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## Histopathological study of lymphocystis disease virus (LCDV) in cultured false clownfish, *Amphiprion ocellaris* (Cuvier, 1830) and true clownfish, *Amphiprion percula* (Lacepede, 1802)

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### PEER REVIEW

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#### Comments

This is an important research work in which authors have studied on the lymphocystis disease in the cultured clownfish. In addition, the results of the present basic research could be helpful for further study in molecular aspects.

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### ABSTRACT

**Objective:** To study the lymphocystis disease virus (LCDV) in two species of marine ornamental fishes through histopathological investigation along with control for differentiating the tissue damage.

**Methods:** Six naturally infected fishes were collected. They were anaesthetized and subsequently killed by organ dissection. The infected fish organs were aseptically cut off and stored with 10% formalin solution for histological study. Samples were examined for gross pathology including location, distribution, shape, size, colour, consistency and special features of typical external lesions by standard method.

**Results:** The diameter of the tumor nodules ranged from 1 to 2 mm (*Amphiprion ocellaris*) and 2.5 to 3.5 mm (*Amphiprion percula*). Light microscopic observation showed over growth of tumor like nodules on the skin or scales and ventral side as well. Numerous hypertrophied cells with basophilic intracytoplasmic inclusion bodies were in the connective tissues of dermis and between scales on two species. The nucleus of lymphocystis cell were enlarged, irregular and containing basophilic margined chromatin. Thus, the similarities among cellular patterns of different fish LCDV isolates from different hosts indicated that these profiles do not depend on the host species.

**Conclusions:** In conclusion, this current study of histopathological statement of LCDV may be adequate for a presumptive diagnosis of lymphocystis disease from both marine as well as fresh water fish species. The findings of asymptomatic carriers by histology using infected skin and fin sampling, which does not imply animal killing, could be important tool to epizootics caused by LCDV. This study may be very useful for further molecular studies.

### KEYWORDS

Clownfish, *Amphiprion ocellaris*, *Amphiprion percula*, Lymphocystis disease virus, Histopathology

## 1. Introduction

Wabnitz *et al.* stated that Anemonefishes are one of the most popular attractions world-wide, due to their adaptability to live in confinement. In the last two decades,

marine aquarium fish trade has been witnessing continuous steady growth, involving major movements of wild reef fishes all over the world<sup>[1]</sup>. Allen *et al.* demonstrated that among the coral associated fishes, clownfishes belonging to the family, Pomacentridae and subfamily Amphiprioninae

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are abundant and about 30 species have been recognized under two genera, *Amphiprion* and *Premnas*[2]. These fishes have some remarkable behavioural characteristics such as symbiotic association with sea anemones[3], formation of a group consisting monogamous pairs and protandrous hermaphrodites[4]. Indeed, Ignatius *et al.* stated that their adaptability to live in captivity, easiness to be fed with artificial diets and their fascinating display behaviour and symbiotic relationship with the sea anemone are the special features[5].

The false clown [*Amphiprion ocellaris* (*A. ocellaris*)] and true clown [*Amphiprion ocellaris* (*A. percula*)] anemonefishes were tropical marine fishes frequently found in Indian Ocean (Andaman Nicobar Islands). They habited coral reefs and sheltered lagoons up to a depth of 20 m. Usually false clown anemone fish appearing in orange to reddish–brown colour with three white bands on the head, body and caudal peduncle. The white bands are outlined in black. The outer margin of fins is white and the inner one is black. The true clown anemonefish can be recognized by three white bands across their bright orange body, with no distinction in colour between sexes. The anterior white band is placed just behind the eye, the middle band goes straight down the middle of the fish and the posterior band occurs near the caudal peduncle. In addition to the white colouring, black edging outlines each fin with varying thickness.

In study of Heppel and Berthiaume, lymphocystis viruses have been implicated as the cause of severe disease, mortality and economic loss in farmed fish and ornamental fish in wild as well as hatcheries. Earlier researches have shown that the genera *Ranavirus*, *Iridovirus* and lymphocystis virus (which is a large virus in this family) include structurally related viruses, all of them are composed of similar protein united which contribute to the icosahedral outline structure[6]. Kitamura *et al.* stated that the first iridovirus disease was described in fish as lymphocystis disease virus (LCDV) which infects a great of fresh water and marine species. LCDV is an icosahedral symmetry virus, approximately 200–300 nm in diameter[7] and LCDV usually appears as a cluster of white or cream coloured warts or flashy growths erupting from the skin or fin of a walleye. The disease has also been found to infect organs that was quite rare. The distribution of LCDV has been reported worldwide such as in Spain[8], France[9], Korea, Japan[10] and China[11].

Mosharrof Hossain and Myung–Joo Oh explained that the virus released into the water when growth on an infected fish ruptures. The virus can infect healthy walleye by entering skin abrasions or cuts and then attacking cells in the connection tissue[12], and this may influenced by some of the factors as water contamination[13], and stress condition caused high population density, nutrition deficiencies,

decreased dissolved oxygen, suboptimal water quality. Also, human manipulation may increase the appearance of LCDV symptoms[8,14–16]. Cano *et al.* reported that *Artemia* sp. might act as a reservoir host of this disease[16]. So, to control the viral diseases, it is important to know the viral dynamics and ecological niches as well as host fish. LCDV most frequently makes an out breaks to the skin and fins of fish and causes economic impacts to the farmers because diseased unsightly fish would not be sold[12], and the LCDV disease shows the impact of eternal growth of tumor likes appearance. Histopathology, lymphocystis disease was characterized by cytomegaly of dermal fibroblasts cells and only rarely has systemic involvement[17–19].

Although in recent years great advances have been made in LCDV studies, the molecular mechanism, virus ecology, replication, spreading and pathogenesis are not clearly understood. The diagnosis of LCDV has been mainly based on the observation of symptoms from the article of Mosharrof Hossain and Myung–Joo Oh[12]. Therefore, the present research aims to study the LCDV in two species of marine ornamental fishes through histopathological investigation along with control for differentiating the tissue damage.

## 2. Materials and methods

### 2.1. Fish sampling

Six infected fishes were collected from ornamental fish hatchery CAS in Marine Biology, Annamalai University, Parangipettai with average total length of 2.5 cm of both species (*A. ocellaris* and *A. percula*). They were transferred into laboratory. The live fishes were anaesthetized and subsequently killed by organ dissection. The infected fish organs were aseptically cut off for histological study.

### 2.2. Sample preparation for histological study

All fish samples were examined for gross pathology including location, distribution, shape, size, colour, consistency and special features of typical external lesions by following method. Lymphocystis tumor tissue were fixed with 10% buffered formalin solution for hematoxylin and eosin and later fixed in 2.5% glutaraldehyde in phosphate buffer saline (0.1 mol/L 1–1, pH 7.4) for 2 h at 4 °C for ultrathin section. Then tissues were post fixed in 1% osmium tetroxide in phosphate buffer saline for 1 h at 4 °C. After fixation sample were dehydrated in ascending ethyl alcohol grade series followed by infiltration and embedded in post fixed Epon–812 epoxy resin according to standard procedures. The section samples were stained with 1% toluidine and methylene blue and observed under ultra–microscopy[13].

### 3. Result

In common LCDV prefers replicate in dermal fibroblasts, resulting in hypertrophied cells and abnormal growth of normal tissue in the outer skin of the fish as white colour nodules (tumor) can easily be detected by necked eye. The primary infection of LCDV was observed in fins and behaviour, food and feeding habits were normal after 20 d of infection. The separated fishes from tanks were used for further studies.

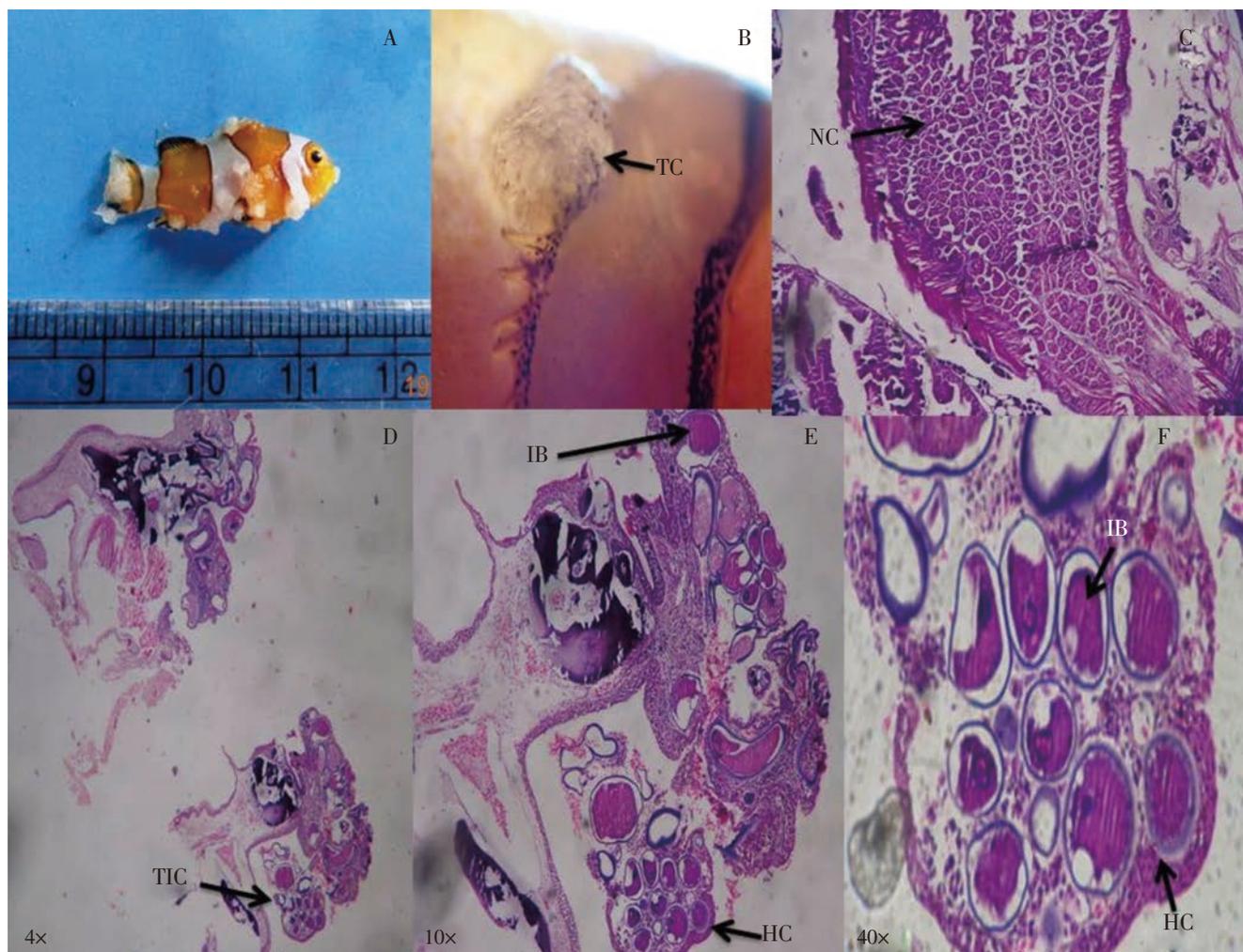
#### 3.1. Macroscopic and microscopic observations

Lymphocystosis disease infected fishes showed multifocal to diffuse white, round, firm, papilloma or tumor like nodules on the skin of the body, fins, eyes and mouth in both species (*A. ocellaris* and *A. percula*). The diameter of the nodules was ranged from 1 to 2 mm in *A. ocellaris* (Figure 1a) and 2.5 to 3.5 mm in *A. percula* (Figure 2a). Most of the fishes were affected at the time of metamorphosis. Light microscopic observation showed over growth of tumor like nodules on the skin or

scales (Figure 1b) as well as ventral side of the fishes (Figure 2b).

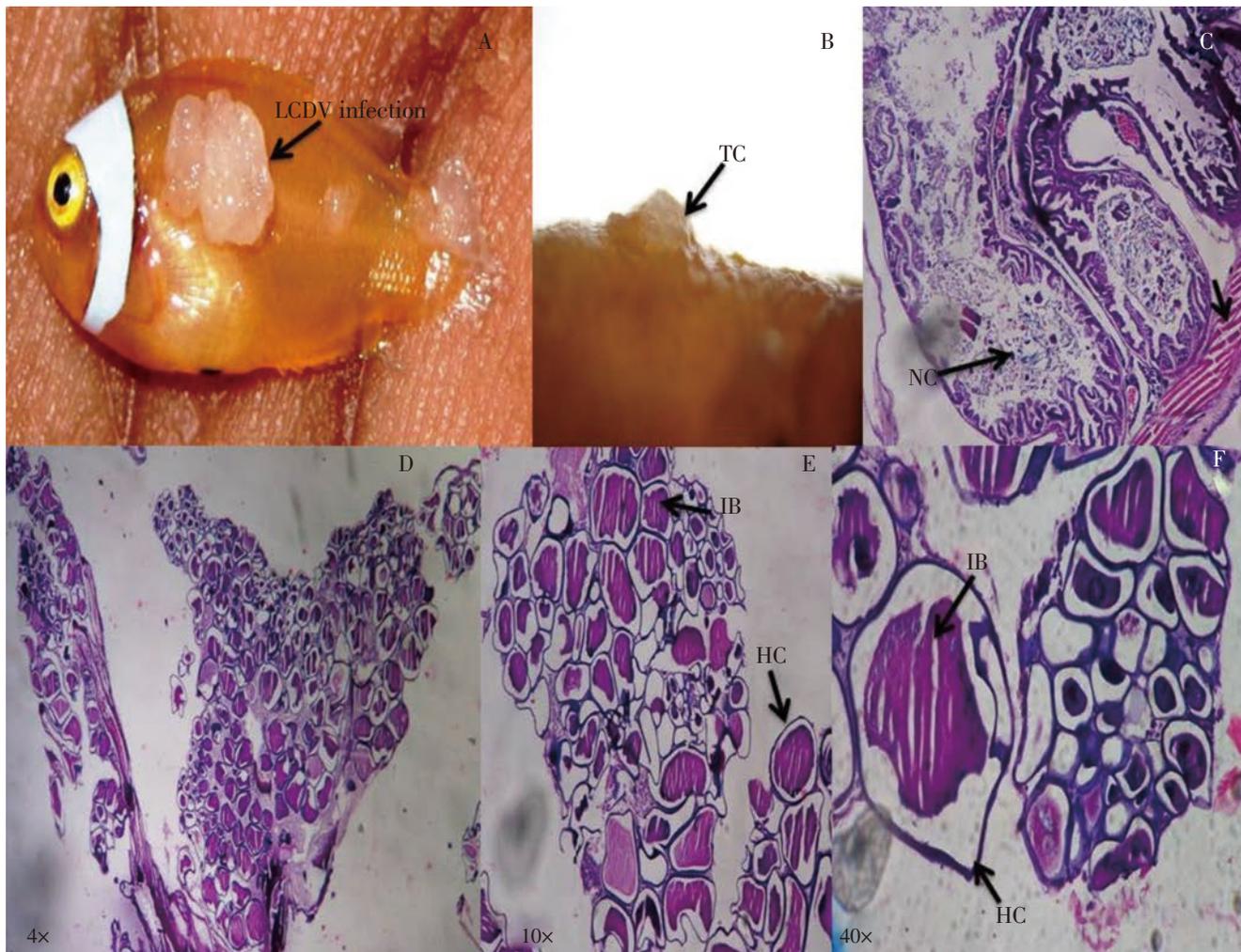
#### 3.2. Histological observations

Many clusters of lymphocystis cells were obtained in the connective tissues of the epidermis at fins and skin. Numerous hypertrophied cells with basophilic intracytoplasmic inclusion bodies were in the connective tissues of dermis and between scales on two species. The lymphocystis hypertrophied cell, was surrounded by a thick smooth hyaline capsule. The nucleus of lymphocystis cell was enlarged, irregular and containing basophilic marginated chromatin. In clown fishes the LCDV cells were irregular, round and connected with each other and some occlusion showed bodies lack of nucleus. Histology results showed that compared to uninfected cells *A. ocellaris* (Figure 1c) and *A. percula* (Figure 2c) infected cell contains irregular shaped cells which were more dominant in *A. percula* compared to *A. ocellaris*. Different magnifications (4×, 10× and 40×) showed clear structure prediction of lymphocystis cells present in the samples.



**Figure 1.** Macroscopic and microscopic view of LDCV infected true clown *A. ocellaris*.

A: Lymphocystis disease infected fish true clown *A. ocellaris* with the range of 2–3 mm in diameter; B: Microscopic figure of tumor infection at pectoral fin; C: Control shows uninfected cells; D: Microscopic structure (4×) of LCDV shows occlusion bodies; E and F: Microscopic structure (10× and 40×) of LCDV shows irregular, many numerous hypertrophied cells (HC) with basophilic intracytoplasmic (IB) inclusion bodies.



**Figure 2.** Macroscopic and microscopic view of LCDV infected true clown *A. percula*.

A: Lymphocystis disease infected fish true clown *A. percula* with the range of 2–4 mm in diameter; B: Microscopic figure of tumor infection in fish ventral side; C: Control shows uninfected cells; D: Microscopic structure (4x) of LCDV shows occlusion bodies; E and F: Microscopic structure (10x and 40x) of LCDV shows irregular, many numerous hypertrophied cells (HC) with basophilic intracytoplasmic (IB) inclusion bodies.

#### 4. Discussion

Mosharraf Hossain and Myung–Joo Oh explained that the virus can infect healthy walleye by entering skin abrasions or cuts and then attacking cells in the connection tissue[12]. Similar observation was observed from the infection of other normal fishes in the same tank. Some of the factors such as water contamination[13], stress condition caused high population density, nutrition deficiencies, decreased dissolved oxygen, suboptimal water quality, or human manipulation may increase the appearance of LCDV symptoms[8,14–16]. LCDV is an opportunity pathogen. When fish undergo stress or injured during fitting by other fishes, the LCDV become more dominant in the tank and it usually prefers true and false clown anemone fishes. Cano *et al.* reported that *Artemia* sp. might act as a reservoir host of this disease[16]. But in the case of current study LCDV was observed after fully metamorphosed form of juveniles; during larva rearing no such disease was observed in ornamental aquaculture. So, it may be the water quality or feed which induce the disease. The virus released into the water when

growths on an infected fish rupture.

The findings in ultra–thin sections of infected cells consensused well with those of previous investigations[20–23]. In addition, they showed more detail of the virus shell and possibly a morphological substrate for the osmiophilia and the other–sensitivity of the virus[18,24]. According to Mosharraf *et al.*, LCDV has two groups: marine and fresh water isolates depending on virus protein profiles which are expensive and it takes long time to detect LCDV[25]. However skin or fin biopsies for histopathology provide a definitive diagnosis of lymphocystis disease which is very easy because tumor cells were totally differentiated by cross pathology. Mosharraf Hossain and Myung–Joo Oh stated that the lymphocystis cells in marine and fresh water fishes as over folded and invaginated, multi–lobular state, inclusion body and hyaline capsules were predominant. Fibroblasts that were infected with *Lymphocystivirus* were continually enlarged or hypertrophy but do not undergo mitosis[12].

Nipodon Pirarat *et al.* explained that the LCDV were detectable not only in the skeletal muscle, gills lamellae but also in visceral organs including spleen, head and

trunk kidney<sup>[23]</sup>. The present study showed the presence of LCDV in the skeletal muscle, gill lamella. The cytoplasm of lymphocystis cells were changed, developing basophilic, intracytoplasmic inclusion bodies that appeared as dense vacuolated bodies. In addition, a thick hyaline capsule surrounding the hypertrophied fibroblast was observed in the cytoplasm, especially in the mature lymphocystis cells observed by Mosharraf Hossain and Myung–Joo Oh<sup>[12]</sup>. Similar observation was observed in the histopathological result of the current study. Nipodon Pirarat *et al.* stated that the senile lymphocystis disease cells became an irregular and broken hyaline capsule. Their nuclei disappeared and cytoplasm were released partly or totally. Similarly, the present study found this was the main reason for spreading LCDV disease to other fishes during culture in aquarium<sup>[23]</sup>.

The previous reports demonstrated the presence of LCDV in cultured false clown fish and the macroscopic finding was similar to the common character of LCDV infection in fish such as infected *Sarcocentrum rubrum* and *Chanda ranga*<sup>[21,26]</sup>. The present study showed the presence of the LCDV by histological findings in cultured anemone fishes of false clown and true clown. To our knowledge this was the first time to observe LCDV in true clown (*A. percula*) anemone fish in captivity. Infection showed same occlusion bodies present in false clown. It resulted that both species consisted of the same pattern of physical appearance. The hosts were frequently affected by lymphocystis disease that occurred in the fins and skin only. This result hypothesized that the lymphocystis disease virus was organ specific and multiply only in the fibroblast cells<sup>[12]</sup>. However in case of present study, it showed different result, LCDV was affected mouth, skeletal muscle, gills lamellae and Nipodon Pirarat *et al.* found affected visceral organs including spleen, head and trunk kidney<sup>[23]</sup>.

According to Mosharraf Hossain and Myung–Joo Oh's observation the ultra–microtome photographs of LCDV shows about 250 nm in diameter within the peri–nuclear cisterna and membrane–enveloped inclusions scattered in the cytoplasm but not in the nucleus<sup>[12]</sup>. The LCDV propagation in the cells has been detected by several methods like PCR, immunoblot or cytometry by other researchers<sup>[16,27,28]</sup>. LCDV will become important because the new group of fishes is affecting, both in marine and fresh water. Although, the fish have different aquatic environment in this study, in marine two different types of species affected by the same virus have been documented by histopathology. These fishes showed the same patterns of viral infected hypertrophied cells in the infected fish. All the figures showed a common cellular pattern of infection in the skin and fins which were fibroblastic in nature. Thus, the similarities among cellular patterns of different fish LCDV isolates from different hosts

indicates that these profiles do not depend on the host species.

In conclusion, this current study of histopathological statement of LCDV may be adequate for a presumptive diagnosis of lymphocystis disease from both marine as well as fresh water fish species. The findings of asymptomatic carriers by histology using infected skin and fin sampling, which does not imply animal killing, could be important tool to epizootics caused by LCDV. This study may be very useful for further molecular studies.

### Conflict of interest statement

We declare that we have no conflict of interest.

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### Comments

#### Background

Clownfish are having good trade value due to their attractive colour and their swimming behavior. But, lymphocystis is a major problem to maintain the fish in captivity. So, there is need to study on the host microbes interaction and overcome this problem.

#### Research frontiers

The present research work was paying attention on the LCDV infection using histopathological investigation.

#### Related reports

Heppel and Berthiaume (1992), Kitamura *et al.*, (2006) and Mosharro and Myung (2011) have conducted various experiments on the LCDV histopathologically.

#### Innovations and breakthroughs

The authors documented lymphocystis disease in the clownfish culturing facility. Number of reports are available on the lymphocystis disease in fresh water fish, but few reports are available in marine fish. So, the study is fine documentation for the pathogenicity of lymphocystis disease of clownfish.

### Applications

Histopathological investigation of the lymphocystis disease will lead to a better understanding of the host–virus interaction.

### Peer review

This is an important research work in which authors have studied on the lymphocystis disease in the cultured clownfish. In addition, the results of the present basic research could be helpful for further study in molecular aspects.

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