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First molecular identification of *Vorticella* sp. from freshwater shrimps in Tainan, Taiwan

Chen-Chieh Liao[a,b], Jyh-wei Shin[c,d], Lih-Ren Chen[a,e], Lynn L.H. Huang[a,b,f,**], Wei-Chen Lin[c,d,*]

[a] *Institute of Biotechnology, College of Bioscience and Biotechnology, National Cheng Kung University, No. l, University Road, Tainan City, 701, Taiwan*
[b] *Department of Biotechnology and Bioindustry Sciences, College of Bioscience and Biotechnology, National Cheng Kung University, No. l, University Road, Tainan City, 701, Taiwan*
[c] *Department of Parasitology, Institute of Microbiology and Immunology, College of Medicine, National Cheng Kung University, No. l, University Road, Tainan City, 701, Taiwan*
[d] *Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan City, 701, Taiwan*
[e] *Physiology Division, Livestock Research Institute, Council of Agriculture, Tainan, Taiwan*
[f] *Advanced Optoelectronic Technology Center, National Cheng Kung University, No. l, University Road, Tainan City, 701, Taiwan*

A B S T R A C T

Freshwater shrimps are the most common crustaceans kept in an aquarium. This study was a survey seeking parasites infecting cultured freshwater atyid shrimps at aquarium stores in Tainan, Taiwan. We observed that atyid shrimps were infested with *Vorticella* and *Scutariella*. *Scutariella* is a common shrimp parasite; thus, we focused on *Vorticella* infection in the atyid shrimps. *Vorticella aequilata*-like pop TW, a freshwater peritrich ciliate, was isolated from the atyid shrimps. The morphological characteristics were investigated using live observations. Specimens from the population showed identical arrangement of the infraciliature and identical ITS1-5.8SITS2 region sequences. The zooids are bell-shaped, 40–58 μm wide and 47–70 μm in long *in vivo*. The food vacuole is variable in shape and is located in the middle of the cell. ITS1-5.8S-ITS2 sequences of *Vorticella aequilata*-like pop TW did not match any available sequences in GenBank. Phylogenetically, *Vorticella aequilata*-like pop TW clusters with the other *Vorticella* within the family Vorticellidae and nests with *Vorticella aequilata* in the subclade. Above all, the morphological characteristics and molecular analyses show that the investigated *Vorticella* is a *Vorticella aequilata*-like species. The phylogenetic analyses of ciliates based on the ITS1-5.8S-ITS2 sequences reveal that the *Vorticella* genus consists of *Vorticella* morphospecies and that taxonomic revision of the genus is needed. Morphometric criteria and molecular analysis were used to describe and identify the *Vorticella* specie and this study presents the first molecular identification analysis of the *Vorticella* species in the cultured atyid shrimps in Tainan, Taiwan.

1. Introduction

Diseases and parasites can be found in all domesticated and farmed plants or animals. Many parasites of freshwater aquarium shrimps are becoming more and more common and are apparently acquired through several species specific for commercial aquaculture, most notably the *Neocaridina* genus. *Neocaridina* spp. is an indigenous species of the Atyidae family, which is distributed throughout China, Vietnam, Korea, Japan and Taiwan (Hung et al., 1993; Shih and Cai, 2007). According to Cai (Cai and Li, 1997), *Neocaridina* spp. originates from ponds, rivers, agricultural canals, mountain streams and reservoirs of its indigenous area. *Neocaridina denticulata* is a very common shrimp species in Taiwan streams. They play an important role in the freshwater ecosystem and are an economical species in the aquaculture (Hung et al., 1993).

The most common external parasites are found on the surfaces and appendages of the animals. These invasions rarely cause death in the wild, but under the stressful conditions often found in densely stocked aquaculture ponds and tanks, they can get out of control and have a negative impact on mobility, molting, growth and function. Breeding and feeding may stop, and this may result in death. In the case of peritrich ciliates, high levels of infection can affect breathing and can be fatal. Parasites are easily transmitted between the species; thus, it is important to isolate any affected specimens in a timely manner and to isolate newly acquired shrimps. Infections by sessile peritrichs, such as *Vorticella* and *Ambiphrya*, are common in many cultured fishes (Abdel-Baki et al., 2014; Woo and Leatherland, 2006) but have not yet been reported in the cultured atyid shrimps (*Neocaridina denticulata*) in Tainan, Taiwan.

Vorticella is a genus with bell-shaped body crowned with a large oral area surrounded by cilia and some stalks to attach themselves to the substrates. The size and shape of the zooid, features of the pellicular surface and stalk, contour and height of the peristomial lip, and number of silverlines have been typically used as taxonomic characters of

* Corresponding author. Department of Parasitology, College of Medicine, National Cheng Kung University, No. l, University Road, Tainan, 701, Tainan, Taiwan.
** Corresponding author. 1. Institute of Biotechnology, College of Bioscience and Biotechnology, No. l, University Road, Tainan, 701, Tainan, Taiwan.
E-mail addresses: lynn@mail.ncku.edu.tw (L.L.H. Huang), wcnikelin@mail.ncku.edu.tw (W.-C. Lin).

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Vorticella. More than 200 species of *Vorticella* have been described using morphological methods. However, the small size of many *Vorticella* species, and its variable zooid size and shape, make it difficult to identify and characterize species of *Vorticella* (Sun et al., 2012; Warren, 1986). In recent studies, the genus are described using gene sequence information (Ji et al., 2015; Liang et al., 2018).

Numerous studies have suggested that phylogenetic analyses incorporating the primary sequences and secondary structures of the most popular markers, nuclear internal transcribed spacers (ITS), produce the most robust trees (Coleman, 2007; Keller et al., 2010; Sun et al., 2013b). In a recent study, Sun et al. provided a new evaluation of the phylogenetic relationship of the *Vorticella* species using the ITS1-5.8S-ITS2 and ITS2 sequences (Sun et al., 2013a). The ITS1 and ITS2 are between closely related genera or at the infrageneric level.

However, compared with genera mentioned above, a number of species of *Vorticellidae* have been studied using modern molecular methods combined with morphological characteristics to properly assess the evolutionary relationships of the species for future taxonomic revisions (Sun et al., 2013a; Wang et al., 2017). Although a certain number of *Vorticellidae* are free-living, most species are epibionts attached to various aquatic organisms. In some cases, some of these species contribute to the mortality of their host (Visse, 2007). A population of *Vorticellidae* was isolated from the freshwater atyid shrimps (*Neocaridina denticulate*) during a systematic survey of the ciliate epibionts of *Neocaridina denticulata* in Tainan, Taiwan. Based on morphological observations and phylogenetic analysis of the ITS1-5.8S-ITS2 region, we reported this species as a member of the *Vorticellidae* genus and assigned it the name *Vorticella aequilata*-like pop TW, with reference to the place where it was detected for the first time. To our best knowledge, it is the first molecular identification of the *Vorticellidae* species in the freshwater atyid shrimps in Taiwan.

2. Material and methods

2.1. Sample collection and identification

The *Vorticella* sp. population was isolated from the atyid shrimps (*Neocaridina denticulate*) in various aquarium stores in Tainan, Taiwan (Fig. 1). We collected the organisms and placed them into a petri dish containing distilled water, pure zooids were isolated by glass micropipettes under a dissecting microscope. Living colonies were observed by using a bright field and differential interference contrast (DIC) microscopy (Olympus MVX10, IX81, Japan) at magnifications between 200x and 1,000x. The morphological characteristics of *Vorticella aequilata*-like pop TW were measured at 600x and 1,000x magnification. Terminology and systematics followed those of Lynn (2008).

2.2. DNA extraction, polymerase chain reaction (PCR), and sequencing

Genomic DNA was extracted with a Genomic DNA Mini Kit (LabPrep, Taipei, Taiwan) according to the manufacturer's instructions. DNA was extracted from *Vorticella* sp. cells collected from the shrimps. Briefly, an abdominal segment with 500 µl phosphate-buffered saline (PBS) was centrifuged at 5000 g for 2 min to harvest the cells. The pellets were washed twice with 1 ml of PBS before resuspension in 100 µl sterile distilled water and 200 µl lysis buffer. The resulting suspension was incubated at 56 °C for 15 min. The lysate was supplemented 200 µl ethanol (95%), briefly vortexed, and centrifuged at 8000 *g* for 1 min. Suspensions were then centrifuged at 20,000 g for 3 min with a genomic DNA spin column. DNA was transferred to a new microfuge tube, and kept frozen until used.

The ITS1-5.8S-ITS2 region, and the 5′ end of the large subunit rRNA were amplified with the ITSF and ITSR primers (Yi et al., 2009). The ITS1-5.8S-ITS2 region was amplified using the ITS-forward (5′-GTTCC CCTTGAACGAGGAATTC-3′) and ITS-reverse (5′-TACTGATATGCTTAA GTTCAGCGG-3′) primers, which are complementary to conserved regions and encompass the 39 end of the SSU rRNA gene, the whole of the ITS1-5.8S-ITS2 region and the 59 end of the large subunit rRNA gene (Diggles and Adlard, 1997; Goggin and Murphy, 2000). The cycling parameters for the ITS1-5.8SITS2 sequence were as follows: 10 min of initial denaturation at 94 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, primer annealing for 30 s at 56 °C, and extension for 30 s at 72 °C; a final extension step was at 72 °C for 10 min. DNA-view stained PCR products separated on gel after electrophoresis in 1.2% agarose (DNA-view, BIOTOOLS CO., LTD, Taiwan) and purified using an EasyPure PCR/Gel extraction Kit (Bioman Scientific Co. Ltd, New Taipei City, Taiwan). Sequencing was performed by Tri-I Biotech, Inc (Tri-I Biotech Inc., New Taipei City, Taiwan).

2.3. Phylogenetic analyses

The SSU rRNA gene sequences of other peritrichs included in the phylogenetic analyses were obtained from GenBank. These include the ITS1-5.8S-ITS2 region sequences (with corresponding accession numbers) of: *Vorticella aequilata* Sc pop (KF524408), *Vorticella aequilata* Se pop (KF524372),V. *convallaria* Ak pop (KF524406), *V. convallaria* Fi pop (KF524407), *V. convallaria* Gz pop (KF524393), V. convallaria Ny pop (KF524404), *V. campanula* Ka pop (KF524426), *V. campanula* Qd pop (KF524418), *V. campanula* Wh pop (KF524421), *V. gracilis* Du pop (KF524409), *V. gracilis* Wh pop (KF524373), *V. nants* Ca pop (KF524383), *V. similis* Mh pop (KF524411), *V. similis* Qd pop (KF524416), *V.* sp18 (KF524424), *Vorticellides infusionum* (KF524368), *V. astyliformis* (GQ872427), *Opisthonecta minima* (KF524429), *O. henneguyi* (KF52430), *Zoothamnium arbuscula* (AF429897), *Z. nii* (EU340859), *Z. plumula* (EU340860), *Z. altemans* (EU340858), *Epistylis chrysemydis* (AF429887), *E. chrysemydis* (GU586187), *E. hentscheli* (AF429889), *E. hentscheli* strain 1 (GU586186), *E. plicatilis* (AF429890), *E. portoalegrensis* (KT358504), *E. galea* (AF429888), *Myoschiston duplicatum* (JN836353), *Myoschiston cf. duplicatum* (JN838354), *Campanella umbellaria* (GU586188), *Trichodina ruditapicis* (FJ499385), and *T._sinonovaculae* (FJ499386).

Maximum likelihood (ML) analysis was performed with PhyML 3.1 software (Guindon et al., 2010), starting from a BioNJ tree and performing an subtree-pruning-regrafting (SPR) branch-swapping (Guindon et al., 2010). For our data, the analysis of the ITS1-5.8S-ITS2 sequences of the peritrich ciliates was conducted with the best model (GTR + G) selected by the AIC criterion in Modeltest 3.7 (Posada and Crandall, 1998) with following parameters: nucleotide frequencies (A = 0.2337, C = 0.0.2883, G = 0.1576, T = 0.3587); substitution rate matrix ([AC] = 0.8947, [AG] = 0.7579, [AT] = 1.1473, [CG] = 0.7474, [CT] = 1.1368, [GT] = 1.0000); gamma distribution shape parameter = 0.5368. Furthermore, the best model for the ITS1-5.8S-ITS2 sequences of *Vorticellidae* was (GTR + G) with the following programmers: nucleotide frequencies ((A = 0.2337, C = 0.0.2883, G = 0.1576, T = 0.3587); substitution rate matrix ([AC] = 0.8947, [AG] = 0.7579, [AT] = 1.1473, [CG] = 0.7474, [CT] = 1.1368, [GT] = 1.0000); gamma distribution shape parameter = 0.486. Bootstrap percentages were obtained after 10000 replicates. Bayesian inference was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) using an evolutionary model as described above. The respective generations of MCMC of ITS1-5.8S-ITS2 sequences were 1,000,000 with sampling every100 generations; the first 25% of the states were discarded as burn-in. Trees were edited and annotated in MEGA 7 (Kumar et al., 2016). Identities of the sequences were calculated by GenBank BLAST, and the genetic distances were calculated using MEGA 7 (Kumar et al., 2016).

3. Results

3.1. Description

We observed that *Vorticella* sp. and *Scutariella* sp. attached to the

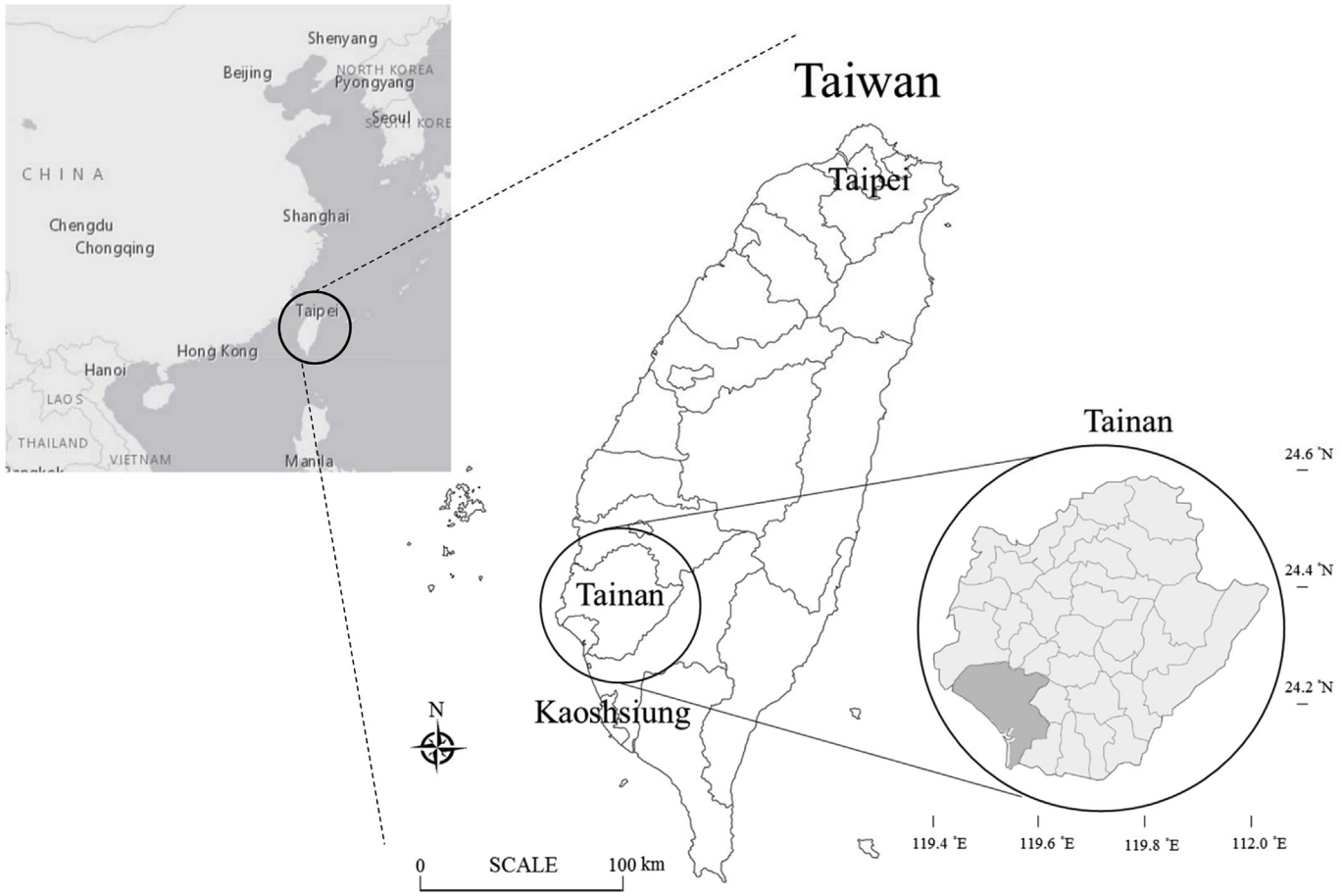

Fig. 1. Map of the Taiwan showing the location of the sampling site.

mantle of the freshwater atyid shrimps. *Vorticella* sp. was observed forming cluster structures on the 5th abdominal segment of the freshwater shrimps (*Neocaridina denticulate*) (Fig. 2) and *Scutariella* sp. was detected on the mantle, rostrum and antenna (Fig. 3). The symptoms of infected freshwater shrimps include loss of appetite and excessive stress or even death. The body of *Scutariella* sp. was 500–689 μm in length and 70–97 μm in width; average length to width ratio of fully extended organism was 6.8:1 (Table 1). In a recent study, *Neocaridina* shrimp were collected from 18 sites in five provinces in Southeastern China and most of them hosted at least one ectosymbiotic worm; the prevalence of *Scutariella* sp. ranged from 25.2 to 100% (Ohtaka et al., 2012). *Scutariella* sp. seem to be the most common shrimp parasite; it is a Temnocephalidan (an order of tubellarian flatworm) that typically lives in the mantle or gills of the shrimp, visible as a series of fingerlike appendages extending from the rostrum (Klotz et al., 2013). There are not many reports about *Vorticella* sp. infection in atyid shrimps. Thus, in this study, we focused on *Vorticella* sp. infection in this study.

Vorticella sp. has a single stalk up to 90 μm in height (Fig. 2). Stalk surface appears hollow with longitudinal striations that are visible at high magnifications. Stalk diameter ranges from 2 to 3 μm. Extended zooids are elongated and bell-shaped; the body is 48–63 μm in length and 29–37 μm in width and the widest part is at a single-layered thin peristomial lip, average length to width ratio of the fully extended zooid is 1.2:1 (Table 2). A number of food vacuoles of variable size were observed in the central part of the zooid (Fig. 2D). The zooid of *Vorticella* sp. responded to the approach of a predator with contraction of the stalk, under the protection of the abdominal segment of the freshwater shrimps.

3.2. Molecular analysis

DNA was extracted and isolated from *Vorticella* sp. population from the atyid shrimps. The following descriptions refer to the topology of the trees resulting from combined primary sequences and secondary structures of the ITS1-5.8S-ITS2 region. The ITS1-5.8S-ITS2 sequences collected from the *Vorticella* sp. population were identical; the sequence length was 360 bp and the GC content was 39% (Fig. S1). According to the results of the BLAST search for the ITS1-5.8S-ITS2 sequence in GenBank, the closest similar species was *Vorticella aequilata* (KF524408, 87% over 311 bp), which differs by 40 substitutions containing 1 gap. The Bayesian inference (BI) and maximum likelihood (ML) trees based on the ITS1-5.8S-ITS2 sequence are almost congruent with respect to topologies, and a consensus tree was reconstructed based on the BI trees. The results indicate that the species of Vorticellidae cluster in two separate clades: clade I consists of the major species of *Vorticella* and *Vorticella aequilata*-like pop TW; and clade II include *Vorticella astyliformis* (Fig. 4). Molecular phylogeny indicates the *Vorticella aequilata*-like pop TW belongs to the Vorticellidae family cluster.

To clearly identify the population isolated from the atyid shrimps, a phylogenetic tree of the *Vorticella* genus was constructed. *Vorticella* species cluster in two separate clades: the four subclades within clade I; subclade 1 consists of *V. gracilis*, *V. campanula* and *V. similis*; subclade 2 consists of *V. convallaria*; subclade 3 consists of *V.* sp18, *V. natans*, *V. aequilata* and *V. aequilata*-like pop TW; subclade 4 consists of *Opisthonecta minima*, *Opisthonecta henneguyi* and *V. infusionum*; and clade II consists of *V. astyliformis* (Fig. 5). The molecular phylogeny analysis reveals that *V. aequilata*-like pop TW clustered with *Vorticella aequilata* to subclade 3, which was in turn nested in the Vorticellidae family.

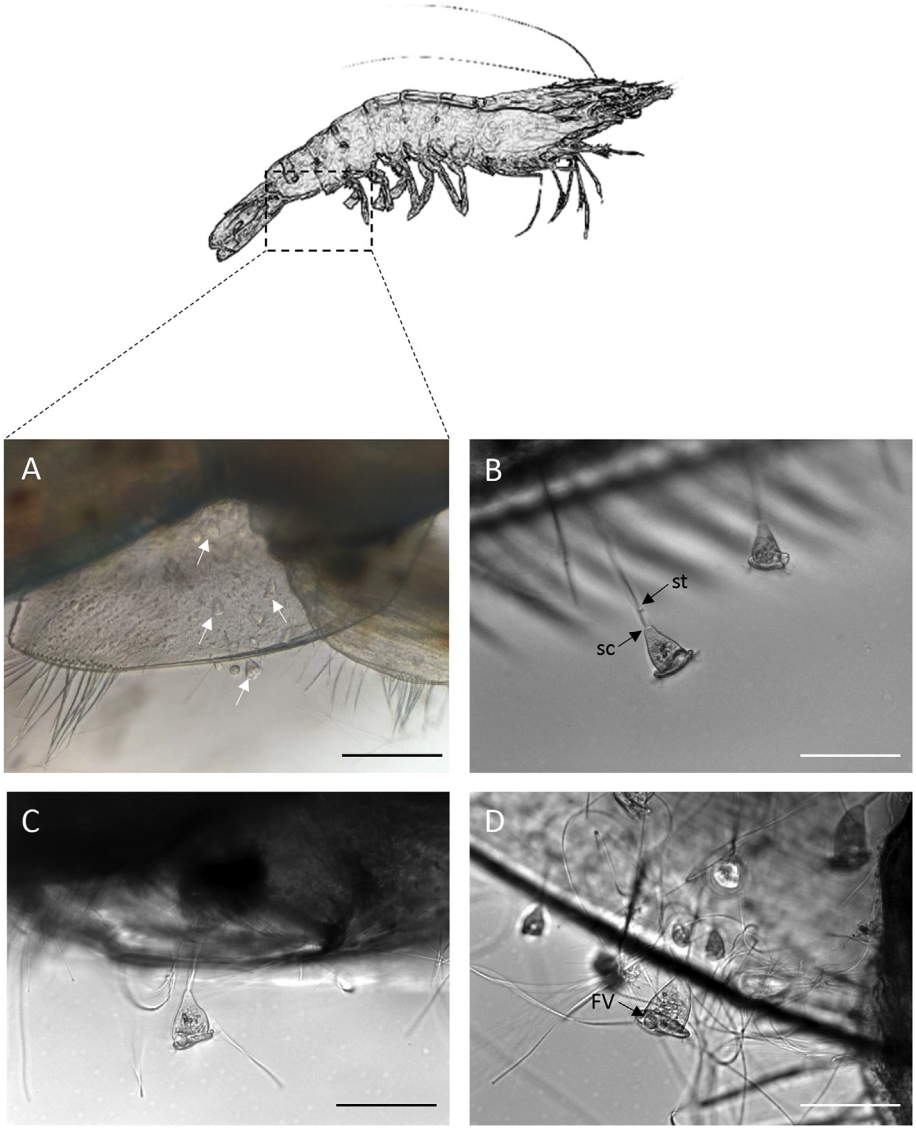

Fig. 2. *Vorticella aequilata*-like pop TW infected freshwater shrimps (*Neocaridina Denticulata*). (st) stalk. (sc) scopula. (FV) food vacuole. Scale bar: A = 500 μm; B-D = 100 μm.

4. Discussion

Freshwater shrimps are the most common crustaceans in an aquarium. The growing market of aquarium shrimps stimulates creation of new aquaculture farms suitable for producing mass quantities of low-cost crustaceans. Aquaculture ponds are usually exposed to the wind and rainwater as they do not have additional filtration; this results in high rates of epibionts found on the bodies of ornamental shrimps in Taiwan. These organisms affect shrimp wellbeing by causing distress which directly weakens of the shrimps, inducing loss of color and even death (Maciaszek et al., 2018). Protozoan parasites are one of the most important groups of pathogens that have a negative impact on the health of farmed and wild shrimps; however, they did not receive a lot of attention because of the technical difficulties inherent in their research compared to larger helminthic parasites (Lom and Dyková, 1992). Current studies of parasites in shrimps are mostly marine species (Chakraborti and Bandyapadhyay, 2011; Lightner and Redman, 1998). Due to the relatively low level of understanding of parasites in freshwater shrimp farms, the lack of effective treatment may lead to escalating problems.

Therefore, increasing our knowledge of protozoan parasites on shrimps will fill long-neglected scholarship gaps and is a prerequisite for rapid and accurate diagnosis of animal epidemics. Parasitic ciliates are one of the most pathogenic protozoa infecting fish (Van As, 1988). Although not generally considered as a problem in the wild, many species cause significant losses under intensive aquaculture conditions (Dickerson, 1996). Infections by sessile peritrichs, such as *Vorticella* and *Ambiphrya*, are common in many cultured fishes (Basson and Van As, 2006); however, thus far, there are no reports on molecular identification of parasites from the cultured freshwater atyid shrimps (*Neocaridina denticulata*) in Taiwan.

The present study explores parasitic fauna of the atyid shrimps in Tainan, Taiwan. We observed that *Vorticella* sp. and *Scutariella* sp. attach to the mantle, rostrum and antenna of the freshwater atyid shrimps. In 2015, Patoka et al. detected numerous protozoans, including *Vorticella* sp., *Caridinicola* sp., and bdelloid rotifer, associated with the atyid shrimps (Patoka et al., 2016). In a previous study, peritrichous ciliate ectosymbionts attached to the limbs and gills. A possible relationship between the peritrich ciliate, *Zoothamnium* sp., and the mortality of the hosts under stress was discussed previously (Overstreet, 1973). Widespread distribution of *Scutariella* sp. on *Neocaridina* spp. in southeastern China was described; the parasite, was

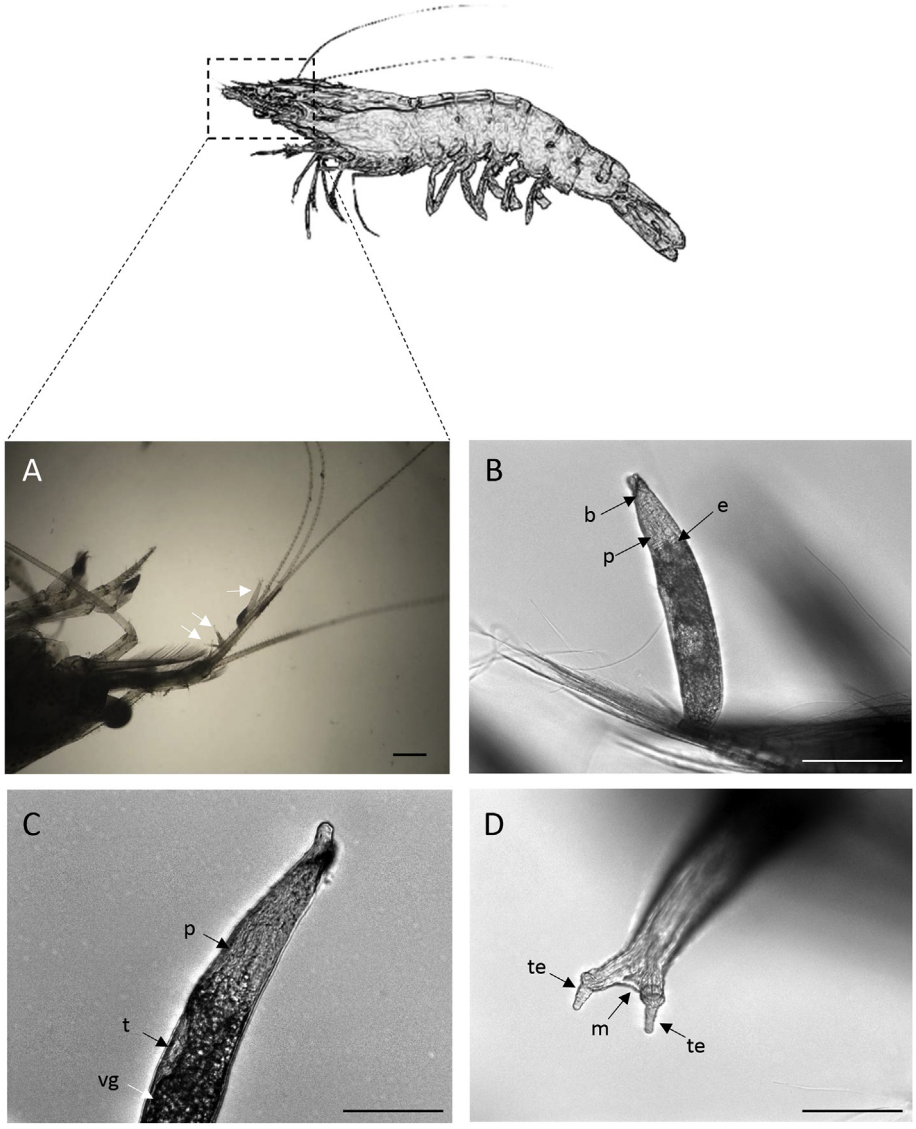

Fig. 3. *Scutariella* sp. infected freshwater shrimps (*Neocaridina Denticulata*). (b) brain. (e) eye. (m) mouth. (p) pharynx. (t) testis. (te) tentacle. (vg) vitelline glands. Scale bar: A = 500 μm; B, D = 200 μm; C = 100 μm.

Table 1
Morphometric data on *Scutariella* sp.; data are from specimens randomly selected in aquarium store of Tainan, Taiwan.

Characters	n	Min	Max	Mean	SD	CV
length	10	500.03	689.46	560.1389	67.83	9.58
width	10	70.25	96.24	82.97111	9.91	7.43

All measurements in μm. n, number of specimens measured; Min, minimum; Max, maximum; Mean, arithmetic mean; SD, standard deviation; CV, coefficient of variation in %; –, data not available; statistically significant different characters are marked with asterisks.

Table 2
Morphometric data on *Vorticella aequilata*-like pop TW; data are from specimens randomly selected in aquarium store of Tainan, Taiwan.

Characters	n	Min	Max	Mean	SD	CV
Zooid length	10	47.91	63.42	54.674	5.24	9.58
Zooid width	10	29.28	36.82	31.923	2.37	7.43
Peristomial disk width	10	40.32	58.48	46.419	5.67	12.21

All measurements in μm. n, number of specimens measured; Min, minimum; Max, maximum; Mean, arithmetic mean; SD, standard deviation; CV, coefficient of variation in %; –, data not available; statistically significant different characters are marked with asterisks.

quite mobile but usually stayed covered within the chamber (Ohtaka et al., 2012). The parasites may even cause casualties since they interfere with breathing and mobility of the host. A recent study showed that infections with peritrichous ciliate ectosymboints of the *Vorticella* sp. genus were frequently recorded in the tiger shrimp (*Penaeus monodon*) in India (Chakraborti and Bandyapadhyay, 2011). In this study, reported species clearly conform to the characteristics of the *Vorticella* sp. genus. The parasite can also affect the larvae and post-larvae of caridean shrimps by inhibiting their feeding activities and movement (Shailender et al., 2012). Therefore, the widespread occurrence of this peritrichous ciliate could be dangerous for the ornamental atyid shrimps produced in intensive aquaculture (Patoka et al., 2016).

Over past years, many freshwater shrimps from the Atyidae family native to Japan, Korea, Vietnam, China and Taiwan have been increasing in popularity in the aquarium industry (Antonio Baeza, 2010; Cai, 1996; Heerbrandt and Lin, 2006). Their small sizes, intensive coloration and large diversity of possible patterns make them valuable to breeders and are the reasons for the rising quantity of color varieties

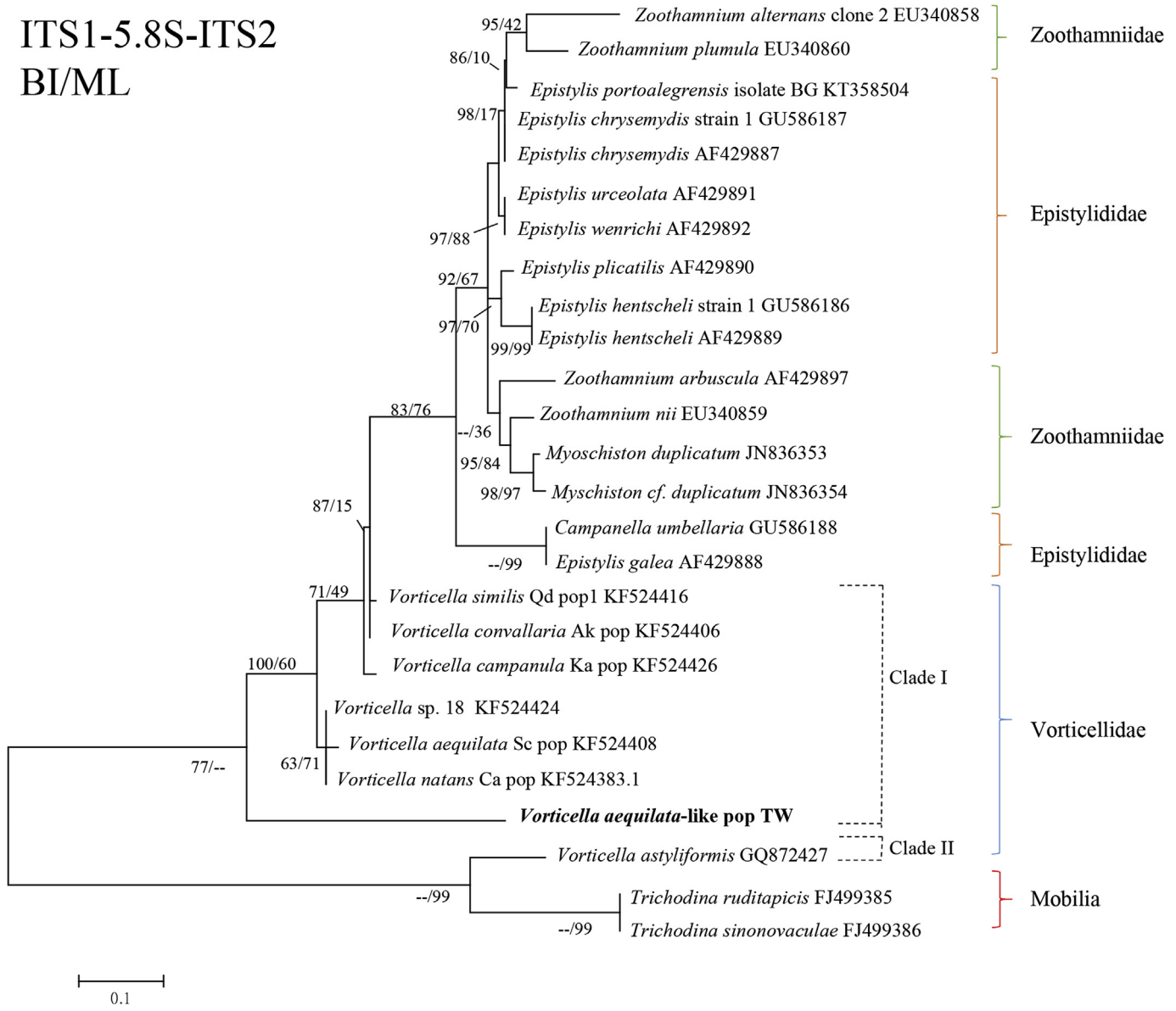

Fig. 4. Bayesian phylogenetic tree of peritrich ciliates constructed from the nuclear ITS1-5.8S-ITS2 sequences. Sequence investigated in this study is in bold. Numbers given at nodes of branches are the posterior probability (BI) and bootstrap (ML) values. The scale bar corresponds to 10 substitutions per 100 nucleotide positions. Classification follows that of Lynn (2008).

available in the global aquarium trade (Maciaszek et al., 2018). Atyid shrimps are not only enriching the appearance of the freshwater tanks but are also an excellent maintenance staff for cleaning of aquatic plants. In a previous study, Niwa and Ohtaka reported that live atyid shrimps imported to Japan from Korea and China were useful in recreational fishing as black rockfish (*Sebastes inermis*) and black sea bream (*Acanthopagrus schlegeli*) baits (Niwa, 2006). Taiwan is playing a leading role in the ornamental shrimp industry by producing six out of every 10 ornamental shrimps. In 2014, the export of ornamental aquarium shrimps from Taiwan reached US$4.72 million, which was mainly to China, Hong Kong, the United States and Singapore (Council of Agriculture, 2015). The export of ornamental aquarium shrimps is important for Taiwan. Despite their potential economic importance on ornamental shrimps, insufficient quantitative information is available on the pathogens, survival and effects of the parasites within these parameters. We anticipate that our study can help to identify the species of parasites that infected the atyid shrimps.

5. Conclusions

Our findings encourage further large-scale Vorticellidae molecular analysis surveys in Taiwan to identify more ciliate parasites present in the freshwater shrimp. Future molecular analysis surveys involving shrimp hosts should also include symptomatic individuals to further highlight the relationship between the ciliate parasites and disease and to provide additional information on the emerging consensus on *Vorticella* pathogenicity.

Conflicts of interest

None.

Acknowledgments

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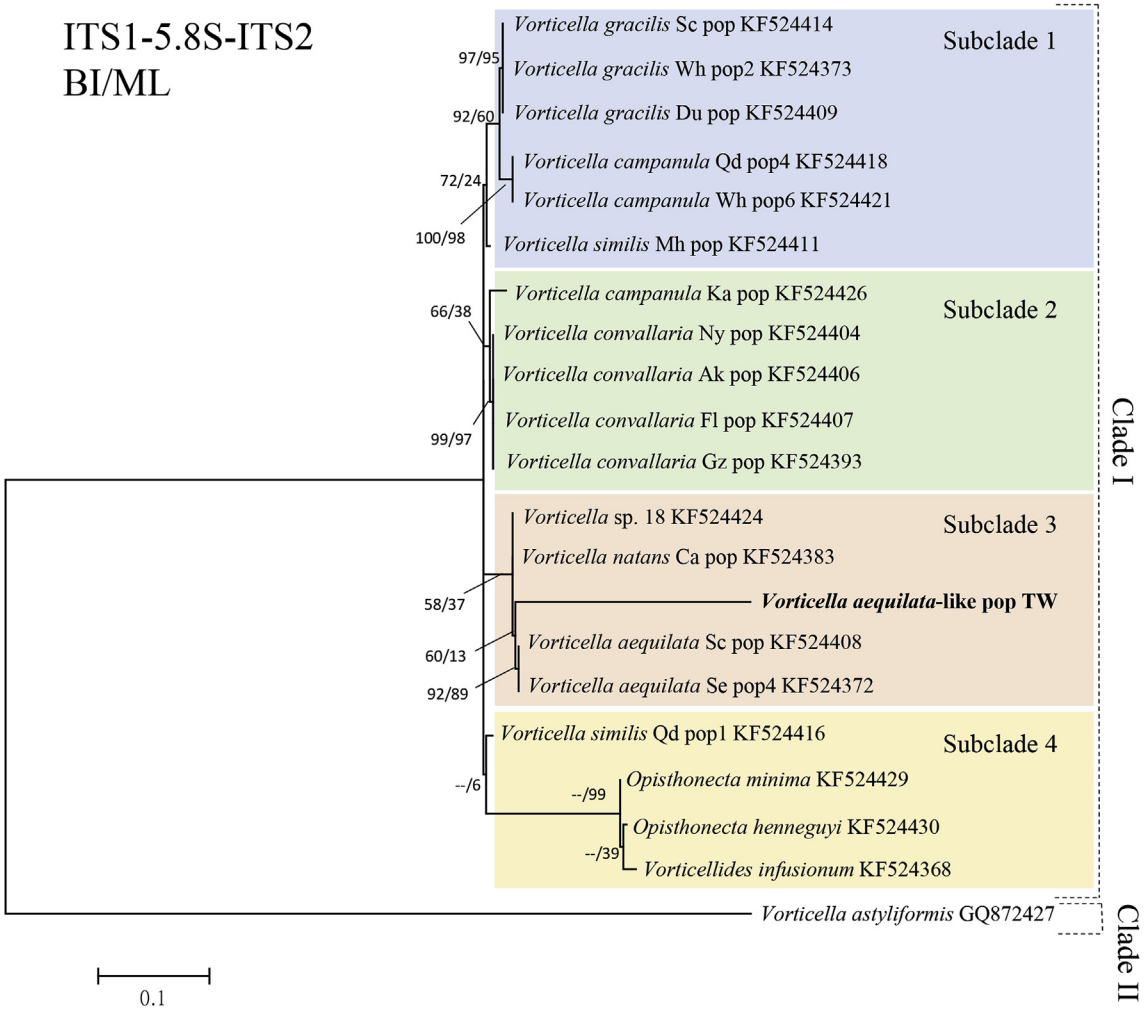

Fig. 5. Bayesian phylogenetic tree of *Vorticella* sp. constructed from the nuclear ITS1-5.8S-ITS2 sequences. Sequences investigated in this study is in bold. Numbers numbers given at nodes of branches are the posterior probability (BI) and bootstrap (ML) values. The scale bar corresponds to 10 substitutions per 100 nucleotide positions. Classification follows that of Lynn (2008).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2018.10.002.

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