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Article

Parasites and Fungi Characteristics on Short Finned Eel *Anguilla marmorata* in Central Sulawesi, Indonesia

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Abstract : Parasitic infections are often not the direct cause of death of fish but the presence of wounds in the fish's body due to parasitic attacks is a trigger factor for secondary infection. Secondary infections can be caused by fungi, bacteria or viruses, which ultimately cause the death of fish. This study aimed to determine the characteristics of parasites and fungi that infected eel (*Anguilla* sp.) in the waters of Poso, Buol, Toli-toli and Donggala, Central Sulawesi as an important part of the diagnosis of fish disease in the framework of teraupetic strategies. Eel were taken as many as 30 individuals / location for observation of parasites and fungi. Parasitological examination was carried out for external and internal parasites on the mucous layer of the body, gills, intestines and stomach, while fungal isolations were carried out on muscles, skin and tissues that have abnormalities. After identification, the prevalence and intensity were carried out. The results showed that the highest prevalence of parasites were *Camallanus* sp (70%), *Proteacephalus* sp (50%) and *Gyrodactylus* sp (40%), and the nematode *Camallanus* sp the tapeworm *Proteocephalus* sp had the highest intensity of 57.5 and 30.8 respectively. Fungal prevalence were found highest in *Saprolegnia* sp (36%), and *Fusarium* sp (32%).

Keywords: parasite; fungi; finned eel; *Anguilla marmorata*; Central Sulawesi

1. Introduction

The eel species widely dispersed from tropical to subtropical region which consisted of 17 species [1]. Nevertheless, the current populations of eel fish including American eel (*Anguilla rostrade*), Japanese eel (*Anguilla japonica*), and European eel (*Anguilla anguilla*) have been reported to less than 10% compared to the eels wild population in 1970 [2,3]. European eels are the most sought–after eel commodity in the market [4]. Consequently, it has been categorised into endangered animal in which its status becoming protected animals due to high exploitation. The decline in number of wild population of eel can also be caused by fishing activity, climate change, pollution, parasites and diseases [5]. In addition, the environmental factors, such as heavy metal contamination can be biomagnification through food chain and reproduction [6], changes in salinity affect the immune response [7].

Several studies on wild eel population have been documented, including the effect of water pollution due to waste contamination on the head characteristic of eel species [8], detrimental effect of heavy metal concentrations on yellow American eel (*Anguilla rostrata*) and European eel (*Anguilla anguilla*) [9]. Hyaluronan accumulation in early ontogeny Japanese eel *Anguilla japonica* [10], accumulation, elimination and neuro-oxidative damage due to lanthanum exposure [11] and estimation of temperature distribution and depth of Japanese eel eggs using otolith oxygen stable

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isotopes [12]; the use of specific recombinant gonadotropins to induce spermatogenesis and spermiation in European eels (*Anguilla anguilla*). Nonetheless, it has not shown yet optimum results in both conservation and reproductive purposes.

Only few studies on parasites and fungi in both wild and cultivated eels have been documented. The parasite found in the eel very diverse and estimated to reach 100,000 species of protozoa parasites and metazoan of marine fish [13]. While fungal infection often occurred in fish and fish eggs as opportunistic secondary infections. Fungal infection most likely occurs to stress and injured fish. Moreover, it has been infected by bacterial, viral, or parasitic diseases. The types of fungi which are often found including *Saprolegniaceae,* such as *Saprolegnia* spp., *Achyla* spp., *Aphanomyces* spp. *Ichthyophonus*, and *Dermocystidium*. Various problems in the cultivation of eels have not been studied, including breeding techniques and disease control. Research on disease prevention is still lacking due to very little information related to disease identification. Although disease outbreak caused by parasites often indirectly increase mortality of fish, it can worsen the condition of fish after infected by fungi, bacteria and viruses. Moreover, fish infected by parasites often experience economic decline and are less safe for humans consumption [14].

Therefore, this study aimed to identify the parasites and fungi on eel (*A. marmorata*.) in the waters of Poso, Buol, Toli-toli and Donggala, Central Sulawesi. This study is an important part of the diagnosis of fish disease in the framework of teraupetic strategies. The results of this research can be used as a basis for studying host-parasite interactions [15], diagnostic development of parasites, application of vaccines [16,17,18], probiotics [19,20,21], and various therapeutic methods for prevention and control of diseases that are cheap and environmentally friendly.

2. Materials and Methods

2.1. Sample collection

This study was conducted from March to November 2018, which located in four different study areas, including, Buol, Donggala, Poso and Toli-Toli, Central Sulawesi Indonesia. The preparation of fish, parasite and fungi identification was carried out at Pangkep State Polytechnic of Agriculture.

2.2. Sampling procedure

A total of 120 short finned eel (*A. marmorata*) obtained from 4 areas (Buol, Donggala, Poso and Toli-toli) which consisted of 30 samples per area. Fish were sampling from natural waters based on sampling locations (purposively), transported to a laboratory using containers equipped with aerators. Fish were anesthetized with MS222 and observed patologicaly on mouth, body, fin, gills, anal and tail. Muscle was cutted from anus to dorsal, down over the body and head in order to investigate parasit and fungi. Research Conduct Permission from the Goverment No.: 1*3/INS-2/PPK/E4/2018 (http://ristekdikti.go.id/wp-content/uploads/2017/03/3.Salinan-penetapan-judul-proposal-PPTI-2017-Gel-1.pdf).

2.3. Identification of parasites

Parasitic examination was carried out by taking the mucus layer of the body's surface, fin, gills and fish organs, including the intestine and stomach. The mucus was properly stirred and observed under microscope. The remain parasites collected are also prepared by semi permanent slides, preserved in 4 % formaldehid and stained with iron acetic carmine.

Parasitic identification was performed based on Fernando et. al. [22-25]. The number of infected fish by parasites was count and parasite intensity and its prevalence were also calculated.

2.4. Identification of fungi

Fungal examination was performed by isolating the fungi from the muscles, skin and tissue that has a disorder or suspected to be overgrown with fungi. The specimen was added with 2% NaCl and centrifuged 2000-3000 rpm for 3 minutes. The 2% NaCl was replaced with 1% formalin and

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centrifuged as in the previous NaCl application. Formalin was replaced with 2% NaCl and centrifuged. Furthermore, NaCl was replaced with 1% Formalin and centrifuged. These process were repeated for 3 times. The specimen was inserted into a glass of sterile grinder and 5-10 drops of distilled water were added and crushed until pulverized. Scouring results were dripped with a pasteur pipette on the cultivation medium and scraped. The scouring results were incubated at room temperature. Fungus colonies that grew from the first isolation were re-cultivated separately from the colonies which grew apart and still in the field of isolation scratches. Isolates were taken with blunt isolate needles and cultured in new culture media, so that pure fungus culture was obtained. It was incubated at room temperature and continued with identification. Identification was according to Hazen and Reed [26,27]. The number of fish infected with fungi was calculated.

2.5. Data analysis

The results of the study consisted of the prevalence and intensity of parasites and fungi were analyzed descriptively using the formula as follows:

$$\text{Prevalence of parasites/fungi} = \frac{\text{Number of infected eels}}{\text{Number of eels examined}} \text{ X } 100\%$$

$$\text{Intensity of parasitic infection} = \frac{\text{Number of parasites}}{\text{Number of infected eels}}$$

3. Results

3.1. Type of parasites

The types of parasites found in four study sites consisted of Nematodes (*Camallanus* sp, *Acanthocephala*), Monogenea (*Dactilogyrus* sp and *Gyrodactilus* sp), Cestoda (*Proteocephalus* sp), Crustacea parasites (*Argulus* sp) and Protozoa parasites (*Trichodina* sp). Based on the territorial waters, there were similarity found in term of types of parasites among eels in different waters. The prevalence and intensity of parasite infection can be seen in the following Table 1.

Table 1. Parasitic organisms, prevalence and intensity of parasitic infection in four study areas of Poso, Donggala, Buol and Toli-toli. The number of samples was 30 in each region and isolated from the skin and intestine organs of the eel (*A. marmorata*).

Study areas	Types of parasite	Prevalence (%)	Intensity
Poso	*Camallanus* sp	50 (15)	57,4 (861)
	Dactilogyrus sp	10 (3)	3 (9)
	Proteocephalus sp	50 (15)	30,8 (462)
	Acanthocephala	30 (9)	4 (36)
	Argulus sp	10 (3)	1 (3)
	Gyrodactilus sp	10 (3)	3 (9)
Donggala	*Proteocephalus* sp	20 (6)	2 (12)
	Camallanus sp	30 (9)	30,3 (274)
	Dactilogyrus sp	10 (3)	8 (24)
	Acanthocephala	10 (3)	8 (24)
	Gyrodactilus sp	10 (3)	1 (3)

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Buol	*Dactilogyrus* sp	10 (3)	2 (6)
	Camallanus sp	70 (21)	1,3 (27)
	Acanthocephala	10 (3)	1 (3)
	Trichodina sp	10 (3)	10 (30)
	Proteocephalus sp	40 (12)	2 (24)
Toli-toli	*Camallanus* sp	30 (9)	1,7 (15)
	Proteocephalus sp	10 (3)	2 (6)
	Dactilogyrus sp	20 (16)	8,3 (50)
	Trichodina sp	30 (9)	3,9 (35)
	Gyrodactilus sp	40 (12)	5,2 (62)

Based on the table above, it can be seen that the four sampling waters of eel fish, including Donggala, Buol and Poso waters showed the same number of parasites.

3.2. The relationship between types of parasit, prevalence and intensity of parasitic infection in different areas.

Figure 1 and Figure 2 illustrated the parasites prevalence and parasite intensity in the four sampling locations. The highest parasite prevalence was parasitic *Camallanus* sp in Buol waters, *Camallanus* sp parasites and *Proteocephalus* sp in Poso waters. The intensity of parasitic infection occurred in *Camallanus* sp parasites in Poso waters and *Proteocephalus* sp in Poso and *Camallanus* sp waters in Donggala waters.

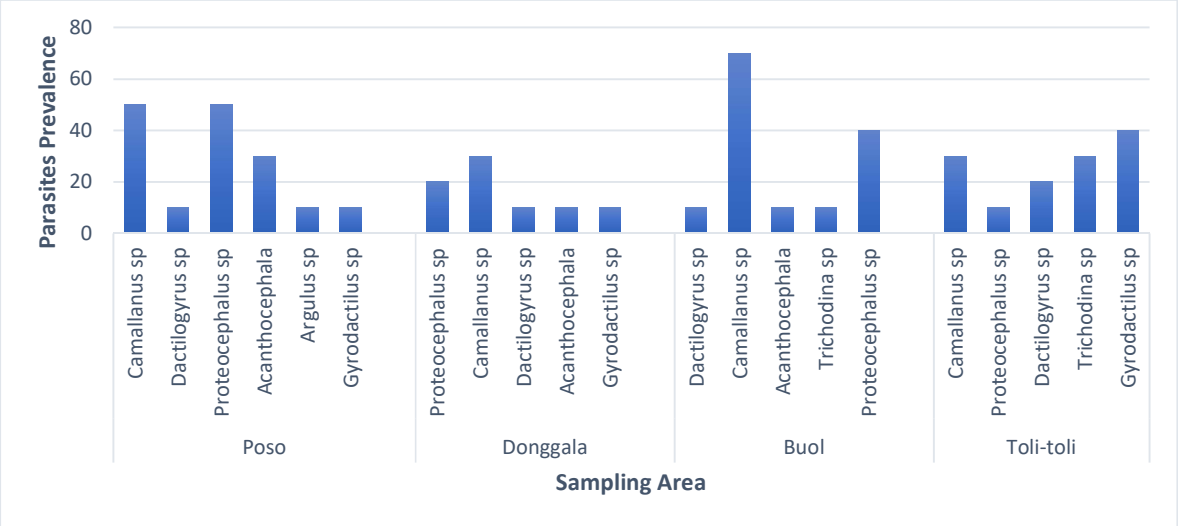

Figure 1. Parasitic prevalence in the waters of Poso, Donggala, Buol and Toli-toli derived Figure 30. samples per area and isolated from the skin and intestine organs of the eel (*A. marmorata*).

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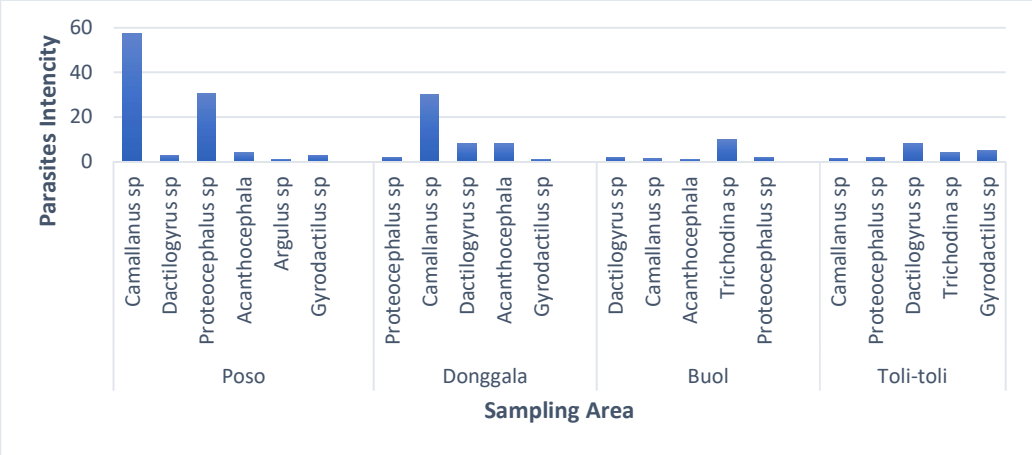

Figure 2. Parasitic prevalence in the waters of Poso, Donggala, Buol and Toli-toli derived Figure 30. samples per area and isolated from the skin and intestine organs of the eel (*A. marmorata*).

Prevalence of parasites in all sampling locations (Figure 3) showed that the *Camallanus* sp parasite, *Proteocephalus* sp and *Dactylogyrus* sp infected eel in all waters of the sample location, followed by *Acanthocephala* and *Gyrodactilus* sp and the lowest in *Argulus* sp and *Trichodina* sp parasites.

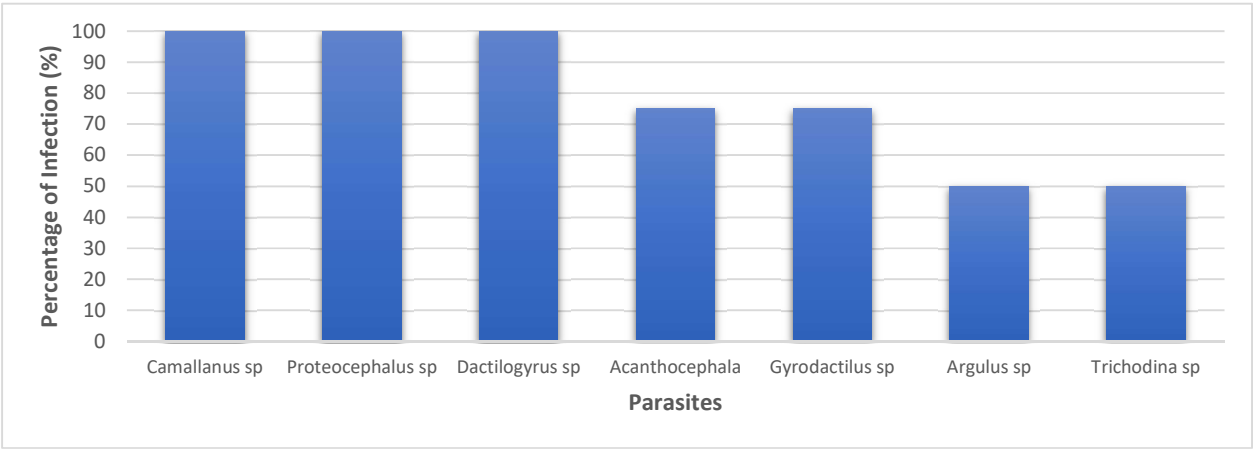

Figure 3. Parasite prevalence in all sampling locations. A number of 30 samples were obtained from each study area, the parasites were isolated from skin organ and intestine of eels (*A. marmorata*).

3. Types of fungi

The type of fungi found in 4 waters consisted of *Aphanomyces* sp, *Penicillium* sp, *Fusarium* sp (1), *Saprolegnia* sp, *Fusarium* sp (2), *Trichoderma* sp and *Achlya* sp. Based on the territorial waters, there were similarities of fungi among eels in different waters. The prevalence of fungal infection from each water location can be seen in the table 1.

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Table 2. species infections and prevalence in short finned eels of Poso, Donggala, Buol and Toli-toli waters. The number of samples was 30 eels per area and the fungi was isolated from the skin and muscle organs of eel (*A. marmorata*).

Study areas	Types of fungi	P (%)
Poso	*Aphanomyces* sp	6 (2)
	Penicillium sp	3 (1)
	Fusarium sp (1)	27 (8)
	Saprolegnia sp	27 (8)
	Fusarium sp (2)	3 (1)
	Trichoderma sp	6 (2)
Donggala	*Saprolegnia* sp	30 (9)
	Achlya sp	10 (3)
	Fusarium sp (1)	27 (8)
	Trichoderma sp	6 (2)
	Aphanocyces sp	13 (4)
Buol	*Fusarium* sp (2)	13 (4)
	Saprolegnia sp	27 (8)
	Trichoderma sp	10 (3)
	Achlya sp	10 (3)
	Aphanomyces sp	10 (3)
	Fusarium sp (1)	17 (5)
Toli-toli	*Penicillim* sp	6 (2)
	Saprolegnia sp	23 (7)
	Fusarium sp (1)	6 (2)
	Fusarium sp (2)	13 (4)
	Achlya sp	10 (3)

4. Relationship between types of fungi, prevelance of fungi and location

Fungal prevalence in four sampling locations illustrated in Figure 4. The highest fungi were *Fusarium* sp and *Saprolegnia* sp which found in Poso waters and *Saprolegnia* sp fungi in Donggala, Buol and Toli-toli waters. Fungal prevalence based on all sampling locations shown in Figure 5 that *Fusarium* sp and *Saprolegnia* sp fungi showed a prevalence of 100%, meaning that these two fungi can infect eel in all sampling stations. The *Penecillium* sp fungi showed a 50% of prevalence with dispersed areas in Poso and Toli-toli waters.

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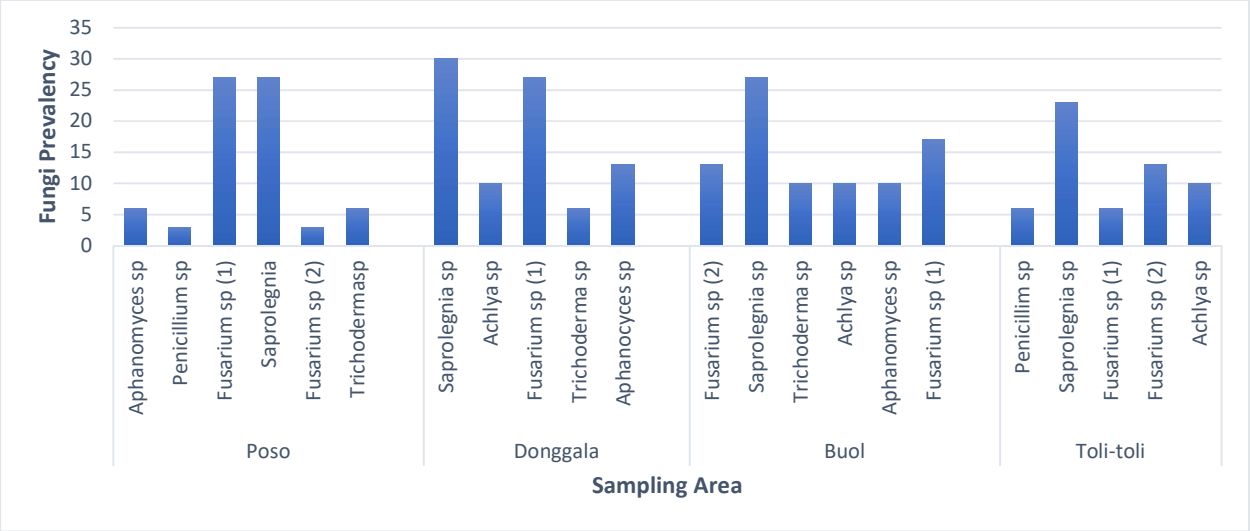

Figure 4. The prevalence of fungi in the waters of Poso, Donggala, Buol and Toli-toli. The number of samples was 30 per each location and the fungi was isolated from the skin and muscle organs of eel (*A. marmorata*).

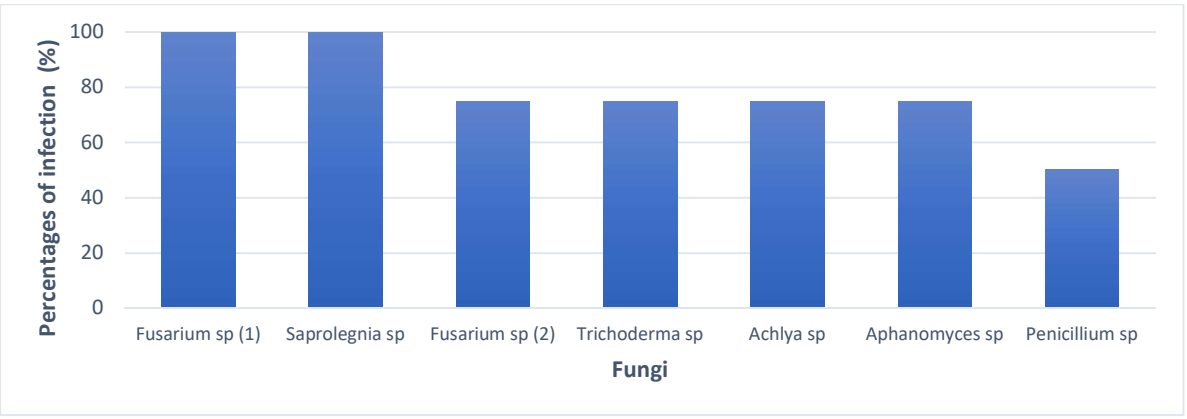

Figure 5. The prevalence of fungi in all location. The number of samples was 30 per each location and the fungi was isolated from the skin and muscle organs of eel (*Anguilla* sp.).

4. Discussion

4.1. Parasites

The results of the present work show that the eel *Anguilla marmorata* was infected by *Camallanus* sp, *Acanthocephala*, *Dactilogyrus* sp, *Gyrodactilus* sp, *Proteocephalus* sp, *Argulus* sp and *Trichodina* sp. The highest parasite prevalence was *Camallanus* sp in Buol waters, *Camallanus* sp parasites and *Proteocephalus* sp in Poso waters. The highest intensity of parasitic infection occurred in Poso waters (*Camallanus* sp parasites) and Poso waters (*Proteocephalus* sp) and Donggala waters (*Camallanus* sp). The highest prevalence of parasitic infection found in all sampling locations was *Camallanus* sp, *Proteocephalus* and *Dactylogyrus* sp, and lowest was *Argulus* sp and *Trichodina* sp parasites. This suggested that *Camallanus* sp, *Proteocephalus* and *Dactylogyrus* sp are considered as common parasites in the eels. The number of parasitic infection in eels depends on the type of food eaten, where the more types of food eaten by fish, the greater the number of parasitic infection in the eel.

Camallanus sp, *Proteocephalus* sp, *Acanthocephala* were found to live in the intestine and stomach, *Gyrodactilus* sp was found in the skin, *Dactilogyrus* sp on the gills and *Argulus* sp live on the skin and gills of fish. The number of nematodes found in this study is in line with the study of Goffredo et al [14] who found all teleostei fish studied in natural (wild) waters infected with nematode larvae. Based

on the data of fish organs infected with parasites, it appears that the parasites only infect specific organs. The target organ of this parasite differs from each parasitic organism, depending on the ability of the parasite to protect itself against the host's immune response and the route of infection [28]. If the host responds to the parasite, it appears that parasite gather in a particular organ [29]. However, if there is no response from the host, the parasite spread throughout the host's body. Based on data on parasite types, it can be seen that the types of parasite found in eel were almost identical, even though the location and characteristics are different.

The presence of parasites in each host is different, for examples *Ichtyophthirius multifiliis* consume the host epithelial cells, leading to enlarged blood vessels around the infected area. This tissue damage disrupts the function of excretion and osmoregulation organs. Similarly, *Gyrodactylus* sp and *Argulus* sp take food from the host by sticking themselves to the host body surface. Argulus pierces the skin with its proboscis, injecting a toxic secretion, resulting in tissue irritation which caused edema and inflammation. In severe infections, *Gyrodactylus* sp causes peeling of the skin and falling of scales, leading to difficulties in the process of respiration and osmoregulation.

Höglund et al. [30] examined the detrimental effect of nematodes *Anguillicola crassus* on silver eels which distrub the spawning process. This parasitic infection is harmful for the host. As a result, the erythrocyte circulation is declining which lead to oxygen depleted in the blood [31]. In addition, the negative impact of the *Anguillicola crassus* parasite on silver fish eels damage the wall of swim bladder of the host. Pathological changes include hemorrhagic, parasitic nodule formation, inflammatory cell proliferation, and connective tissue hypertrophy, necrotic areas and edema. Thischanges is eventually caused a large thickening of the swim bladder wall [32] and a large reduction in the volume of the swim bladder.

Further research was carried out by Palstra et al [33], showing that the infected eel with *Anguillicola crassus* nematode parasite experiences slow swimming due to swim bladder damage. At a severe level of parasitic infection, the eel is difficult to swim lead to difficulty to migrate to spawning ground. Monogenean ectoparasites are a significant threat to many cultivated fish species. The most pathogenic monogeneans are identified from *Capsalidae, Diplectanidae, Anoplodiscidae* and *Gyrodactylidae* families in the *Monopisthocotylea* group, and *Microcotylidae, Heteraxinidae* and *Diclidophoridae* in the *Polyopisthocotylea* group [34[. *Capsalids* like Neobenedenia spp. has caused the death of many aquaculture fish species [35]. Hematological parameters of infected fish are the main parameters that experience abnormalities, such as lack of blood or osmotic imbalance in fish due to parasitic infection [36]. Monogeneans have a short life cycle. Adult parasites continue to lay eggs and hatch into larvae and reach the stage of adolescence and adulthood after settling into the fish as their host.

Changes in abundance or community structure of parasitic organisms can be used as indicators of changes in environmental chemistry [37,38]. The use of Acanthocephalus lucii and cestoda Proteocephalus percae in the host intestine as indicators of heavy metal bioaccumulation has been carried out by Sures and Siddall [39,40]. The use of both types of parasites is likely to be related to their ability to accumulate pollution material throughout the body surface [41].

The success or failure of parasites in a host is determined by the success of parasites in avoiding the introduction of the host immune response, parasite survival and persistence in the host and avoidance of host death from the inflammatory response induced by pathogenic parasites. The body's defense response to parasitic infection and the parasite strategy to avoid the host's defense response is a form of continuously evolution [42]. If the parasite succeeds in avoiding the host's defense response, the parasite may infect the host and cause infection or illness. On the other hand, vaccination is not available to increase the specific body defenses of fish [43], so it depends on the natural immune system of fish.

When the parasite can infect fish, the parasite enters through the skin of the fish and enter the network. If the tissue enters the protected area by the body's defenses, parasite infection can be eliminated immediately [44] but only a few types of parasites are eliminated, such as cercariae. The success of eliminating this host is determined by several factors, including the position where the

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parasite attacks the host. According to [45] the immune response in the host's eyes and brain is less effective against worm invasion.

Harmful parasite worms including *Digenea, Cestoda, Acanthocephala* and *Nematoda* according to Dezfuli et al. [46]. Digenea endoparasites such as flatworms or platyhelminths, when they mature stage, they produce larvae. Flatworms have two sucker-like organs. At least this digworm attaches one sucker to the mucosal surface of the digestive tract of fish. This digenea worm causes damage to the host, especially in mucosal lumen, mucosa or epithelial tissue [47]. In general, digenea worms that live in the living intestine around the mucosa or epithelial tissue, mucus, blood, host digestive products, and the histolitic secretion product of the worm itself [48], thus destroying the mucosal epithelium covering the villi [47].

2. *Fungi infection*

The type of fungus that infects eel in four different waters consisted of *Aphanomyces* sp, *Penicillium* sp, *Fusarium* sp (1), *Saprolegnia* sp, *Fusarium* sp (2), *Trichoderma* sp and *Achlya* spp. The prevalence of fungus infection in the four sampling locations was found high in *Fusarium* sp and *Saprolegnia* sp fungi in Poso and Saprolegnia fungi in Donggala, Buol and Toli-toli waters. Fungal prevalence based on all sampling locations showed *Fusarium* sp and *Saprolegnia* sp had a prevalence of 100%, meaning that these two fungi could infect eel fish at all sampling stations. While the *Penecillium* sp has the lowest prevalence (50%) with the area of attack of eel on Poso and Toli-toli waters.

Pathogenic fungi and parasites, along with pathogenic bacteria and viruses, infect various types of hosts including fish. This fungal disease causes significant damage to cultivated fish and wild fish around the world. When it is compared to other animals, only a few types of fungi infect the fish. The Fungi, from the Family of Saprolegniaceae in Oomycetes class is the most frequently infected fish [49]. Saprolegniaceae, is opportunistic, infects fish when stressed or there is a decrease in immune response due to environmental conditions, or the presence of bacterial or viral infections, or when fish have lost non-specific body defenses such as mucus due to trauma or improper handling [50]. However, there are types of fungi from the Saprolegniaceae Family that cause primary infections that cause disease without predisposing factors.

Fungal infections can be diagnosed. Nonetheless, it is different from diagnosis in bacteria, parasites or viruses. Fungal classification is based on the life cycle, morphology of hyphae, and reproductive methods, as well as the types of spores produced. The fungi cannot synthesize their own nutrients. Hetrofotrof requires preformed organic matter for growth and reproduction. They can be categorized as saprophytes, which use dead organic matter or as parasites that infect living organisms for food. Most fungi are facultative compared to obligate parasites or saprophytes.

Khoo [51] has described the clinical symptoms of fungi in the class of Oomycetes, Saprolegniasis, Aphanomyces and Branchiomycosis. Saprolegniasis infection is characterized by white fibers or like cotton, Aphanomyces infection accompanied by skin ulcers can fuse with bacterial ulcers, Branchiomycosis infection is characterized by the presence of cytology in infected fish gill tissue and the presence of skin lesions due to Dermocystidium spp.

Oomycetes are a group of pathogenic fungi class that most commonly found in the fish which live in freshwater, estuary and sea. Almost all freshwater fish are considered vulnerable to be infected with at least one species of oomycetes [52]. A distinctive feature of Oomycetes is the production of heteroconous zoospores, namely biflagellate motile spores with different flagella, a whiplash type and a tinsel type [53]. Oomycetes have mi- chondrial cristae with tubular confirmation that distinguishes them from other fungi, which have platelike cristae [54] Oomycetes also have cellulose as a component. cell wall, which also helps distinguish from other fungi.

Saprolegniasis infection in fish and eggs caused by members of the genera *Saprolegnia, Achyla,* and *Dictyuchus* [52]. *Saprolegniasis* infect the fish especially in winter season or low temperatures. *Aphanomyces astaci* is a type of fungus that is pathogenic in crayfish, while other species of the Aphanomyces group are pathogenic in fish [55]. Both in freshwater and brackish waters, wild fish or

cultured fish, the infected fish by Oomycetes, shows lesions, including granulomatous mycosis (GM), epizootic ulcerative syndrome (EUS), red sore disease (RSD), and ulcerative mycosis (UM).

Branchiomycosis is also known as gill rot, this fungus mostly infects the gills of fish, especially freshwater fish. Infected fish with this fungus experience respiratory problem and lethargy disorders [52]. The fungi is able to infect the host at water temperatures above 20 °C, and mortality can reach 50% [56]. Morbidity can reach 100%, but not all fish species in the pond are infected. Fungus infection occurs quickly, when the fish stocking density is high, algal blooms, or an increase in non-ionized ammonia.

5. Conclusion

Eel (*A. marmorata*) was contaminated by the parasites: *Camallanus* sp, *Dactilogyrus* sp, *Proteacephalus* sp, *Acanthocephala*, *Argulus* sp, *Gyrodactilus* sp. The type of fungus that commonly infected eel in all waters of sampling sites consist of *Aphanomyces* sp, *Penicillium* sp, *Fusarium* sp. (1), *Fusarium* sp. (2), *Saprolegnia* sp, Trichoderma and Achlya. The parasites: *Camallanus* sp, *Proteacephalus* sp, *Dactilogyrus* sp, and the fungi : *Fusarium* sp and *Saprolegnia* sp. attakced the eel in all stations. The highest prevalence of parasites were *Camallanus* sp (70%), *Proteacephalus* sp (50%) and *Gyrodactylus* sp (40%). The parasites: *Camallanus* sp and *Proteacephalus* sp were the most frequent parasites (57,4% and 30,8%, respectively). Fungal prevalence were found highest in *Saprolegnia* sp. (36%) and *Fusarium* sp. (32%). It can be concluded from this study that the parasites: *Camallanus* sp, *Proteacephalus* sp, *Dactilogyrus* sp, and the Fungi: *Fusarium* sp and *Saprolegnia* sp represent the major microorganisms to obvious attacked the eel in the natural waters of Central Sulawesi, Indonesia.

Author contributions: AMR, ER, HT, AR and WH participated field work on fish sampling; AMR, ER and AR investigated parasites and fungi of fish; HT and WH collected and interpreted data; HT, WH and AR developed concept of the study and working hypotheses; AMR dan ER performed numerical calculations and provided critical review on the paper drafts.

Conflicts of interest: The authors declare that they have no conflict of interest.

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