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DNA identification of ciliates associated with disease outbreaks in a New Zealand marine fish hatchery

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ABSTRACT: Ciliates associated with fish mortalities in a New Zealand hatchery were identified by DNA sequencing of the small subunit ribosomal RNA gene (SSU rRNA). Tissue samples were taken from lesions and gill tissues on freshly dead juvenile groper, brain tissue from adult kingfish, and from ciliate cultures and rotifers derived from fish mortality events between January 2007 and March 2009. Different mortality events were characterized by either of 2 ciliate species, *Uronema marinum* and *Miamiensis avidus*. A third ciliate, *Mesanophrys carcini*, was identified in rotifers used as food for fish larvae. Sequencing part of the SSU rRNA provided a rapid tool for the identification and monitoring of scuticociliates in the hatchery and allowed the first identification of these species in farmed fish in New Zealand.

KEY WORDS: Small subunit ribosomal RNA gene · Scuticociliatosis · *Uronema marinum* · *Miamiensis avidus* · *Mesanophrys carcini* · Groper · *Polyprion oxygeneios* · Kingfish · *Seriola lalandi*

INTRODUCTION

The scuticociliates are major pathogens in marine fin-fish aquaculture and have caused mass mortalities in a range of farmed fishes (Kim et al. 2004a, Jung et al. 2005, 2007, Paramá et al. 2006) and crustacea (see Jung et al. 2005). Ciliates infect the gills, skin, muscles and internal organs and can be highly histophagous, destroying infected tissues. Several species of opportunistic scuticociliates have been implicated in systemic infections of farmed fish (Paramá et al. 2006), although identification of the parasite species involved in scuticociliatosis can be difficult with traditional microscopy methods (Song & Wilbert 2000, Paramá et al. 2006). DNA methods are increasingly used for specimen identification of a wide range of taxa (e.g. Hebert et al. 2003). The small subunit ribosomal RNA (SSU rRNA, 18S) region has been used for the identification of ciliate pathogens in fin-fish farms (Kim et al. 2004a,b, Jung et al. 2007) and in crustacea (Ragan et al. 1996), and for phylogenetic and taxonomic studies of ciliates (Hirt et al. 1995, Affa'a et al. 2004). Jung et al. (2005) have described the complete SSU rRNA sequence of the pathogenic scuticociliate *Miamiensis avidus*; subsequently synonymy between *Philasterides dicentrarchi* and *M. avidus* was suggested based on SSU rRNA sequences (Paramá et al. 2006). There is a growing DNA database of ciliate SSU rRNA sequences available on GenBank which can be utilised to match sequences and identify unknown ciliates.

Most DNA studies have used cultured ciliates or cloned DNA product to avoid amplification of host tissues (Kim et al. 2004a, Song et al. 2009). Here we evaluate the application of DNA sequencing for the identification of ciliates in direct amplification of fish tissues. We report the DNA identification of ciliates associated

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with fish mortalities in a marine fish hatchery in northern New Zealand, in particular hatchery-reared juveniles of groper *Polyprion oxygeneios* (Schneider et Forster, 1801) and adult kingfish *Seriola lalandi* Valenciennes, 1833, and demonstrate that different mortality events were characterized by 2 ciliate species over a 2 yr time period. This is the first record of these ciliate species associated with mortalities in farmed fish in New Zealand.

MATERIALS AND METHODS

Small subsamples of tissue (~200 mg) were taken from skin lesions and gill tissues of juvenile groper (22 to 60 mm fork length) that had been frozen at –20°C or fixed in 5 volumes of 90% ethanol soon after mortalities occurred in the hatchery. Adult kingfish which had died in the hatchery were dissected and small subsamples of tissues fixed in 5 volumes of 90% ethanol. Live ciliates isolated from dead groper and from water samples taken from groper juvenile tanks exhibiting a high fish mortality, were cultured in L-15 (Leibovitz) medium with L-glutamine and filtered seawater following methods described by Iglesias et al. (2002). Ciliates reached densities of 10^4 l^{-1} seawater in the groper juvenile tanks. Cultures were centrifuged to a pellet and resuspended in 90% ethanol for DNA analysis. Rotifers used as live food for larval groper, also exhibited mortalities associated with ciliates and were fixed in 90% ethanol. Muscle tissue samples were taken from wild-caught specimens of groper and kingfish, and DNA extracted following methods used for ciliates.

Total genomic DNA was extracted from fish tissues, rotifers, and ciliate pellets by homogenisation and overnight digestion with Proteinase K at 55°C, followed by a phenol-chloroform-ethanol procedure (Taggart et al. 1992). The universal primer pair designed to amplify the SSU rRNA gene of ciliates (Sogin 1990) was trialled and the internal primers developed by Jung et al. (2005) used to amplify smaller subregions of the SSU rRNA gene (Table 1). DNA amplification procedures followed those used at NIWA, using a Cetus 9600 DNA thermocycler (Perkin-Elmer). Amplifications were carried out using an initial denaturation of 94°C for 2 min, followed by 40 cycles of 94°C for 60 s, 50°C for 60 s, and 72°C for 120 s, and a final extension at 72°C for 7 min. The sizes of the amplified products were checked by electrophoresis in 1.5% agarose gels, stained with ethidium bromide. Those amplifications that produced a single amplified product were selected for sequencing (see Table 2). DNA products were labeled using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and sequenced using an ABI 3730 capillary sequencer following manufacturer's instructions. Sequencing was conducted in both directions, and the 2 sequences joined and aligned. The sequences were edited in CHROMAS (Technelysium, Queensland), aligned in CLUSTAL in MEGA version 3 (Kumar et al. 2004) and stored in BIOEDIT (Hall 1999). The sequences were submitted to GenBank in a BLAST search to identify the closest matching species; only matches greater than 98% identity were considered. Sequences derived from tissue samples from dead fish, dead rotifers, the cultured ciliates, and fish hosts were aligned against GenBank SSU rRNA entries for ciliates and marine fishes. Currently there are no SSU rRNA sequences available for groper and kingfish.

A neighbour-joining (NJ) tree was created using the software MEGA3 (Kumar et al. 2004) to provide a graphic representation of the divergence among the unknown ciliates from New Zealand and the 98%+ matched sequences derived from GenBank. Modeltest version 3.7 (Posada & Crandall 1998) was used to determine the best-fit model using likelihood ratio tests, and the Tamura-Nei (TrN) distance model selected (Tamura & Nei 1993); bootstrapping was performed with 1000 replications.

Table 1. Primers used to amplify the small subunit (SSU) rRNA gene in ciliates and host fishes in New Zealand

Code	Sequence	Source
Cil A	5′-ACC TGG TTG ATC CTG CCA GT	Sogin (1990)
Cil B	5′-TGA TCC TTC TGC AGG TTC ACC TAC	Sogin (1990)
Cil 2	5′-CTA TCA GCT TTC GAT GGT	Jung et al. (2005)
Cil 3	5′-GTA GGC TCT TTA CCT TGA	Jung et al. (2005)
Cil 4	5′-CAA ATC ACT CCA CCA ACT	Jung et al. (2005)
Cil 5	5′-ACG ACT TCT CCT TCC TCT	Jung et al. (2005)

RESULTS AND DISCUSSION

Several primer pairs trialled on the tissue samples, cultured ciliates, and rotifers produced a single amplified fragment. However, following sequencing, not all of these fragments were from ciliates. The universal primer pair Cil 2/Cil 5 and the CilA/Cil 4 pair (Table 1) amplified single fragments with most tissue samples, but preferentially amplified fish host tissue, groper or kingfish; only samples of cultured ciliates and rotifers produced ciliate-specific fragments. The primer pairs Cil 3/Cil 4 and Cil 2/Cil 4 always amplified a ciliate fragment, even in tissue samples taken from fish lesions and

Table 2. Small subunit (SSU) rRNA results for fish tissues, ciliate cultures, and rotifers tested from fish mortality events between February 2007 and March 2009. All juvenile groper were derived from one hatchery in Bream Bay, but the 2008–09 juveniles were on-grown in 2 locations: BB: Bream Bay hatchery; GP: Greta Point aquarium facility. Numbers in parentheses: number of specimens or cultures

Specimen	Tissue source	Sample date	Sequence length (bp)	Primer pair	Identity (%)	Identification
Kingfish	Brain	Feb 07	500	Cil 3/Cil 4	98	*Miamiensis avidus*
Kingfish	Brain	Nov 08	518	Cil 3/Cil 4	99	*Miamiensis avidus*
Juvenile groper (2)	Skin lesions	Jan 07	505–527	Cil 3/Cil 4	98–99	*Miamiensis avidus*
Juvenile groper (2)	Skin lesions	Dec 07	505	Cil 3/Cil 4	99	*Uronema marinum*
Ciliate	Cultures (4)	Dec 07	1300	Cil 2/Cil 5	99–100	*Uronema marinum*
Juvenile groper	Skin lesion	Jan 08	500	Cil 3/Cil 4	99	*Uronema marinum*
Juvenile groper (4)	Skin lesions	Mar 08	510–525	Cil 3/Cil 4	99–100	*Miamiensis avidus*
Ciliate	Cultures (6)	Mar 08	1360	Cil 2/Cil 5	99–100	*Miamiensis avidus*
Rotifer	Larval tank	Sep 08	1080	Cil 2/Cil 5	99	*Mesanophrys carcini*
Rotifer	Larval tank	Sep 08	1200	Cil 2/Cil 5	99	*Mesanophrys carcini*
Juvenile groper BB (3)	Gills	Jan 09	523	Cil 3/Cil 4	99	*Miamiensis avidus*
Juvenile groper BB (5)	Skin lesions	Jan 09	490–510	Cil 3/Cil 4	99	*Miamiensis avidus*
Juvenile groper BB (3)	Skin lesions	Feb 09	511–520	Cil 3/Cil 4	99	*Miamiensis avidus*
Juvenile groper GP (4)	Gills	Jan 09	508–512	Cil 3/Cil 4	99	*Miamiensis avidus*
Juvenile groper GP (4)	Gills	Feb 09	497–505	Cil 3/Cil 4	100	*Miamiensis avidus*
Juvenile groper GP (8)	Gills & skin lesions	Mar 09	505–529	Cil 3/Cil 4	99	*Miamiensis avidus*

gills. Sequences for the New Zealand ciliates have been deposited in GenBank, Accession Nos.: *Miamiensis* FJ936000, *Uronema* FJ936001, and *Mesanophrys* FJ936002.

The suspect ciliates could be identified to the species level with 99 to 100% identity matches against sequences held in GenBank; results are summarized in Table 2 and Fig. 1. Sequences from the host fish, groper and kingfish, matched against marine fishes, with 97 and 96% identities to *Epinephelus moara* (GenBank Accession No. FJ176794.1) and *Auxis rochei* (GenBank Accession No. AB193747.1), respectively, reflecting the limited number of fish species sequenced for this DNA region.

Three species of scutiociliate were identified from the New Zealand SSU rRNA gene sequences (Table 2 and Fig. 1), but only 2 species were associated with fish mortalities. *Uronema marinum* was the only ciliate identified in dead juvenile groper in the hatchery during the southern summer 2007–08, while *Miamiensis avidus* was associated with juvenile groper mortalities in summer 2006–07 and summer 2008–09, and adult kingfish mortalities in February 2007 and November 2008. Even though the ciliates were identified in dead fish, they might not have been the primary cause of mortalities but a secondary invasion in stressed or damaged fish. Fin damage is common in hatchery reared juvenile groper, especially the spiny dorsal and pectoral fins, and opportunistic ciliates may invade damaged tissues. Other ciliates are known to enter fish hosts via existing lesions (Paramá et al. 2003), and both *U. marinum* and *M. avidus* are known pathogens in marine fish farms and coastal waters in the northern hemisphere (e.g. Jung et al. 2005, 2007). The first signs of scuticociliatosis in juvenile groper were skin lesions, typically haemorrhagic lesions at the base of the fins, with moribund fish exhibiting loss of balance (L. Tubbs, NIWA, pers. comm.). Once external lesions were noted the progression of the disease was rapid with death occurring within 24 to 48 h (Y. Gublin pers. obs.).

Uronema marinum and *Miamiensis avidus* have not been previously identified in New Zealand waters and might be cosmopolitan species, alternatively the New Zealand specimens might be closely related species that have not diverged from their northern sister taxa in the SSU rRNA gene. Some species such as *U. nigricans* appear to be cosmopolitan and adapted to live in marine and freshwater environments (Crosbie & Munday 1999) as are other ciliates (Finlay et al. 1999). Faster-evolving regions of DNA will be required to assess the phylo-geographic relationships among the northern and southern scutiociliates. In this respect, DNA fingerprinting of the morpho-species *Parauronema virginianum* has indicated a possible species complex (Shang & Song 2005).

A third scutiociliate, *Mesanophrys carcini*, was associated with rotifer but not groper mortalities in the hatchery. *M. carcini* is known to cause scuticociliatosis in farmed turbot *Scophthalmus maximus* in China (Wang et al. 2005), where it is a common ciliate in coastal waters and in marine farms (Shang et al. 2003),

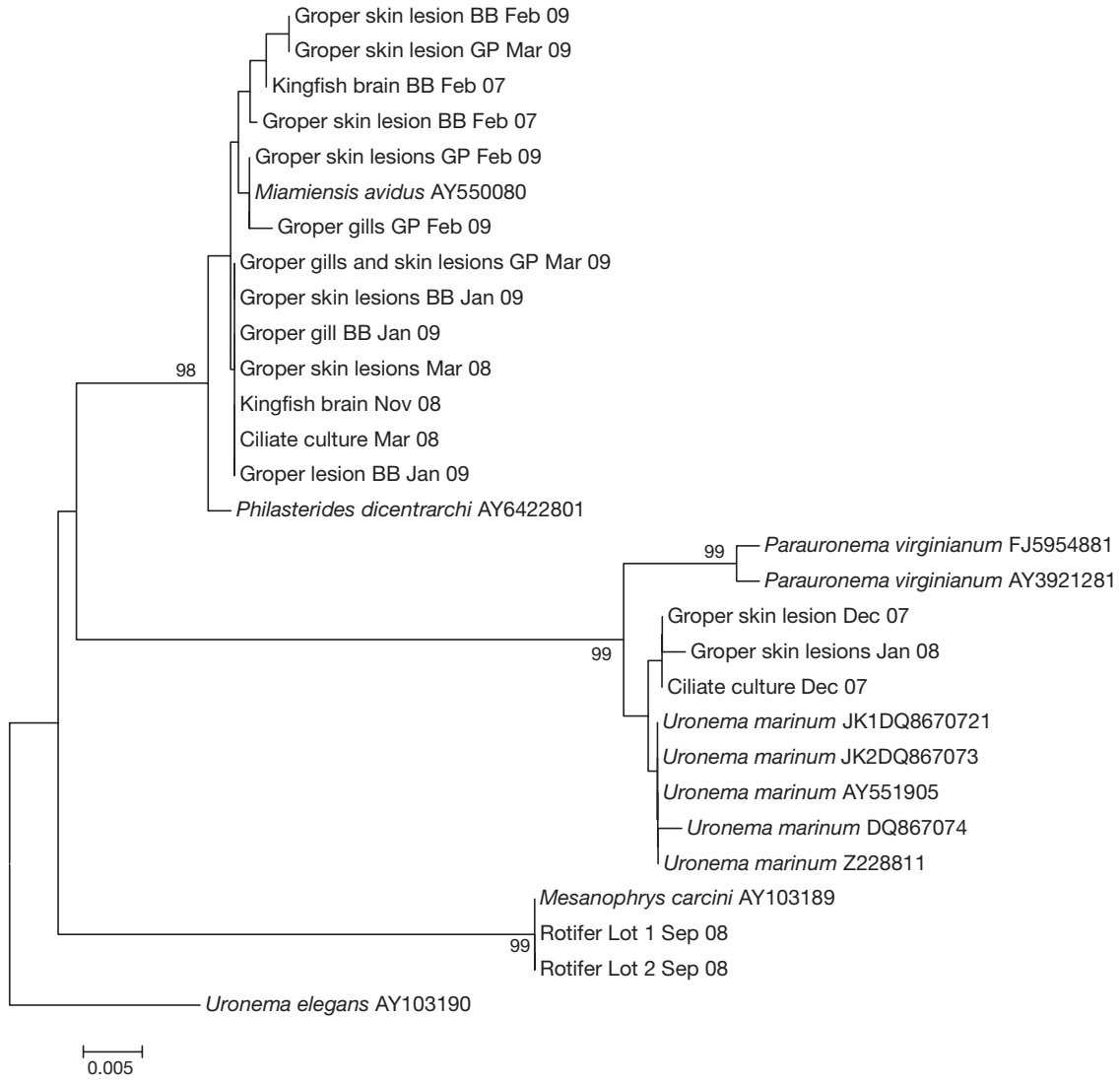

Fig. 1. Small subunit (SSU) rRNA neighbour-joining tree based on Tamura-Nei (TrN) distances used for identification of unknown ciliates in fish hatcheries in New Zealand. The tree has been rooted with *Uronema elegans*. Numbers at nodes are bootstrap percentages (>75%) after 1000 replicates. BB: Bream Bay hatchery; GP: Greta Point aquarium. GenBank Accession Nos. are given after species names

and is a known pathogen in the northern hemisphere crab *Cancer pagurus* (Bower 1996), and will need to be monitored in future fish mortalities in the New Zealand hatchery.

Use of the ciliate specific primers allows direct amplification from fish tissues taken from dead and dying fish with out the need for cloning or culturing, and although shorter DNA sequences are obtained with the ciliate specific primers (400 to 500 bp), there was sufficient divergence in this DNA subregion to identify ciliates to the species level. Paramá et al. (2006) were similarly able to identify *Miamiensis avidus* in juvenile turbot with a 350 bp product of the 18S rDNA. An RFLP test, based on presence and absence of restriction enzyme sites, identified with the programme Webcutter 2.0 (http://users.unimi.it/~camelot/tools/cut2.html) has been designed to distinguish the 3 ciliate species identified in the 2007–09 mortalities and will provide a more rapid molecular identification tool for ciliates in New Zealand fish hatcheries, but may not discriminate additional species. DNA sequencing will be required to confirm ciliate identities at the start of fish mortality events. The ~500 bp sequence of the SSU rRNA gene, amplified with the Cil 3/Cil 4 primer pair that selectively amplify ciliate DNA in fish tissues, provides diagnostic bases that distinguish recognised taxa, and will be used as a monitoring tool to identify ciliates in the hatchery.

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