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Controlling parasitic dinoflagellates of fish，with special emphasis on molecular genetics and immunity *

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Abstract Parasitic dinoflagellates，as exemplified by the marine pathogen *Amyloodinium ocellatum*，cause major losses in cultured fish. Some new treatments for *Amyloodinium* have emerged in recent years，including chloroquine，hydrogen peroxide and 3，N-methylglucamine lasalocid. However，copper remains the most useful treatment. Recent advances in molecular genetics and immunology have the potential to greatly enhance our understanding of the epidemiology and control of these important pathogens. Molecular phylogenetic analyses suggest that some parasitic dinoflagellates (e.g.，*Amyloodinium ocellatum*) might form a highly homogeneous taxon while others (e.g.，*Piscinoodinium pillulare*) probably constitute more than one species or even higher taxa. These molecular analyses have also allowed the development of highly sensitive probes that can detect very small numbers of parasites in environmental samples. Studies of the immune response to *Amyloodinium* have revealed that fish can mount a strong and highly protective specific response to parasite exposure. A significant part of this response is mediated by antibody. Fish also constitutively express endogenous，host-produced polypeptide antibiotics (histone-like proteins) in the skin and gills that are highly lethal to *Amyloodinium* at concentrations that are well within the levels present in host tissues. Utilization of these specific and nonspecific defenses has the potential to greatly enhance our ability to protect against these devastating pathogens [*Acta Zoologica Sinica* 51 (4)：550－553，2005].

Key words Parasitic dinoflagellates，Finfish，Diagnosis，Control，Molecular phylogeny

用分子遗传学及免疫学方法控制鱼类寄生性甲藻 *

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摘 要 寄生性甲藻，例如 *Amyloodinium ocellatum*，给鱼类养殖带来严重的危害。虽然，近年来针对 *Amyloodinium* 的药物治疗有一些新的进展，如使用氯喹、过氧化氢和 3，N-泛影葡胺拉沙洛西等，然而，含铜类药物仍然是最有效的。近年来分子遗传学和免疫学的进展，使得我们能够更好地了解寄生性甲藻的流行病学及其防治方法。分子系统学研究认为某些寄生性甲藻，如 *Amyloodinium ocellatum*，可以聚类为高度同源性的一支；而其它的如 *Piscinoodinium pillulare*，则可以认为不止一种或更高的分类阶元。这些分子分析也发展出一些高灵敏的检测技术，可以检测出环境中极少量的寄生性甲藻。通过对 *Amyloodinium* 的免疫学研究表明，鱼类能对寄生物的感染产生强烈的高度特异的保护性免疫应答，其中主要是抗体介导的免疫应答。鱼体皮肤和鳃也能表达内源非特异性多肽抗生素（类组蛋白），它们能对 *Amyloodinium* 造成致命的破坏。利用这些特异或非特异的免疫防御，将更有助于我们控制这些具有严重危害性的寄生性甲藻 [动物学报 51 (4)：550－553，2005]。

关键词 寄生性甲藻 有鳍鱼 诊断 控制 分子系统发育

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1 Introduction

Dinoflagellates are a very diverse group of aquatic protozoa. Their most prominent role is as important components of the food chain, where they function as either free-living autotrophs (primary producers) or heterotrophs (grazers). Others are important as endosymbionts of invertebrates, such as corals. Although most of the 140 or so parasitic species infect invertebrates, those belonging to the genera *Amyloodinium*, *Pisicinoodinium*, *Crepidoodinium* (all currently in classified in the Family Blastodiniphyceae) and *Ichthyodinium* (Family Syndiniaceae) are parasites of fish. While there is evidence that the toxic dinoflagellate *Pfiesteria* also can exhibit parasitic tendencies, it is not a typical parasite and thus will not be considered further in this review. The life cycle of the first 3 genera is triphasic, consisting of a parasitic, pyriform trophont, a reproductive tomont (palmella stage), and a free-swimming infective dinospore stage. In contrast to these 3 ectoparasites of skin and/or gills, *Ichthyodinium* is an endoparasite that infects the eggs of marine fishes. The parasitic stages of this dinoflagellate consist of 3 generations of schizogony within infected eggs, although descriptions of the exogenous stages and mode of infection remain uncertain.

Although the ectoparasites are apparently widely distributed in nature, host and dinospore dispersal as well as natural predation upon the infective dinospores by planktivores probably maintains parasite densities on host fish below the level required to cause clinical disease. However, under the often crowded conditions of aquaculture, rapid increases in parasite numbers may occur under favorable conditions, and it is under these circumstances that the pathogenicity of *Amyloodinium* and *Piscinodinium* is most evident, see Noga and Levy (1995) for a review. In rare cases, feral fish populations have experienced severe morbidity and mortality due to ectoparasitic dinoflagellates. Thermal stress, high levels of ammonia, and an incompletely developed immune response in young fish might have contributed to several of these natural outbreaks. Among the parasitic dinoflagellates, *Amyloodinium* is by far the most significant pathogen in aquaculture and most published data is focused on this parasite.

2 Traditional diagnosis

Fish with heavy ectoparasitic dinoflagellate infections on their gills typically exhibit rapid respiration as large numbers of parasites compromise gill function. Fish with skin infections may exhibit 'flashing' behavior. Grossly, they may have a whitish, brownish, or golden sheen on the skin. This surface sheen is easiest to observe by placing the fish in the dark and shining a beam of light through the water parallel to the surface of the skin. Anesthetized or recently euthanized individuals can also be examined in this way. Trophonts can also be dislodged by placing the fish in a small container and subjecting them to osmotic shock (e.g., add marine fish to freshwater). The detaching trophonts and newly formed tomonts can then be collected from the bottom of the container and observed microscopically. Alternatively, microscopic examination of gill or fin clips, or skin scrapings, serves the same purpose. Positive identification requires observation of appropriately stained thin sections, and/or the use of scanning or transmission electron microscopy in order to identify key structures. However, such methods are cumbersome, expensive and not very sensitive when dealing with small numbers of parasites. Furthermore, they may fail to reveal important genetic differences among various isolates that are not discernable by morphological analyses. Thus, recent advances in molecular phylogeny and diagnostic methodologies are helping to clarify the relationships both between and within the various taxa (Litaker et al., In Preparation).

3 Phylogeny and molecular diagnosis

Effective control of parasitic dinoflagellates requires a thorough understanding of their epidemiology, including host and geographic range, as well as other factors affecting transmission. These factors can only be discerned via a thorough understanding of the phylogenetic relationships among the different parasite isolates. Such studies may also lead to more effective methods for diagnosis of specific pathogens.

Traditionally, attributes such as the parasite's environment (marine vs. freshwater), mode of attachment, and presence or absence of chloroplasts or stomopode in the trophont, have been used to classify the three ectoparasitic genera as follows: *Crepidoodinium*, found in estuarine environments, possesses chloroplasts, lacks a stomopode, and is attached to the host by a large flat projection-bearing adhesive surface that touches but fails to penetrate into the host cells; *Piscinoodinium*, a chloroplast-containing parasite of freshwater fish, lacks a stomopode and has a well-developed attachment disk from which rhizocysts firmly anchor it into the host cells; *Amyloodinium*, a parasite of marine fish, does not contain chloroplasts, has a stomopode, and has attachment structures (rhizoids) that penetrate deeply into the host cells.

All three genera are at present monospecific. However, recent evidence suggests that some taxa might be represented by multiple strains, if not multiple species. In other cases, the data support a mon-

specific phylogeny. Phylogenetic analysis of the small subunit (SSU) ribosomal gene sequenced from dinospores of the DC-1 isolate has confirmed the placement of *A. ocellatum* in the Family Blastodiniphyceae (Litaker et al., 1999). As a prelude to the development of sensitive and specific PCR diagnostic assays for *A. ocellatum*, the SSU of 3 different isolates (DC-1, Florida and Red Sea) were sequenced (Levy et al., Unpublished Data). There was >99% sequence identity, strongly suggesting that these isolates were probably identical species. *Amyloodinium*-specific oligonucleotide primers derived from these molecular analyses could detect as few as 1 dinospore/ml of water when processed using the Mobio® Soil Extraction Kit followed by PCR. This assay was found to be species-specific (Levy et al., Unpublished Data). However, in contrast to the SSU data suggesting a homogeneous taxon, scanning electron microscopy studies by Landsberg and co-workers have shown that the morphology of the gymnodinoid-type dinospores of *Amyloodinium ocellatum* appears to vary among isolates; these data suggest that there may in fact be more than a single strain or species of *Amyloodinium*. Although the taxonomic significance of dinospore morphology remains controversial, it suggests that further studies need to be done to correctly discern phylogenetic relationships.

In striking contrast to the molecular phylogenetic findings with *Amyloodinium* that support its placement along with *Pisicinoodinium* and *Crepidoodinium* (all currently in classified in the Family Blastodiniphyceae), data just obtained from the SSU rDNA gene of an isolate that has many typical features of *Piscinoodinium* (i. e., typical trophont infecting skin of freshwater tropical fish) places this organism in the Family Gymnodiniphycidae, making it closely related to such dinoflagellates as the symbiont *Symbiodinium pilosum* and the free-living *Gymnodinium simplex*, rather than the distantly related Blastodiniphyceae. In addition, closer morphological examination via ultrastructure does not appear to correspond to published descriptions of *Piscinoodinium*. These molecular and morphological data suggest that the taxonomic status of this parasite requires significant revision.

4 Immunoprophylaxis

At present, there are no practical vaccines available for treatment of any parasitic dinoflagellates. However, recent studies have identified important defensive mechanisms that might be used to enhance protection to these pathogens.

In terms of specific immunity, *Amyloodinium ocellatum*-infected fish develop protective resistance following both natural and experimental infections (Noga and Levy, 1995; Cobb et al., 1998). An ELISA has been developed for the detection of specific fish anti-*A. ocellatum* antibody following injection of live or killed organisms. Immune serum having these antibodies affected the growth, motility and infectivity of cell-cultured parasites. Antibodies were also detected following a natural outbreak of amloodiniosis in cultured hybrid striped bass. Cobb and co-workers demonstrated that, following weekly sublethal challenges with *A. ocellatum* dinospores, tomato clownfish *Amphiprion frenatus* developed significant immunity to infection in about one month. This immunity was associated with an antibody response as measured by ELISA (Cobb et al., 1998). This antibody reacted with specific parasite antigens in Western blots, reacted with trophonts in an indirect fluorescent antibody test, and could be passively transferred to naÏ ve fish, providing some measure of resistance.

In terms of nonspecific immunity, *Amyloodinium* is also highly sensitive to natural, host-produced antibiotics, known as histone-like proteins (HLPs). These HLPs, present in high concentrations in the skin and gills, are highly lethal to trophonts (Noga et al., 2001). This preferential toxicity for trophonts was unexpected, since virtually all drugs act only against the dinospore (infective stage) and trophonts are typically highly resistant to therapy.

5 Drug treatment

Parasite burdens on individual fish can be rapidly reduced by osmotic shock if the fish can be handled and will tolerate such treatment. However, this is not practical for large numbers of fish or fish that cannot easily be handled or otherwise captured for treatment. Dinospores are usually the stage that is most susceptible to a variety of interventions including chemotherapy, predation by filter feeders such as *Artemia*, treatment with ultraviolet radiation, rapid flushing of the environment, or altering either temperature or salinity beyond that tolerated by the parasite. Although copper is the most widely used drug, especially against *Amyloodinium* dinospores, its use can be problematic against *Piscinoodinium* in freshwater due to its toxic effects on fish in soft, acidic conditions. Formalin baths are somewhat effective against trophonts but require rather prolonged immersion and may not completely kill the tomonts, which may resume development once the formalin has dissipated. Chloroquine phosphate, as first discovered by Carol Bower, is highly effective against *A. ocellatum* but is expensive. Hydrogen peroxide has been reported to be effective against *Amyloodinium* trophonts infecting the Pacific threadfin *Polydactylus sexfilis*. Another drug that might also be effective against

A. ocellatum is 3, N-methylglucamine lasalocid.

6 Propagation of *Amyloodinium* under controlled conditions

Studies of immunoprophylaxis and drug treatments for *Amyloodinium* have been greatly accelerated by the development of in vitro culture systems. Early investigation required either collection of naturally infected fish or examination of infected and/or diseased fish arising from spontaneous outbreaks in aquaculture facilities. Later, a standardized method for *in vivo* propagation on clownfish (*Amphiprion* spp.) was developed. Soon afterwards, parasites were successfully propagated on gnotobiotic (germ-free) guppies *Poecila reticulata* and in organ culture. Later, propagation entirely in cell culture greatly enhanced the ability to study this parasite. Briefly, parasites are propagated using a gill cell line (G1B) as host cells, while the culture is maintained in an artificial seawater solution. This system has allowed the continuous *in vitro* propagation of *A. ocellatum* for over 15 years (Noga and Levy, Unpublished Data). Interestingly, these parasites have remained pathogenic to fish. The G1B culture system has been used to successfully propagate *Amyloodinium* isolates from the Red Sea, Mediterranean Sea, and Gulf of Mexico (Florida). Others have developed an alternative cell culture method using aggregates of dorsal fin cells derived from redfish *Scianenops ocellatus* (Oestmann and Lewis, 1996).

7 Conclusions and future directions

Because natural fisheries have reached or surpassed their sustainable harvest capabilities, the gap between supply of fish and consumer demand must be met by aquaculture. It is under these intensive cultivation conditions that parasitic dinoflagellates exert their greatest impact. The majority of experimental work has been performed on *A. ocellatum* due in part to its economic importance but also because of the relative ease that it can be maintained *in vivo* or *in vitro*. Clearly, efforts towards development of similar methodologies for *Piscinoodinium* and other harmful species are warranted. Molecular biology will continue to clarify the taxonomic relationship among the genera, provide for rapid and standardized diagnosis and provide information needed to manage these infections, especially in cultured species. The extensive evidence for a protective immune response against *A. ocellatum* holds great promise for eventual development of protective vaccines.

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