural ponds for the less expensive shrimps that are not subject to intensive selection, while a controlled environment, such as aquaria, is used for the finest and priciest selections (Maciaszek *et al.*, 2018). However, not all ornamental shrimps are bred in captivity, as collection in the wild for some species is often more profitable (Calado, 2009; De Grave *et al.*, 2015). Since colouration and price generally dictate how worth an animal is, cheap and poorly colored wild-caught species have higher chances of getting abandoned by their owners and are often used as live food in aquaculture as well as in indoor aquaria (Hung *et al.*, 1993).

Caridina formosae (Hung *et al.*, 1993) from the family Atyidae is a dwarf shrimp native to inland waters of Taiwan. This generally transparent olive, brown, or light pinkish-red species reaches up to 1.7 cm in body length (Hung *et al.*, 1993). Thanks to its various colouration and characteristic dark spots, *C. formosae* is occasionally kept in, “so-called”, biotope aquaria, aimed to recreate natural ecosystems in aquarium conditions. However, the species is not common in the aquarium trade, as its coloration is less attractive to breeders in comparison to certain other dwarf shrimps (Weiperth *et al.*, 2019b; authors' observations). Therefore, *C. formosae* has not been a subject to selective or intensive breeding, in contrast to the often naturally coexisting *Neocaridina davidi*.

Unfortunately, aquarium shrimps, same as crayfish, are known to be vectors of several diseases and pests, which in

some cases can have detrimental effect. For instance, the invasive North American crayfish, *Procambarus clarkii* and certain other decapods, have been reported as non-symptomatic carriers of the crayfish plague, a disease that is lethal to crayfish of non-North American origin (Svoboda *et al.*, 2014; Putra *et al.*, 2018). However, health issues of intensively farmed low-cost shrimps are often overlooked by the breeders (Maciaszek *et al.*, 2018). This has largely contributed to the presence of other, undesirable species, such as epibionts in the breeding ponds. In some cases, unchecked imported shrimps, become the source of the accidental introductions of epibionts in aquaria and seminatural pond farms (Patoka *et al.*, 2016a; Maciaszek *et al.*, 2018) as well as within natural water bodies (Niwa *et al.*, 2005; Ohtaka *et al.*, 2012).

Branchiobdellidans are leech-like obligate epibionts of crustaceans, mainly crayfish (Gelder and Williams, 2015). Most of them were historically endemic; however, human-mediated transportation of crayfish and shrimps, unintentionally translocated these associated organisms alongside into new locations (Niwa and Ohtaka, 2006; Ohtaka *et al.*, 2012; Gelder and Williams, 2015). They are opportunistic omnivores, hunting for plankton and algae, but also feed on detritus as well as host hemolymph. Among the branchiobdellidans, *Holtodrilus truncatus* is, at present, the only member reported in Atyidae shrimp of South-East Asia (Gelder and Williams, 2015; Ohtaka *et al.*, 2015). To date, *H. truncatus* has been found in *Neocaridina* spp. in Japan, Korea, China, and Taiwan (Niwa and Ohtaka, 2006; Ohtaka *et al.*, 2012, Ohtaka *et al.*, 2015; Ahn and Min, 2016), *Caridina pseudodenticulata*, in Taiwan (Ohtaka and Chen 2010), *C. leucostica*, *C. multi-dentata*, *C. rubella*, *C. rapaensis*, *C. typus*, and *Paratya compressa* in Japan (Fujita *et al.*, 2010; Tanaka *et al.*, 2016).

To our knowledge, *H. truncatus* has never been reported in *C. formosae*. Moreover, this epibiont has not yet been reported among imported shrimps destined to the aquarium hobby. To date, the only reported epibiont in *C. formosae* is the microsporidium *Triwangia caridinae* (Wang *et al.*, 2013). With the present paper, we contribute with a new addition to the list of epibionts of *C. formosae*, providing the first confirmed evidence of *H. truncatus* occurring on that species. This is also the first report of the presence of this epibiont in imported shrimps destined to the aquarium hobby.

2 Material and methods

2.1 Epibiont detection and observation

We examined a total of 120 *Caridina formosae* adults (total length ~1.6 cm) from a shipment of aquarium fauna imported from Taipei, Taiwan, to Warsaw, Poland, in March 2019. Shrimps were transported in four aquarium bags containing 30 individuals each. All individuals were identified using morphological descriptions provided by Hung *et al.* (1993). No ovigerous female was present. The sample included 74

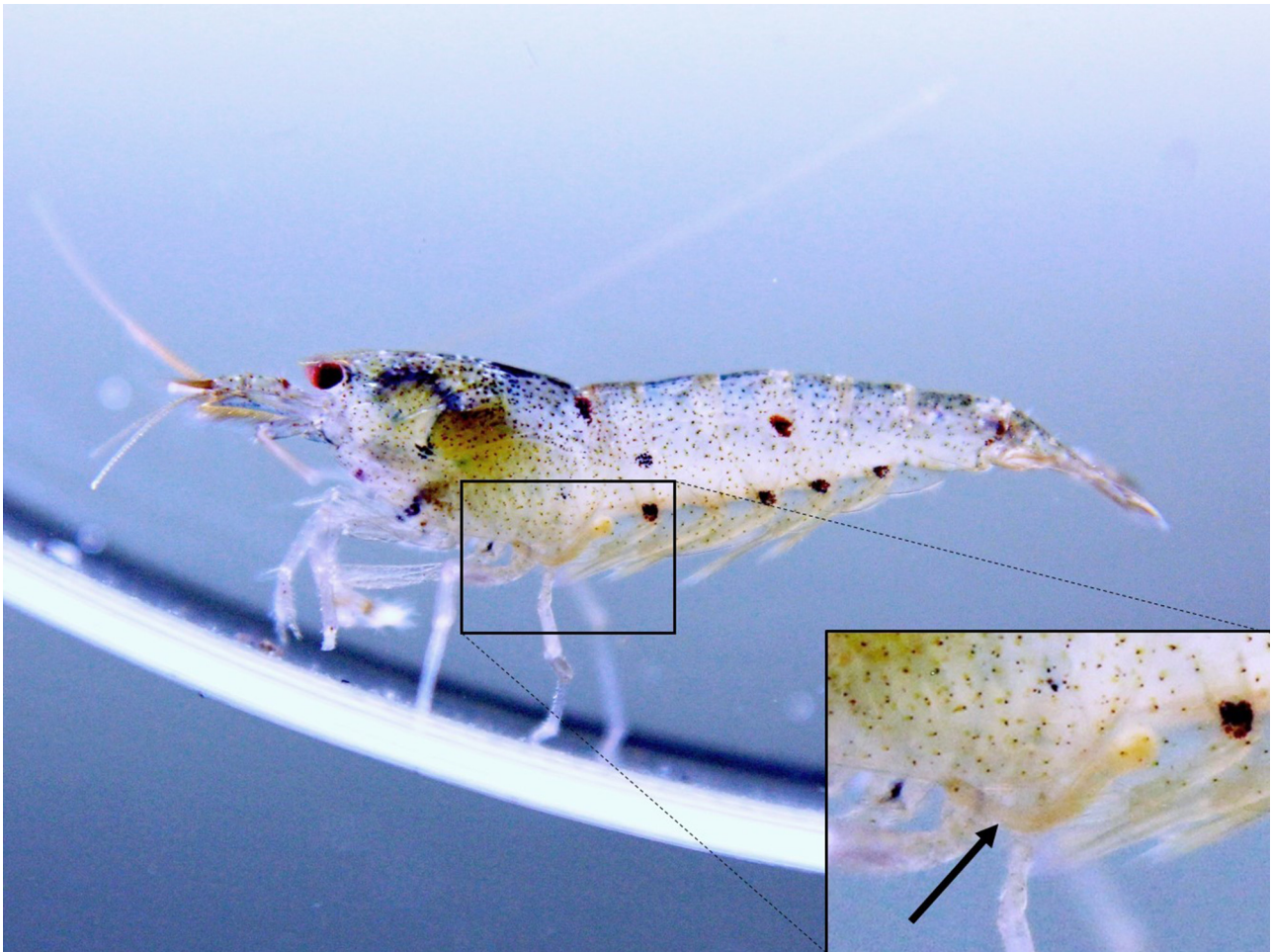


Fig. 1. Adult *Holtodrilus truncatus* (black arrow) penetrating *C. formosae* body (pereopodal area).

females, 38 males, and 8 individuals with no evident sexual dimorphism. After arrival, shrimps were acclimatized in four separate aquaria of $20 \times 10 \times 10$ cm filled with the water of the relative transport bag (~ 1.5 l) and illuminated using 6500 K 21W fluorescent lamp. After 10 hours, each shrimp was captured and analyzed for epibionts presence or traces of activity in four different microhabitats defined according to Maciaszek *et al.* (2018) as rostrum area, gills, pereopods area, as well as pleopods and abdomen area. Preliminary observations were carried out directly in each aquarium by secluding each individual shrimp with a petri dish pushed against the inner part of the wall (Fig. 1). To constrain movement of epibionts among different shrimp individuals, infected shrimps were separated for further inspection under the microscope, Leica DM50000B (Leica Camera AG, Wetzlar, Germany). Isolated epibiont individuals were counted, photographed with Canon DR-E[^] DC-Coupler EOS 5D (Canon Inc., Tokio, Japan) and identified using available literature (Ohtaka and Chen, 2010; Ahn and Min, 2016) as well as DNA barcoded.

2.2 Molecular analyses

Two individuals of episympiotic branchiobdellidans were DNA barcoded with cytochrome C oxidase subunit I (COI)

marker. The whole bodies were used for DNA extraction with the phenol-chloroform method (Hillis *et al.*, 1996). The polymerase chain reaction (PCR) was conducted according to the protocol provided by Hou *et al.* (2007) with the primer pair HCOJJ/LCOJJ (Astrin and Stuben, 2008). PCR products were then purified using Exonuclease I and FastAP alkaline phosphatase (Werle *et al.*, 1994) and subsequently sequenced by Macrogen Inc., Korea. Obtained sequences were verified by BLAST search (Altschul *et al.*, 1990) and deposited in BOLD Systems (Ratnasingham and Hebert, 2007) as well as in the GenBank database (Benson *et al.*, 2005). Haplotypes were detected in DnaSP software (Librado and Rozas, 2009). The phylogenetic tree was built with Maximum Likelihood method in MEGA 7.0 using substitution model and bootstrap test performed on 1000 replicates (Kumar *et al.*, 2016). The sequence of *H. truncatus* was retrieved from GenBank database and used for the comparison (accession number KX683299, deposited by Ahn and Min (2016). Additionally, sequences of *Xironogiton kittikasi* deposited in GenBank by Williams *et al.* (2013) (accession number JQ821632) and *Branchiobdella pentadonta* deposited in GenBank by Šarić *et al.* (2018) with accession number KY775124, were used as outgroups.

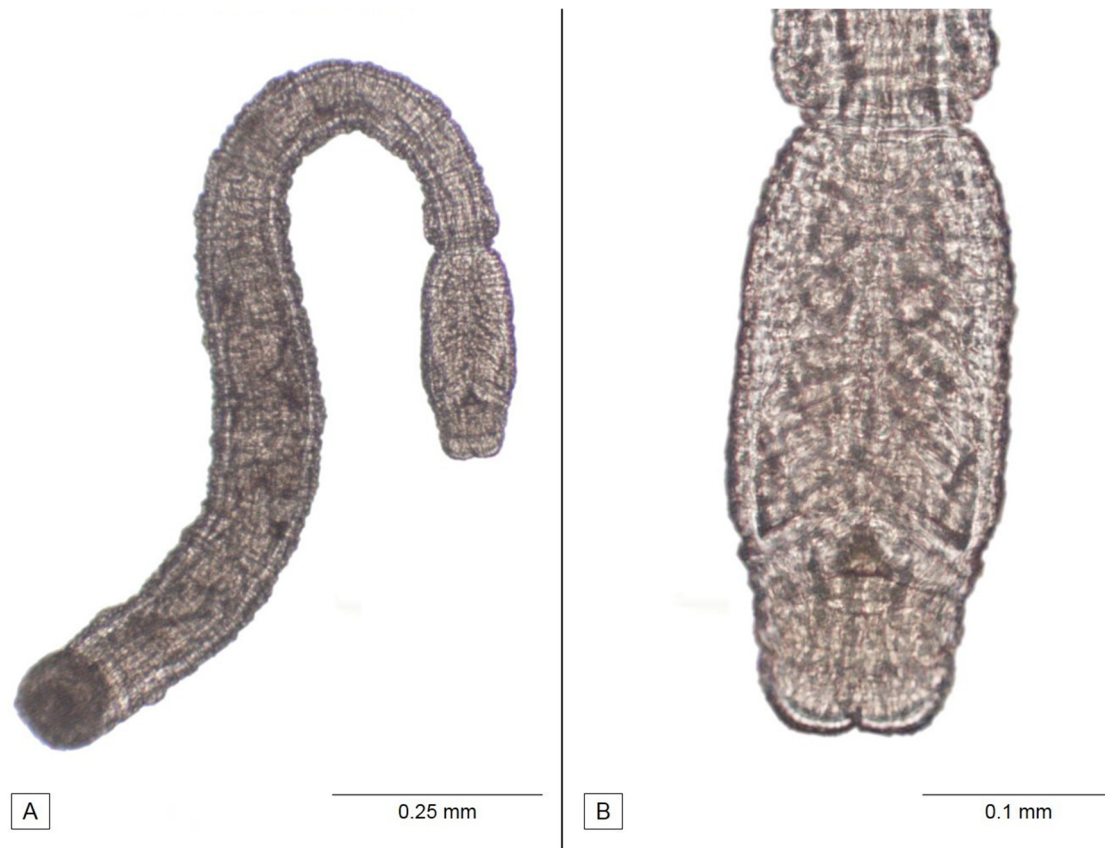


Fig. 2. (A) Whole-body image of *Holtodrilus truncatus* adult isolated from *Caridina formosae* and (B) close-up image of *H. truncatus* head.

2.3 Statistical analyses

Descriptive and statistical analyses were conducted in R 3.6 (R Core Team, 2020). To investigate differences in *H. truncatus* load between *C. formosae* sex, the quantitative parameters prevalence, intensity, and mean intensity were analyzed. Eight individuals without evident sexual dimorphism were not infected and excluded from statistical analysis. Prevalence is the proportion of infected hosts in the host sample and expressed in percentage. The prevalence of *H. truncatus* in the host sample was compared between male and female hosts using Fisher's exact test. Intensity is the number of parasite individuals in an infected host individual. Mean intensity is the average number of parasite individuals found in all infected hosts (non-infected hosts were excluded). A 95% confidence interval for mean intensity was obtained by using the bias-corrected and accelerated (BCa) bootstrap with 10,000 replications. To assess microhabitat preferences of *H. truncatus* among female and male shrimps, we used generalized linear models (GLM) with binomial distribution for prevalence and negative binomial distribution for intensity followed by ANOVA. Correlations among damaged areas, prevalence, and intensity were analyzed with Spearman's rank correlation coefficient.

3 Results

A total of 122 adult branchiobdellidans and two cocoons were found among the shrimp sample and identified as *H. truncatus* (Fig. 2, Tab. 1). Representatives of this epibiont

species had a brownish-transparent body ranging from 1.5 to 2.5 mm in length when alive. Peristomial lobe and dorsal segmental appendage absent (Fig. 2A). The head measured between 0.15 and 0.24 mm in width and was always broader than the first segment (Fig. 2B). The dorsal and ventral jaws were similar in size (~30 μm in width) and shape: orangish-brown, triangular, with a large median tooth and three pairs of smaller lateral teeth [3-1-3/3-1-3], sometimes a single lateral tooth was missing. Cocoons were transparent, ~0.5 mm in height, ovoid in shape, with a peduncle cemented onto the gill surface. Molecular analysis revealed that both individuals belong to one haplotype (Fig. 3).

In all examined *C. formosae*, 23.3% were infected by at least one *H. truncatus* individual. Overall, the mean intensity of *H. truncatus* was higher in females than males, while males had higher epibiont prevalence (Tab. 1). However, differences in prevalence between male and female shrimps were not significant (Fisher's exact test, $p=0.498$). At the shrimp population level, *H. truncatus* showed marked microhabitat preferences with 44.3% occurrence in the pereopods area, 22.1% in the rostrum area, 21.3% in the pleopods and abdomen area, and 12.3% in the gills. Similar to what observed in the combined sample, *H. truncatus* specimens were more frequent in the pereopods area of both females and males of *C. formosae* (43.5% and 46.7% respectively). However, with the exception of gills area, the occurrence of *H. truncatus* across other microhabitats differed among male and female shrimps. In females, 29.3% of *H. truncatus* occurred in rostral area, and 14.1% in pleopodal and abdominal area, whereas in

males, the epibiont was absent in rostral area and was more frequent in pleopodal and abdominal area (43.3%). Differences in microhabitat utilization among males and females of *C. formosae* were also apparent in the prevalence and mean intensity of *H. truncatus* (Tab. 2). The prevalence of

H. truncatus was higher in pereopodal area of females and pleopodal and abdominal area of males. Pereopodal area of females also had the highest mean intensity of *H. truncatus*, while in males, the highest mean intensity was observed on pereopods. In males, the prevalence of *H. truncatus* significantly differed among microhabitats (ANOVA, χ^2 (3)=14.672, $p=0.002$), but not in females (ANOVA, χ^2 (3)=0.109, $p=0.991$). In contrast, differences in mean intensity among microhabitats were significant for females of *C. formosae* (ANOVA, χ^2 (3)=15.369, $p=0.001$) but not for males (ANOVA, χ^2 (2)=4.1173, $p=0.1276$). Moreover, two females harbored one cocoon each in their gills chambers.

Table 1. Prevalence and mean intensity of *Holtodrilus truncatus* in the *Caridina formosae* sample.

| | <i>C. formosae</i> | | |
|-------------------------|--------------------|---------------|---------------|
| | All | Females | Males |
| Sample size | 112 | 74 | 38 |
| Prevalence (%) | 23.3 | 23.0 | 28.9 |
| Mean intensity (95% CI) | 4.4 (3.3–6.1) | 5.4 (3.8–7.9) | 2.7 (1.9–3.5) |

4 Discussion

Here we provide additional evidence on the occurrence of *H. truncatus* in atyid shrimp, as well as the first report of this

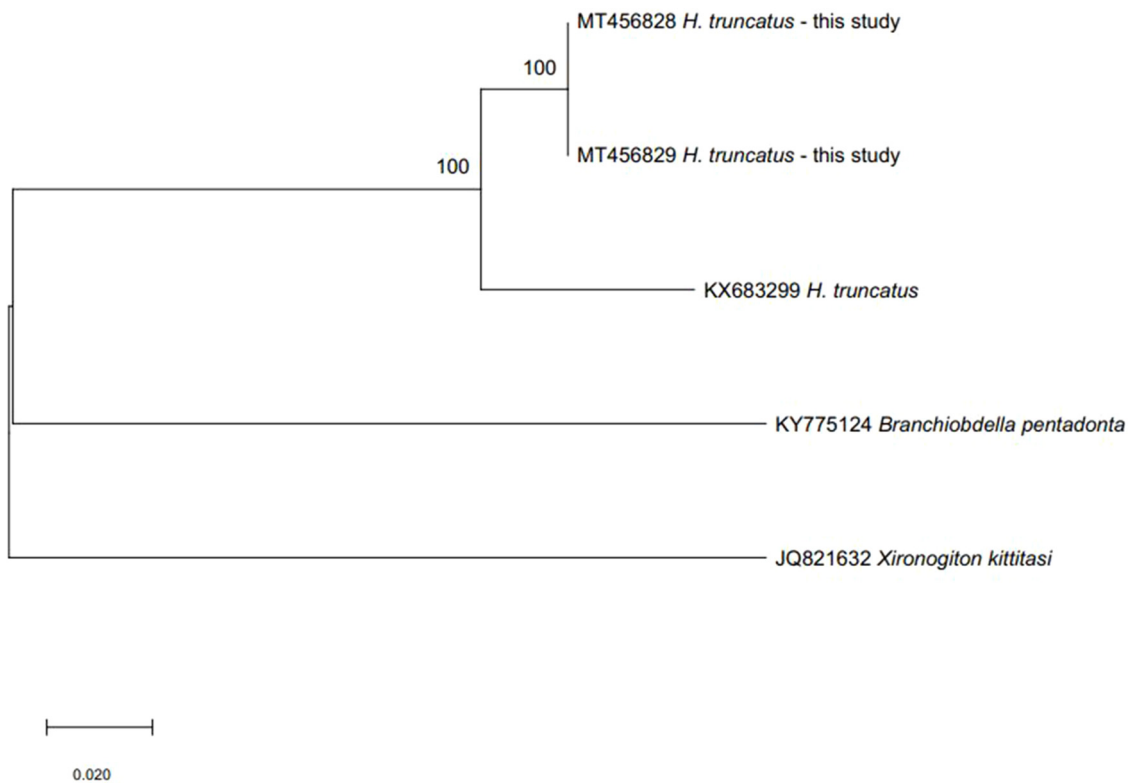


Fig. 3. Maximum-likelihood tree for examined *Holtodrilus truncatus* and GenBank-stored sequences.

Table 2. Prevalence and mean intensity of *Holtodrilus truncatus*.

| Microhabitat | Females (n = 74) | | Males (n = 38) | |
|----------------------|------------------|-------------------------------------|----------------|-------------------------------------|
| | Prevalence | Intensity (95% confidence interval) | Prevalence | Intensity (95% confidence interval) |
| Rostrum | 10.8% | 3.38 (2.25–4.25) | – | – |
| Gills | 10.8% | 1.5 (1.12–1.75) | 2.6% | 3 |
| Pereopods | 12.2% | 4.44 (2.67–7.78) | 10.5% | 3.5 (1–4.5) |
| Pleopods and abdomen | 10.8% | 1.62 (1.12–2) | 21.1% | 1.62 (1.12–1.88) |
| Entire body | 23% | 5.41 (3.76–7.88) | 28.9% | 2.73 (1.91–3.45) |

branchiobdellidan in *C. formosae*. Moreover, the presence of cocoons in the shrimp gills indicated that the reproduction of *H. truncatus* occurs in *C. formosae*, proving that this shrimp species is a suitable host and, consequently, a potential vector for this epibiont. In this study, the overall majority of *H. truncatus* were found in pereopodal area, suggesting at first glance a microhabitat preference toward this area. Contrary to what observed in female *C. formosae*, *H. truncatus* displayed a preference for pleopodal and abdominal area of males. Microhabitat preference toward pereopodal area as well as pleopodal and abdominal area is also in agreement with the finding of Niwa *et al.* (2014), who identified the preferred attachment location in *Neocaridina* spp. between the fifth pereopod and the first pleopod. The choice of this area may not be casual, as it facilitates access to the gill chamber, carapace, and eggs when ovigerous females are present (Niwa *et al.*, 2014).

In accordance with the observation of Ohtaka *et al.* (2012) on *Neocaridina* spp. we detected damages in gills as well as pleopodal and abdominal area of *C. formosae*. Females more likely presented damages in both areas, suggesting that the activity of *H. truncatus* might be higher in them than in males. Accordingly, cocoons were only found on *C. formosae* females. At a similar body length, females generally have a larger surface area than males, due to a more robust body, thus offering higher opportunity for movement and attachment. This might explain why females showed damages in multiple locations, harbored cocoons, and overall had higher intensities of *H. truncatus* inhabitation. However, it is not excluded that other factors such as differences in molting frequency between male and female shrimps, if present, may also play a role in *H. truncatus* infestations. The high mobility of the epibiont can explain the absence of correlation between prevalence and intensity of *H. truncatus* in these areas. According to the effects caused by other shrimp epibionts, having similar microhabitat preferences such as Scutariellidae (Platyhelminthes: Rhabdocoela) members (Ohtaka *et al.*, 2015), it is not excluded that the occurrence of *H. truncatus* on aquarium shrimps may have a detrimental effect on host fitness. Weakened shrimp may also have lower reproduction frequency and less intense colouration resulting in loss of income for breeders. However, without rigorous investigations on the nature of the relationships between *H. truncatus* and *C. formosae*, the potential adverse effect on shrimp fitness remains speculative (Gelder and Williams, 2015).

In Taiwan, *C. formosae* coexists with other freshwater shrimp species such as *N. davidi* on which *H. truncatus* has also been reported (Ohtaka *et al.*, 2015), suggesting that this epibiont may potentially migrate among different crustacean species. Migrations of *H. truncatus* among different species of crustaceans, including the red swamp crayfish *P. clarkii*, proved to occur under experimental conditions (Niwa *et al.*, 2014). We, therefore, can not exclude that under favorable conditions, this might happen as well in the natural environment. It is not clear if our sample of *C. formosae*, was breed in captivity or was wild-caught; therefore, we can not identify if our sample was in touch with *H. truncatus* in the wild, in the shrimp farm or at the wholesaler facility. Although preventive measures such as quarantine and bath in a saline solution (Tanaka *et al.*, 2016) might prevent the spread of *H. truncatus*, these are scarcely implemented by shrimp farms

and wholesale facilities. If unnoticed, the usually high density of shrimp in farm and wholesaler facilities might facilitate the spread of *H. truncatus* among different species of shrimp and, eventually, crayfish that finally end up in private aquaria. Moreover, as shrimp females are bigger and more colourful than males, imported ornamental freshwater shrimps typically display a skewed sex ratio toward females, which according to our results, harbor a higher burden of *H. truncatus* and cocoons, potentially enhancing the spread of this epibiont to other decapod crustaceans.

Branchiobdellidan representatives are often endemic; however, human-mediated transportation of crustaceans may enhance their distribution range through releases of infected individuals into new locations. In certain areas, competitive interactions with alien branchiobdellidans have already impacted native species (James *et al.*, 2017). Moreover, the ability of *H. truncatus* to infect different crustacean species, including the crayfish *Procambarus clarkii*, one of the most invasive species in the world (Gherardi and Acquistapace, 2007; Johović *et al.*, 2020), might potentially enhance the spread of this epibiont.

Among European crustaceans, branchiobdellidans are found only on crayfish (Longshaw, 2011; Skelton *et al.*, 2013; Subchev, 2014). However, *H. truncatus* may be transmitted to native atyid shrimp species (Tanaka *et al.*, 2016), including *Atyaephyra* sp., as well as *Dugastella valentina*, which is an endangered species (Christodoulou *et al.*, 2016; Jabłońska *et al.*, 2018b). Limited information is available on the influence of *H. truncatus* on its hosts (Tanaka *et al.*, 2016), and the occurrence of this epibiont species may have both positive as well as negative effect on populations of European crustaceans in case of its accidental introductions to natural water bodies. Although cohabitation between branchiobdellidans and other epibionts such as the European members of the family Scutariellidae which also use shrimps as specific hosts might occur (Matjašič, 1990; Ohtaka *et al.*, 2015; Pešić *et al.*, 2018), interactions among *H. truncatus* and other native epibionts are also unpredictable.

To sum up, the occurrence of *H. truncatus* in imported ornamental shrimps creates opportunities for its global spread via the aquarium trade. Due to its small size, and transparent colouration, the presence of *H. truncatus* may go unnoticed, particularly in shrimp with intensive pigmentation. Its spread might be facilitated by possible migration to other more popular crustacean species kept by aquarium hobbyists and accidentally spread to native and other highly invasive species once ornamental crustaceans are released in the natural environment. We, therefore, urge for more control over this epibiont to limit its diffusion. Preventive measures such as quarantine and bath in a saline solution are recommended.

Acknowledgments. This work was supported by the Warsaw University of Life Sciences under Grant No. 505-10-072500-Q00390-99.

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Cite this article as: Maciaszek R, Jabłońska A, Prati S, Świderek W. 2020. First report of freshwater atyid shrimp, *Caridina formosae* (Decapoda: Caridea) as a host of ectosymbiotic branchiobdellidan, *Holtodrilus truncatus* (Annelida, Clitellata). *Knowl. Manag. Aquat. Ecosyst.*, 421, 33.