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Zschokkella hildae Auerbach, 1910: Phylogenetic position, morphology, and location in cultured Atlantic cod

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ABSTRACT

The myxozoan *Zschokkella hildae* Auerbach, 1910, was detected with a prevalence of 100% in cultured Atlantic cod, *Gadus morhua* L. aged 1+ from a culture facility on the west coast of Scotland. Sporogonic stages of *Z. hildae*, plasmodia producing 2–5 mature spores, were located predominantly in the collecting ducts and ureters of the kidney, and spores were present in the urine collected from the bladder. Less frequently, plasmodia were detected in the interstitial tissue of the kidney. The parasite prevalence in cultured fish was considerably higher than reported in wild fish but no obvious signs of pathology were detected. SSU rDNA sequencing and phylogenetic analysis showed that *Z. hildae* is closely related to a *Sinuolinea* sp. from the urinary system of turbot, *Psetta maxima* (L.), and that these two species, together with other myxozoans from the urinary system of marine fish cluster together in a sub-clade of the recognised marine clade of myxozoans. This sub-clade is characterised by a specific linear expansion segment, helix E23_15 in the secondary structure of variable region V4 of the SSU rDNA. *Z. hildae* and *Sinuolinea* sp. show extraordinary large linear expansion segment in both V4 and V7 and an important number of complementary base changes in the conservative regions of the SSU rDNA, indicating considerable evolutionary changes in the SSU rDNA of these species when compared with other myxozoans from the marine environment.

1. Introduction

Myxozoans are highly specialised metazoan parasites and some members represent important pathogens of cultured marine and freshwater fish. The myxozoan *Zschokkella hildae* was first described by Auerbach in 1910 [1] from the kidney and the urinary bladder of three fish hosts belonging to the order Gadiformes: the greater forkbeard, *Phycis blennoides* (Brünnich) (Phycidae), and two Gadidae, the Atlantic cod, *Gadus morhua* L., and saithe, *Pollachius virens* (L.). In subsequent years *Z. hildae* has been reported from several other gadids, i.e. blue whiting, *Micromesistius poutassou* (Risso) [2]; whiting, *Merlangius merlangus* (L.), haddock, *Melanogrammus aeglefinus* (L.) [3]; Alaska pollock, *Theragra chalcogramma* (Pallas), Pacific cod, *Gadus macrocephalus* Tilesius, saffron cod, *Eleginus gracilis* (Tilesius), polar cod, *Boreogadus saida* (Lepechin) [4]; and Arctic cod, *Arctogadus glacialis* Peters [5], thereby demonstrating its broad host spectrum within the gadids and the closely related phycids. Despite the recent increase in aquaculture of Atlantic cod, most publications mentioning *Z. hildae* refer to its use as a biological tag in wild cod (e.g. [6,7]). Recently MacKenzie et al. [8] mentioned the high level of infection with *Z. hildae* in cage-cultured cod, identifying the parasite as a potential problem for cod farming. Thus, although *Z. hildae* is the type species of the genus *Zschokkella*, little is known about it apart from target organ and host range. The current study aimed to provide a detailed morphological description and comparison with published reports, to determine the exact localisation in the host organs and the phylogenetic position within the Myxozoa, based on SSU rDNA sequences.

2. Materials and methods

2.1. Parasite origin, sampling regime and methodology

Atlantic cod, *Gadus morhua* L. of age 1+ were obtained from a tank-based farm on the Mull of Kintyre, Scotland (55°25′N, 5°44′W) in December 2006 ($n=20$; total length 25.5–29 (27.8) cm) and July 2007 ($n=25$; total length 29–34 (30.9) cm). All fish were transported in local water and were killed by overdose of the anaesthetic (MS222) within 3 days of arrival at the laboratory and processed immediately thereafter as follows: kidney smears of all fish were examined for the presence of myxozoan parasites by light microscopy and parts of infected kidneys were fixed for 24 h in neutral buffered formalin for histology. Thereafter, they were dehydrated through a graded alcohol series and transferred into xylene and then paraffin. Five μm sections were cut and stained with Giemsa. Urine was collected exteriorly from some fish with a pipette after slight pressure on the abdomen in

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the area of the urinary bladder. From other fish, whole urinary bladders were dissected, fixed and processed for histology as described above.

Fresh smear preparations of five kidneys in which *Z. hildae* spores occurred in high numbers were used for plasmodia and spore measurements and the remainder of the organ was used for spore concentration and subsequent DNA extraction. Measurements were taken on digital images (1000× magnification) of 5 plasmodia and 5 spores from each fish (total of 25 spores and plasmodia) according to the recommendations of [9] and using the computer software UTHSCSA ImageTool Version 3.0.

For DNA extraction, spores were concentrated using a 2 phase system of 3.76% polyethylene glycol/4.8% dextran as described previously [10]. This did not allow for complete separation of the spores from the host tissue but concentrated them considerably. The spore samples were then transferred to Eppendorfs containing 300 µl TNES urea buffer (10 mM Tris–HCl (pH 8), 125 mM NaCl, 10 mM ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulphate, 4 M urea) for the extraction of DNA using a standardised phenol-chloroform protocol [11] after digestion with 100 µg ml^{-1} proteinase K overnight at 55 °C.

2.2. Amplification and sequencing of SSU rDNA

The SSU rDNA was chosen as the gene region for determining the phylogenetic position of *Z. hildae* amongst the myxozoans as the SSU rDNA database contains the largest number and variety of myxozoan sequences when compared with other genes and gene regions. Furthermore, the main evolutionary trends within the myxozoans determined by SSU rDNA analyses have been confirmed by LSU rDNA sequences [12] and elongation factor 2 data [13].

The extracted DNA was re-suspended in RNAse/DNAse free water and left to dissolve overnight in a refrigerator. Polymerase chain reactions were performed in a final volume of 30 µl containing 0.01 U$µl^{-1}$ of Thermoprime Plus DNA polymerase and the related 10× buffer with 1.5 mM $MgCl_2$ (ABgene, Epsom, UK), 0.2 mM of each dNTP, 0.5 mM of each primer and 50–100 ng of DNA. Amplification of the SSU rDNA of *Z. hildae* was attempted using a PCR assay previously described [14] which applies universal SSU rDNA primers ERIB1 and ERIB10 [15] and subsequently the specific myxozoan primers MyxospecF and MyxospecR [14]. However, this was unsuccessful. Thus, various other combinations of universal and myxozoan-specific primers were tried and a combination of two PCR reactions successfully amplified the SSU rDNA of *Z. hildae*. The primers used successfully were MyxspecF [14] and 18R [16], as well as 18e [17] and ZHR (designed in the current study and on the basis of the amplicon produced and sequenced using MyxspecF and 18R). All primer sequences, the annealing temperature for primer pairs and the origin of the primers are documented in Table 1. The PCR products of the 5 samples were sequenced after purification by spin columns (High Pure PCR Product Purification Kit, Roche Diagnostics, Mannheim, Germany). Sequencing was conducted in a 48 capillary ABI 3730 sequencer (Applied Biosystems) using the BIG Dye Terminator v 3.1 Ready Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions and applying the same primers as used for PCR.

Table 1
Primers used in this study for amplification and sequencing of the SSU rDNA of *Zschokkella hildae* and other myxozoan SSU rDNA isolates from the kidney of *Gadus morhua* from Scotland with PCR annealing temperature for different primer combinations indicated.

	Primer	5′ to 3′ sequence	Author	
48°C	ERIB1	ACCTGGTTGATCCTGCCAG	[15]	
	ERIB10	CTTCCGCAGTTCACCTACGG	[15]	
	MyxospecF	TTCTGCCCTATCAACTWGTTG	[14]	
56°C	MyxospecR	GGTTTCNCDGRGGGMCCAAC	[14]	
	18e	TGGTTGGATCCTGCCAAGT	[17]	64°C
60°C	18R	CTACGGAAACCTTGTTACG	[16]	
	ZHR	GAGACAGATCCTGAGTCACAGAGCA	This study	

2.3. Sequence alignments, SSU rDNA secondary structure analysis and phylogeny

Submission of the consensus sequence obtained to the BLAST on GenBank™ showed that it had not been published previously and identified one myxozoan with considerable sequence identity. For phylogenetic analysis, SSU rDNA sequences of *Z. hildae*, of all other *Zschokkella* spp. sequenced to date, as well as of various representatives of the morphologically similar genus *Myxidium* Bütschli, 1882 and of important matches from the BLAST at GenBank were aligned to SSU rDNA sequences of representatives of all major phylogenetic clades as defined by Fiala [14]. A subset of SSU rDNA sequences from a previous alignment [18] was used as a profile for alignment of the selected sequences in Clustal X version 1.18 [19]. Due to the presence of large inserts in the SSU rDNA of *Z. hildae* and a high percentage of sequence divergence when compared with other myxozoans, secondary structure analyses of the SSU rRNA of *Z. hildae* and its closest relative, *Sinuolinea* sp., were conducted. Some additional structures for different variable regions of the SSU rDNA of related species, *Zschokkella* sp. (DQ377705), *Zschokkella lophii* Freeman, Yokoyama and Ogawa, 2008 (DQ301509) and *Gadimyxa* spp. (EU163421, EU163425, and EU163428) were also obtained. Modelling of blocks of high positional variation by energy minimisation was carried out using the RNA structure program version 4.5 [20]. The obtained structures were compared with known SSU rRNA models ([21]; http://www.psb.ugent.be/rRNA/index.html) and with previously predicted myxozoan models [18], and they were manually adjusted to ensure retention of conserved core elements, taking into account predicted tertiary interaction [22]. Sequences were coded with a dedicated comparative sequence editor (DCSE) secondary structure notations using TextPad 4.7.3 according to the obtained structures, and structural models were displayed using RnaViz2 [23].

For phylogenetic analysis only helical and non-helical structures which could be homologised with certainty were used and all species- or group-specific expansion segments which were not present in all myxozoan taxa were excluded (see [18]). Other ambiguous regions of the alignment were identified and removed by choosing the following strict parameters in the computer program GBlocks 0.91b [24]: minimum number of sequences for a conserved position = 33, minimum number of sequences for a flanking position = 55, maximum number of contiguous non-conserved positions = 8, minimum length of a block = 5, and allowed gap positions = half. Phylogenetic tree inference was carried out by a likelihood-based Bayesian tree sampling procedure (BI) using MrBayes v 3.0 [25] as well as a maximum parsimony (MP) approach using PAUP Version 4.0b10 [26]. The general time-reversible model (GTR + I + Γ [27]) with nst = 6, rates = invgamma, ngammacat = 4 was applied in the BI analysis and posterior probability distributions were generated using the Markov Chain Monte Carlos (MCMC) method. The MCMC was run for 500,000 generations sampling every 100th tree and burn-in was set at 12,000 generations, when the process had reached a stationary state. MP used a heuristic search with tree bisection–reconnection (TBR) branch swapping, random addition of taxa ($n = 10$) and the ACCTRAN option. Bootstrapping was conducted using 1000 replicates. Transition/tranversion ratio was 1:2 and gaps were treated as missing data. All trees were unrooted. The malacosporean *Tetracapsuloides bryosalmonae* Canning, Tops, Curry, Wood and Okamura, 2002 was used as outgroup in both analyses.

3. Results

3.1. Morphological description and location of Z. hildae spore forming stages

Microscopically, different developmental stages of spore-forming plasmodia (Fig. 1a) and spores (Fig. 1b) of *Z. hildae* were detected in