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Research paper

Evidence of multiple species of *Chilodonella* (Protozoa, Ciliophora) infecting Australian farmed freshwater fishes

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ABSTRACT

Parasitic *Chilodonella* species, *Chilodonella piscicola* and *Chilodonella hexasticha,* cause considerable economic losses globally to freshwater farmed fish production. Some genetic studies of *Chilodonella* spp. have indicated that many species within the genus may form cryptic species complexes. To understand the diversity of *Chilodonella* spp. infecting Australian freshwater farmed fish, specimens were isolated from infected barramundi (*Lates calcarifer*) and Murray cod (*Maccullochella peelii*) from fish farms in tropical north Queensland (QLD), temperate Victoria (Vic) and New South Wales (NSW) for genetic and morphological analysis. Parasites were stained and measured for morphological description and comparative phylogenetic analyses were performed using the mitochondrial small subunit (mtSSU) rDNA marker. Morphological analyses revealed four distinct morphotypes of *Chilodonella* infecting farmed barramundi and Murray Cod. Three putative species were isolated from barramundi (*Chilodonella hexasticha, C. acuta* and *C. uncinata*) and one from Murray cod (*C. piscicola*). However, phylogenetic analyses detected only three distinct genotypes, with the putative *C. hexasticha* and *C. piscicola* sharing 100% sequence identity. This suggests that Australian isolates of *C. hexasticha* and *C. piscicola* could represent the same species and may exhibit phenotypic plasticity. Further molecular analysis, including isolates from the type localities, should be performed to support or refute the synonymy of these species.

1. Introduction

Parasitic ciliates are considered some of the most harmful parasites of cultured fishes (Lom and Dyková, 1992; Padua et al., 2013; Pinheiro and Bols, 2013). The genus *Chilodonella* (Phyllopharyngea: Chilodonellidae) contains many free-living ciliate species, but only two *Chilodonella piscicola* ((Zacharias 1894; syn. *C. cyprini* (see Moroff 1902), and *Chilodonella hexasticha* (Kiernik 1909) are reported to be opportunistic parasites on freshwater fishes (see Bastos Gomes et al., 2016). Chilodonellosis (infections caused by *Chilodonella* spp.) may result in rapid epizootic events on fish farms with mortalities within two or three days of infection and losses of 50–95% of fish stocks (Paperna and Van As, 1983; Karvonen et al., 2010). It is hypothesised that parasitic *Chilodonella* spp. are generally opportunistic and only cause disease and mortalities when fishes are affected by stressors (Lom and Nigrelli, 1970; Egusa, 1983; Urawa and Yamao, 1992). Changes in environmental conditions (temperature, sunlight, oxygen levels and pH) have been associated with changes in *Chilodonella* spp. numbers as these conditions impact reproduction, generation time and the ability to obtain food from the environment (Rintamäki et al., 1994; Shukla and Gupta, 2001).

Recently, it was proposed that some *Chilodonella* species may comprise cryptic species complexes given discordance between morphological descriptions and genetic analyses (Mitra et al., 2013; Warren, 2013). Cryptic species can be grouped by shared morphology, but advances in molecular studies have revealed divergence in genetic loci characterising multiple genetic and/or biological varieties (Blaxter, 2004; Finlay et al., 2006; Katz et al., 2011). *Chilodonella* species have been traditionally distinguished based on the number of ciliary rows present in the left (longer cilia bands),

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or right (shorter cilia bands) kinety bands (cilia arranged closely in longitudinal rows), the morphology of the cytostome and the size of mature cells (Ashburner and Ehl, 1973; Nikolic et al., 2006; Mitra et al., 2013; Warren, 2013). However, genetic analysis of *C. uncinata* (Ehrenberg 1838) has shown that considerable cryptic species diversity exists within North America *Chilodonella* strains (Dunthorn et al., 2011). Therefore, Australian *Chilodonella* species could also represent a cryptic species complex.

Fish infections caused by *Chilodonella* spp. have attracted increasing research interest as the farming of freshwater fish for food production expands globally. In Australia, freshwater fish farms are affected by frequent parasitic *Chilodonella* spp. outbreaks that can cause losses of up to 10% of revenue for each production cycle (Bastos Gomes et al., 2016). However, until now only two species (*C. hexasticha* and *C. piscicola*) have been associated with infections in Australian fishes. Furthermore, limited investigation into the taxonomy of Australian parasitic *Chilodonella* has been performed to confirm that these infections are caused by only two species. The aim of this research was to use a combined morphological and molecular approach to resolve the taxonomic status of *Chilodonella* impacting freshwater farmed fish in Australia.

2. Materials and methods

2.1. Chilodonella *spp. collection*

Chilodonella spp. were collected for species characterisation from two commercially farmed fishes, Murray cod, *Maccullochella peelii* (Mitchell 1838), and Barramundi, *Lates calcarifer* (Bloch 1790), from eastern Australia between March 2013 and November 2014. Fish hosts of both species ranged from 100 g to 3 kg. For *M. peelii*, parasites were scraped from gills and skin mucus of fish showing behavioural signs of infection (*i.e.* not feeding and gasping at tank edges) from three Victorian farms (27 samples) and one farm in New South Wales (3 samples). These parasites were preserved in RNAlater® (Life Technologies Australia Pty Ltd.30-32 Compark Circuit, Mulgrave Victoria 3170) and 80% ethanol. In the case of *L. calcarifer*, fish showing behavioural signs of infection were selected for sampling from a single farm from tropical north Queensland. Five to 10 fish per pond (126 fish over a period of 20 months) were sedated using the anaesthetic AQUI-S® (Aqui-S New Zealand Ltd; AQNZ) according to the manufacturer's instructions. Mucus from the skin and gills was carefully removed using the blunt edge of a scalpel blade to prevent excess blood in the sample. Mucus was spread on a glass slide using scalpel blades and the fresh mounts examined under a light microscope (40X objective) to confirm infection. Sixty slides (from *L. calcarifer* samples) containing *Chilodonella* cells were selected for silver staining and morphological description. An additional 60 samples containing part of the gill or mucus with *Chilodonella* spp. were also preserved in 80% ethanol for genetic analysis. A detailed workflow for the putative *Chilodonella* spp. isolated from Australian freshwater farmed fishes is found in Table 1.

2.2. Chilodonella *spp.* in vitro *culture*

Chilodonella spp. cells isolated from the gills and skin of infected freshwater *Lates calcarifer* from various epizootic events between October and November 2013 were maintained *in vitro* to enable a surplus of specimens for morphological characterisation and molecular analyses. Ciliates were maintained in six-well plates (FalconTM) in the dark at room temperature. Each well contained 6 mL of filtered, autoclaved pond water obtained from the fish farm of origin. A sterile rice grain was added to each well to support bacterial growth (modified from Bellec et al., 2014).

Initially, polycultures were established from all ciliates present on *L. calcarifer* gills and skin. After a period of 24 h in culture plates in the dark, ciliate cultures were examined under a compound microscope (20×). A single *Chilodonella* cell from mixed cultures was collected using a clean micropipette and placed into the well of a new culture plate (containing 6 mL of filtered, autoclaved pond water and a single sterile rice grain) for specific species isolation. This process was repeated every third day for six months. *Chilodonella* cells were transferred into new plates for continuing *in vitro* culture growth. For fresh preparations, single cells were collected from culture plates and placed on glass slides with two drops of 4% formalin (to stop cell movement).

2.3. *Morphological diagnosis of* Chilodonella *spp.*

The *Chilodonella* spp. ex *L. calcarifer* were isolated from infected farmed fish from tropical north Queensland and freshly prepared by placing parasite cells directly onto glass slides. *Chilodonella* sp. ex *M. peelii* were isolated from infected fish from New South Wales and Victorian farms and stained using Klein's dry silver impregnation (Lom and Dyková, 1992) to observe informative morphological characters for species level identification. For the silver impregnation method, slides were covered with 2% aqueous solution of silver nitrate ($AgNO_3$) for approximately 8 min and rinsed in distilled water. Slides were placed under shortwave UV light for 1 h. Dried slides were mounted with Canada balsam using a coverslip and analysed using compound microscopy (100X objective). Images were taken using a UC50 camera fitted to an Olympus BX53 compound light microscope. Line drawings were produced by examining specimens and photomicrographs. Morphological measurements were performed using LabSens software (Olympus Soft Imaging Solutions). Cell measurements (width and length) are represented in micrometres (μm).

2.4. *Molecular identification of* Chilodonella *spp.*

2.4.1. *DNA extraction and amplification*

DNA was extracted from *Chilodonella* cells obtained from farmed fish and from monocultures maintained *in vitro*. Before DNA extraction *Chilodonella* cells collected from fish farms and preserved in 80% ethanol were concentrated to facilitate cell manipulation. Tubes containing cells were pelleted by spinning at 16,000 g for 15 min, whereas *Chilodonella* obtained from monocultures were pelleted by spinning at 6000 g for 20 min. DNA was extracted using a CTAB (Cetyl Trimethyl Ammonium Bromide) protocol modified from Edwards et al. (1991).

PCR reactions were carried out in a final volume of 10 μL, containing 2 μL of each DNA sample. When PCR inhibitors were present (from fish mucus) DNA samples were diluted 1 in 10 and 2 μL of diluted DNA added to the PCR reaction. A negative (H_2O) and a positive control (*Chilodonella* spp. DNA sourced from infected farmed fish) were used in each extraction. Q5 hot start high fidelity DNA polymerase (New England BioLabs, MA) was used (volumes according manufacturer's instructions). Three markers, SSU rRNA, mtSSU rDNA and ITS1-5.8S-ITS2 were amplified for species identification and for phylogenetic analyses. These markers were chosen based on other studies trying to understand the morphological and molecular phylogenetic relationships among ciliates (Dunthorn and Katz, 2008; Lynn, 2008; Dunthorn et al., 2011; Deng et al., 2015). Each marker was amplified using 10 μM of each gene specific set of primers; mtSSU rDNA primers were mtSSUF and mtSSUR (Dunthorn et al., 2011), with PCR cycling conditions of 1 min at 98 °C; 35 cycles of 5 s at 98 °C, 10 s at 60 °C, 20 s at 72 °C; 1 min of final extension at 72 °C; SSU rRNA was amplified using universal eukaryotic primers Euk A and Euk B (Medlin et al., 1988); ITS-5.8S-ITS2 was amplified using ITS-F and ITS-R (Yi et al., 2009).