Acta Oceanol. Sin., 2017, Vol. 36, No. 10, P. 115–119
DOI: 10.1007/s13131-017-1050-y
http://www.hyxb.org.cn
E-mail: hyxbe@263.net

Pathogenic bacterium *Vibrio harveyi*: an endosymbiont in the marine parasitic ciliate protozoan *Cryptocaryon irritans*

QIAO Ying[1, 2], WANG Jun[2], MAO Yong[2], LIU Min[2], CHEN Ruanni[2], SU Yongquan[1, 2*], KE Qiaozhen[1], HAN Kunhuang[1], ZHENG Weiqiang[1]

[1] State Key Laboratory of Breeding of Larimichthys crocea, Ningde 352103, China

[2] College of Ocean and Earth Sciences, Xiamen University, Xiamen 361102, China

Received 29 April 2016; accepted 3 August 2016

Abstract

Vibrio harveyi, known as a pathogenic bacterium caused severe secondary bacterial infections of the large yellow croaker *Larimichthys crocea*, was identified as an endosymbiont in the marine parasitic ciliate protozoan *Cryptocaryon irritans*. Meta 16S sequencing method was used to identify the bacterial flora in *C. irritans*, and *V. harveyi* was isolated via culture-dependent method. *Vibrio harveyi* was observed in cytoplasm of *C. irritans* at the stage of tomont both by transmission electron microscopy and by Fluorescence *in situ* hybridization; no signal, however, was detected in nucleus area. The relationship between *V. harveyi* and *C. irritans* and the role of endosymbiotic *V. harveyi* in *C. irritans* merit further investigation.

Key words: endosymbiotic bacterium, protozoan, *Vibrio harveyi*, *Cryptocaryon irritans*, Meta16S sequencing

Citation: Qiao Ying, Wang Jun, Mao Yong, Liu Min, Chen Ruanni, Su Yongquan, Ke Qiaozhen, Han Kunhuang, Zheng Weiqiang. 2017. Pathogenic bacterium *Vibrio harveyi*: an endosymbiont in the marine parasitic ciliate protozoan *Cryptocaryon irritans*. Acta Oceanologica Sinica, 36(10): 115–119, doi: 10.1007/s13131-017-1050-y

1 Introduction

Cryptocaryon irritans is an obligate parasitic ciliate protozoan which causes severe marine cryptocaryonosis (white spot disease), and results in huge economic losses in aquaculture sector of the large yellow croaker *Larimichthys crocea* in China (Sun et al., 2006; Luo et al., 2008). This parasitic ciliate protozoan undergoes four stages, including the theront (the infective stage), the trophont (the parasitic/feeding stage), the protomont (the pre-reproductive stage) and the tomont (the reproductive stage known as the cyst) (Colorni, 1987; Colorni and Diamant, 1993a; Burgess et al., 1994). Excessive amount of parasitic trophonts infect the underneath epithelium of skin and gills, and usually cause the asphyxiation, osmotic imbalance and secondary bacterial infections in fishes (Colorni, 1987; Diamant et al., 1991); the last was considered as a direct factor lead to the death of *L. crocea* (Liu et al., 2012).

Bacteria frequently colonize in the cells or on the cell surface of the ciliates. Intracellular bacteria are known as “symbionts” or “endosymbiont” (Preer et al., 1974), and this phenomenon widely spreads in various protistan groups. Characteristically, bacteria have two types of endosymbiosis: cytoplasmic endosymbiosis and intranuclear symbiosis. Relatively large ciliate cells were considered as a vast well-structured microcosm for bacterial immigrants, for protecting the endosymbiont from environmental influences and providing substances and energy which the endosymbiont required (Fokin, 2004). The vast majority of bacterial symbionts of ciliates comprise a variety of bacteria in many genera of different subgroups of proteobacteria. (Schweikert et al., 2013).

In the present study, we isolated *Vibrio harveyi* from *C. irritans*, inferred the possible endosymbiosis phenomenon. Furthermore the bacteria isolated from the skin ulcer of *C. irritans* infected *L. crocea* confirmed to be homology to the isolated endosymbiosis. However, the physiological relationship between *V. harveyi* and *C. irritans* is still not clear. Further studies are needed to clarify whether *V. harveyi* is a simply endosymbiont in the parasitic ciliate protozoan *C.irritans* or contributes to the growth and parasitism of *C. irritans*.

2 Materials and methods

2.1 *Cryptocaryon irritans*

Cryptocaryon irritans isolates were propagated by a serial passage on juveniles of *L. crocea* from Ningde aquaculture region (Fujian Province, China) as previously described (Dan et al., 2006). The tomonts of *C. irritans* were properly conserved in the sterilized seawater at 12 °C.

2.2 *Fish and seawater samples*

Uninfected individuals of *L. crocea* (n=3–5, weight (85.5±15.1) g, body length (19.2±1.3) cm) were reared in an aerated 150 L FRP-tank using sand-filtered seawater at 20 °C and fed daily with formulated diets for two weeks. Subsequently about 7 000 theronts per individual were added into the tank for *C. irritans* challenge. The experiment lasted for nine days in order to induce the secondary bacterial infection. Seawater was exchanged 50% twice a day. On the ninth day post challenge, infected individuals (n=3) were anesthetized with 200 mg/L MS-222, and the tomonts at the bottom of the tank were collected.

One liter of seawater before challenge (I.Water) and at the ninth day after challenge (F.Water) in the FRP-tank were filtered using 0.22 μm pvdf microporous membrane and stored at –80 °C

Foundation item: The National Natural Science Foundation of China under contract Nos 31372504, 41176115 and 41476118.
*Corresponding author, E-mail: yqsu@xmu.edu.cn

until the DNA isolation.

The tomonts were collected from the glass plates and incubated in 0.1% (v/v) hydrochloric acid for 24 h to kill the extracellular bacteria. The treated tomonts were washed three times using sterilized sand-filtered seawater (SSFW) and transferred to a 1.5 mL microcentrifuge tube. The tomonts collected were stored at –80 °C for the DNA isolation.

Swimming theronts were collected after hatched in the SSFW, washed three times in 50 mL SSFW by centrifugation at 3 500 r/min for 10 min, and then preserved in 1.5 mL centrifuge tubes for further analysis.

2.3 *DNA extraction and Meta 16s rRNA sequencing analysis*

Total genomic DNA from the tomont, I.Water and F.Water samples were extracted and amplified using bacteria barcoding primers (515F-806R) targeting the V4 region of 16S rRNA genes. Sequencing libraries were generated and sequenced on an Illumina MiSeq platform at Novogene Bioinformatics Technology Co. Ltd. (Beijing, China). The generated raw reads were qualitatively analyzed and taxonomically annotated via Mothur software (Schloss et al., 2009).

2.4 *Isolation and identification of the endosymbiotic bacteria*

In order to verify the results of Meta 16S rRNA sequencing, the tomonts collected were treated in 0.1% (v/v) hydrochloric acid to kill the extracellular bacteria and then ground with SSFW using aseptic grinding rod. The cell debris were plated on Thiosulfate citrate bile salts sucrose agar culture medium (TCBS), and then the hydrochloric acid solution which treated the tomonts was plated in the corresponding solid medium as the negative control. Plates for each preparation were incubated aerobically at 28 °C for 24 h. The bacterial isolates were identified and classified using 16S sequencing as Yoon J H described (Yoon et al., 1996). The amplified sequences of 16S rRNA gene were performed and identified using BLAST (http://blast.ncbi.nlm.nih.gov/BLAST).

2.5 *Bacteria isolated from infected individuals*

Bacteria were isolated from skin ulcer of the infected individuals using repeated plate streaking (Benediktsdóttir and Sigurjónsdóttir, 1998; Yin-Chun, 201). The culture and 16S rRNA identification methods as mentioned in Section 2.4.

2.6 *Ultrastructural observation*

The tomonts and theronts collected were fixed in 2.5% glutaraldehyde seawater at 4 °C for 24 h, washed in 0.15 mol/L PBS (pH 7.2) at 4 °C and postfixed in 1% osmium tetroxide for 1 h. Scanning electron microscope (SEM) and transmission electron microscopy (TEM) steps were subsequently operated with minor modification as Diggles BK described (Diggles, 1997).

2.7 *Probes and FISH*

Fluorescence *in situ* hybridization method (FISH) was adopted to analyze the distribution of the endosymbiotic *Vibrio* within the theront of *C. irritans*. The GAM42a-probe (Manz et al., 1992) was adopted for the FISH experiments which bonded a highly conserved region within the 23S rRNA gene of *Gammaproteobacteria*. The GAM42aN-probe was designed as the negative control to confirm the nonspecific binding. The probes were synthesized and fluorescently labeled at 5′- termini with Cy3 fluorophore (Invitrogen, Shanghai).

Approximately 4×10^3 theronts of *C. irritans* after prefixation treatement with 8.0% aqueous paraformaldehyde in 50 μL of SSFW were pipetted onto poly-L-lysine-treatedslides, and then follow the operation guide of the FISH Kit (EXONBIO, Guangzhou, China). As the theronts were incubated with the CY3-labelled GAM42a-probe and then poststained with DAPI solution, the slides were observed in a phase contract microscope.

3 Results

3.1 *Meta 16S rRNA sequence*

Meta 16S rRNA sequences revealed the distribution of environmental microorganism in *C. irritans* and seawater. In the tomont samples (TOMONT), 98.6% of the OTUs were annotated as the phylum Protobacteria; Protobacteria counted for 22.8% in I.Water and 11.2% in F.Water. In I.Water samples, 65.8% of the OUTs were annotated as the phylum Crenarchaeota, and 78.9% of the OUTs in F.Water samples were the phylum Bacteroidetes (Fig. 1).

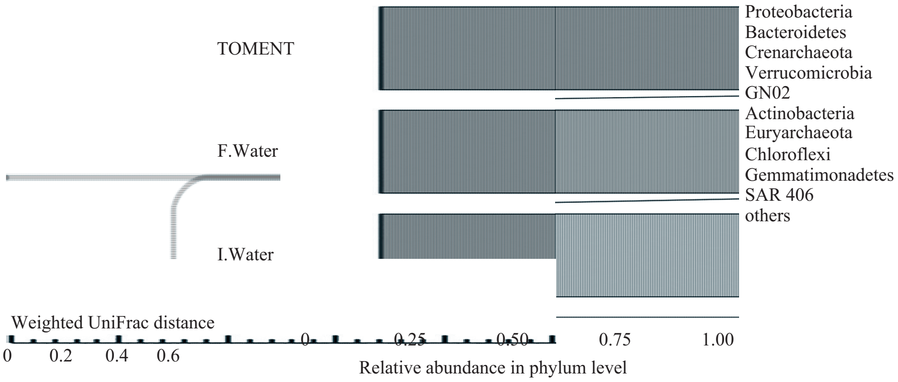

Fig. 1. UPGMA clustering tree based on the weighted UniFrac distance. The left is UPGMA clustering tree structure, and the right is the relative abundance at the phylum level.

At the family level, Vibrionaceae accounted for 66.8% in the tomont sample, and following Campylobacteraceae for 4.5%, Cohaesibacteriaceae for 2.0% and Flarobacteriaceae for 0.4%. In I.water sample, Cenarchaeaceae accounted for 65.8% and Saprospiraceae was 77.4% in F.Water sample (Fig. 2). As at the genus level, the most abundant genus existing in the tomont sample was *Aliivibrio* (34.5%) and following *Vibrio* (32.1%). In I.Water sample, *Nitrosopumilus* which belonged to Cenarchaeaceae accounted for 65.8% (Fig. 3).

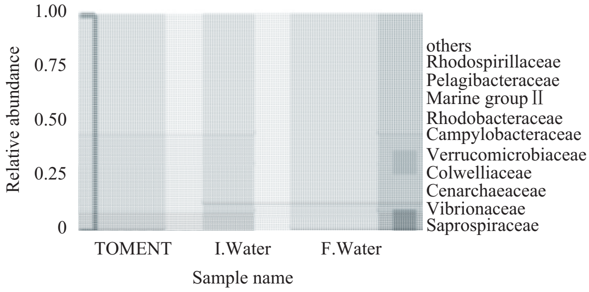

Fig. 2. Relative abundance of the bacteria at the family level in seawater and tomont.

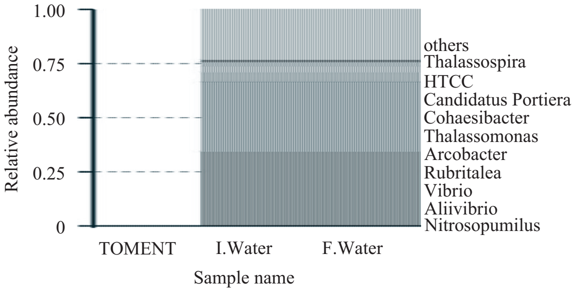

Fig. 3. Relative abundance of the bacteria in at the genus level.

3.2 *Identification and bioinformation of isolated bacteria*

The tomont lysates of *C. irritans* were plated on TCBS agar medium, incubated at 28 °C for 24 h. Green colonies were observed on TCBS agar; the bacterial isolates were Gram-negative with motile rods through gram stain and microscopic examination. Bacteria isolated from skin ulcer of infected individuals presented the same results on TCBS agar. The 16S sequencing result of the isolated bacteria confirmed the accuracy of Meta 16S RNA sequencing, the two isolates showed high sequence similarity with 16S rRNA genes from *V. harveyi*. The phylogenetic tree were constructed using MEGA6 with Minimum evolutionary distance algorithms, and the bacteria clusters isolated from the tomont and the infected individuals were most closely with *V. hareyi*, and less closely with *Aliivibrio* species (Fig. 4).

3.3 *In situ identification*

SEM clearly showed the outside details of the tomonts of *C. irritans* with many impurities and bacteria adhering to the outside cyst wall of the tomont (Fig. 5a), and a mass of bacteriform objects aggregated on the cyst wall (Figs 5b and c). TEM sections of the bacteria showed rod-shaped and oblong bacteria (Fig. 6), and the sections of the tomont also showed the rod-shaped or oblong endosymbionts throughout the cytoplasm of the ciliate (Fig. 6c).

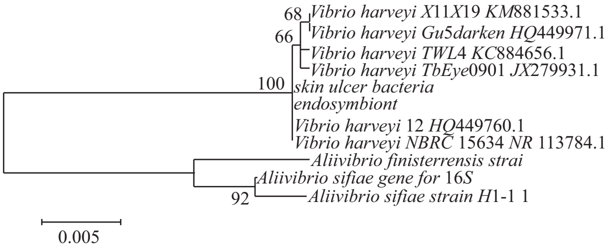

Fig. 4. Phylogenetic relationships of *Cryptocaryon irritans-Vibrio* endosymbionts. Minimum evolutionary distance trees were calculated using 16S rRNA gene sequences. Numbers at nodes are percentages of bootstrap values based on 1 000 samplings.

The theronts showed DAPI-positive, classic "four macronucleus and several micronucleus" of *C. irritans*, and the DAPI-stained endosymbionts were blurry (Fig. 7). As the theronts were incubated with the CY3-labelled GAM42a-probe and then poststained with DAPI solution, the DAPI staining nucleus area and the surrounding hybridization patterns were observed by fluorescence microscope. The micronucleus and macronucleus of theronts which took up almost the whole cell were strongly stained with DAPI. The *Vibrio*-positive endosymbionts were located near the anterior and posterior ends of the theront cells, and the nucleus area was surrounded by endosymbionts (Fig. 8). No positive signals were observed in the nucleus area, either in the micronucleus or in the macronucleus. No signal was detected in the theronts when using the GAM42aN-probe (not shown).

4 Discussion

The present study employed the sensitive Meta 16S sequencing method to examine the microbial flora of the aquaculture seawater and the marine parasite *C. irritans*. The most abundant bacteria in the seawater before fish culture (I.Water sample) was Cenarchaeaceae. In the seawater after fish culture and ciliate challenge, Saprospiraceae was the most, follow by Verrucomicrobiaceae. Saprospiraceae is considered as the organic disintegrator of the water environment, and Verrucomicrobiaceae is usually found in water and faeces of the animals. For the tomont of *C. irritans*, the most abundant bacterium was from Vibrionaceae, account for 66.8%, and followed by Altheromonadeles for 12.5%, Campylobacteraceae for 4.5%, Coheasibacteriaceae for 2.0% and Flarobacteriaceae for 0.4%. SEM of the tomont also revealed some unidentified bacteria adhered to the surface of the cyst wall (Fig. 5).

One strain of bacteria was isolated via plate culture method

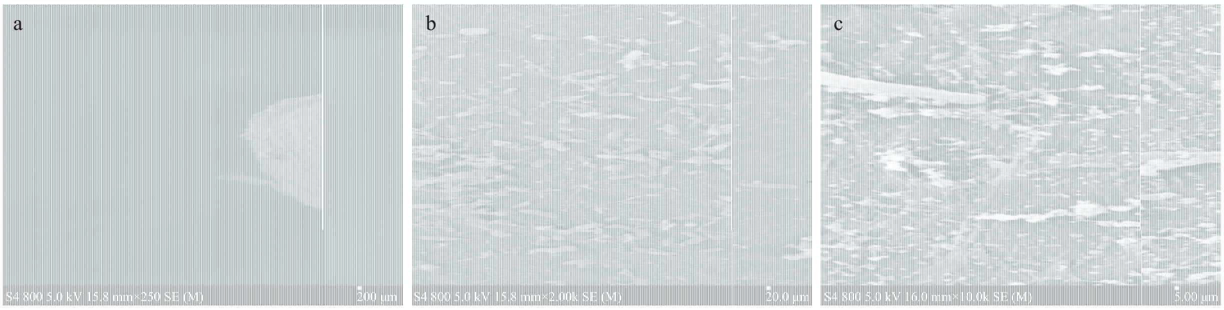

Fig. 5. SEM of the tomont of *Cryptocaryon irritans*. a. Cyst wall, noted the tomont appears devoid of any characteristic cortical structure, and b and c. higher resolution of the cyst wall. The arrows indicate the unknown rod-shaped bacteria.

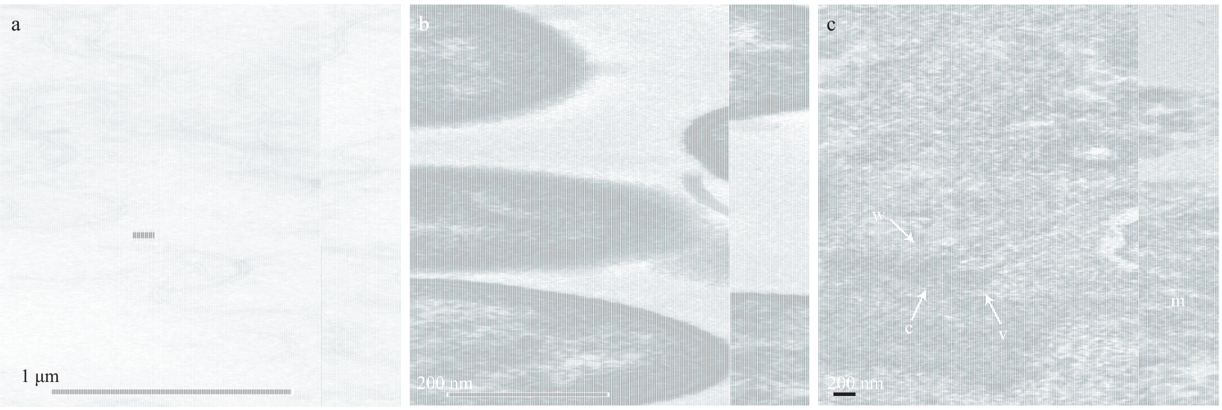

Fig. 6. TEM of endosymbionts isolated from the tomont of *Cryptocaryon irritans*. a. Endosymbionts using negative stained method (arrow indicates the flagellum of the endosymbionts), b. Endosymbionts via microtomy, and c. the tomont (v indicates the vacuole, w the cell wall, c the cell membrane, and m the mitochondrion).

Fig. 7. Fluorescence images of the theront of *Cryptocaryon irritans* stained with DAPI. a. The white light vision of the theront (arrow indicates macronucleus), b. DAPI-stained theront showing the macronucleus and micronucleus (blue, the arrow indicates macronucleus and the white triangles indicate the micronucleus), and c. merged image of Figs 7a and b.

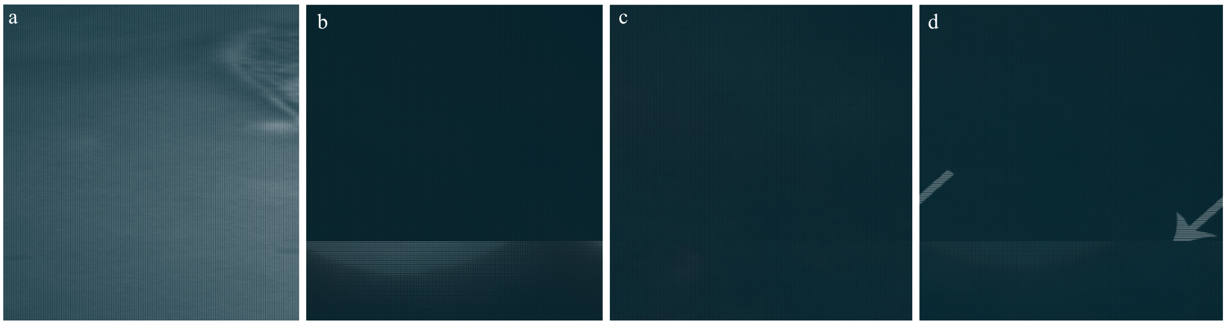

Fig. 8. FISH analysis of the theront of *Cryptocaryon irritans* labeled with GAM42a-probe and counterstained with DAPI. a. The white light vision of the theront, b. DAPI-stained theront showing the macronucleus and micronucleus (blue, the white arrow indicates macronucleus and the white triangles indicate the micronucleus), c. fluorescence image of the theront showing GAM42a-probe endosymbionts (red), and d. merged image of Figs b and c showing endosymbionts stained with red fluorescence (the white arrow indicates macronucleus and the white straight arrows GAM42a-labeled endosymbionts).

and identified as the pathogenic bacterium *V. harveyi* by 16S rRNA sequencing. The TEM (Fig. 6) and the FISH (Fig. 8) results demonstrated that the isolated *V. harveyi* was an endocytobionts of the ciliate *C. irritans*, and it distributed in the cytoplasm of the cell. Amir Saeed (Amir Saeed, 2007) also found that *Vibrio cholerae* grew in the cytoplasm of the free-living amoeba *Acanthamoeba castellanii*, showing an endosymbiont-host relationship. Nevertheless, we found that not all the tomonts or theronts of *C. irritans* were endosymbionts-carriers, and *V. harveyi* were detected only in a small amount of *C. irritans*. This revealed that *V. harveyi*-*C. irritans* endosymbiosis-relationship was not a common phenomenon.

The bacteria isolated from the fish skin ulcer was identified as the same species as the *V. harveyi* endosymbiont from the tomont of *C. irritans*. The two isolates have high sequence identity with 16S rRNA genes and clustered with some other *V. harveyi* strains (Fig. 4). The results suggested that *V. harveyi* which causing secondary bacterial infection on *L. crocea* may not only come from the water environment, but also from the infection of *C. irritans*. It is still not clear whether the endosymbionts play a role in the pathogenesis of *C. irritans* infections or if they affect the immune-response system of the infected *L. crocea*.

A greater majority of ciliates are heterotrophes, and their heterotrophic feeding is usually implemented via phagocytosis. The ciliates feed on bacteria, algae, fungi and even some smaller ciliates. All the feeding process is inevitably associated with a highly differentiated and complex cortical structure: cytostome (Verni and Gualtieri, 1997).

In *C. irritans*, only the trophont could feed on histocyte and tissue fluid cells of the hosts, such as *L. crocea*, using cytostome. The oral apparatus of the theront has not yet been fully developed and the theront is not feeding at this stage (Colorni and Diamant, 1993b), and revealed that the only source of intracellular endosymbiont is the phagocytosis process of the trophont at the infected site and the skin ulcer area. *Vibrio harveyi* from water environment or the surface of the theront infected *L. crocea* and caused secondary bacterial infections, suggesting the latter led to the death of *L. crocea*. The parasitic trophont then feeds on the *Vibrio harveyi* infected tissues, and swallows *V. harveyi* as its endosymbiont. Whether the regression infection could occur causing by the endosymbiont *V. harveyi* is still unknown.

Though these endosymbiosis phenomena were widely observed in ciliates, either interactions or relationships between partners in ciliophoran symbiotic systems have not yet been investigated in detail. The physiological relationship between *V. harveyi* and *C. irritans* is still not understood. Whether the endosymbionts play a critical role in growth or parasitic process of *C. irritans* or if they have some pathogenic effects on *L. crocea* are still need further studied.

Acknowledgements

The authors thank the State Key Laboratory of Breeding of *Larimichthys crocea* (Ningde, Fujian Province, China) for providing the experimental fish and equipment.

References

Benediktsdóttir, Helgason, Sigurjónsdóttir. 1998. *Vibrio* spp. isolated from salmonids with shallow skin lesions and reared at low temperature. J Fish Dis, 21(1): 19–28

Burgess P J, Matthews R A. 1994. *Cryptocaryon irritans* (Ciliophora): photoperiod and transmission in marine fish. J Mar Biol Assoc U K, 74: 535–542

Colorni A. 1987. Biology of *Cryptocaryon irritans* and strategies for its control. Aquaculture, 67(1–2): 236–237

Colorni A, Diamant A. 1993. Ultrastructural features of *Cryptocaryon irritans*, a ciliate parasite of marine fish. Eur J Protistol, 29(4): 425–434

Dan X M, Li A X, Lin X T, et al. 2006. A standardized method to propagate *Cryptocaryon irritans* on a susceptible host pompano *Trachinotus ovatus*. Aquaculture, 258(1–4): 127–133

Diamant A, Issar G, Colorni A, et al. 1991. A pathogenic Cryptocaryon-like ciliate from the Mediterranean Sea. Bull Eur Assoc Fish Pathol, 11(3): 122–124

Diggles B K. 1997. Some information on the morphology of *Cryptocaryon irritans* from South-East Queensland, Australia. Eur J Protistol, 33(2): 200–210

Fokin S I. 2004. Bacterial endocytobionts of ciliophora and their interactions with the host Cell. Int Rev Cytol, 236: 181–249

Liu Zhenyong, Lin Xiaojin, Xie Youquan, et al. 2012. Research on death caused by secondary bacterial infection of *Cryptocaryon irritans* on *Pseudosciaena crocea*. J Fujian Fish (in Chinese), 34(1): 11–15

Luo X C, Xie M Q, Zhu X Q, et al. 2008. Some characteristics of host-parasite relationship for *Cryptocaryon irritans* isolated from South China. Parasitol Res, 102(6): 1269–1275

Manz W, Amann R, Ludwig W, et al. 1992. phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst Appl Microbiol, 15(4): 593–600

Preer J R Jr, Preer L B, Jurand A, et al. 1974. Kappa and other endosymbionts in *Paramecium aurelia*. Bacteriol Rev, 38(2): 113–163

Saeed A, Abd H, Edvinsson B, et al. 2007. *Vibrio cholerae-Acanthamoeba castellanii* interaction showing endosymbiont-host relation. Symbiosis, 44(1-3): 153–158

Schloss P D, Westcott S L, Ryabin T, et al. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol, 75(23): 7537–7541

Schweikert M, Fujishima M, Görtz H D. 2013. Symbiotic Associations Between Ciliates and Prokaryotes. Berlin Heidelberg: Springer, 427–463

Sun H Y, Zhu X Q, Xie M Q, et al. 2006. Characterization of *Cryptocaryon irritans* isolates from marine fishes in Mainland China by ITS ribosomal DNA sequences. Parasitol Res, 99(2): 160–166

Verni F, Gualtieri P. 1997. Feeding behaviour in ciliated protists. Micron, 28(6): 487–504

Yoon J H, Lee S T, Shin Y K, et al. 1996. Rapid identification of *Saccharomonospora strains* by multiplex PCR using species-specific primers within the 16S rRNA gene. J Microbiol Methods, 27(1): 89–95

Yu Yinchun, Han Yanmei, Wang Xinyi, et al. 2015. Identification of pathogenic from *Carassius auratus* triploid with skin bleeding and ulcers and its prevention of drug screening. J Agric Catastrophol, 5(3): 28–30, 62