Comparative analysis of 18S rRNA genes from *Myxobolus aeglefini* Auerbach, 1906 isolated from cod (*Gadus morhua*), plaice (*Pleuronectes platessa*) and dab (*Limanda limanda*), using PCR-RFLP

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Abstract

The myxosporean parasite *Myxobolus aeglefini* is a marine species, which can be found in the cartilage of mainly gadid fish species. The parasite has, however, been recorded in the flatfish plaice (*Pleuronectes platessa*) and dab (*Limanda limanda*). It is not clear if isolates from unrelated hosts represent the same species. Therefore a molecular study was conducted to reveal differences at the DNA level between these isolates. PCR was successfully conducted on three different isolates of *Myxobolus aeglefini* sampled from cod (*Gadus morhua*), plaice and dab respectively, using 18S rDNA as template. A PCR product of approx. 1600 base pairs was obtained and RFLP (Restriction Fragment Length Polymerase) was conducted on the fragment with the restriction enzymes *Hinf* I, *Msp* I and *Hae* III. No differences between the isolates were found, suggesting that the three isolates represent the same species.

Introduction

The marine myxozoan *M. aeglefini* was originally described from the whiting *Melanogrammus aeglefini* (L.). It parasitizes the cartilage of other gadid fishes including cod *Gadus morhua* (L.) and hake *Merluccius merluccius* (L.) and has also been found in plaice *Pleuronectes platessa* (L.) (Kabata, 1957; Karasev, 1988; Lom & Dyková, 1992). Mellergaard & Nielsen (1984) recorded it from dab *Limanda limanda* (L.). However, Gaevskaya & Kovaleva (1976) noticed that *M. aeglefini* from gadid fishes differs from specimens described from plaice in a number of morphological details. Morphological distinction of myxospores isolated from hosts (the most widely used diagnostic method) is difficult, but it has been suggested that parasites from distantly related hosts could represent different species (Molnár, 1994; Molnár & Székely, 1999). New methods for molecular diagnosis of myxosporeans have been introduced (Andree et al. 1998; 1999a,b; Eszterbauer et al., 2000; Kent et al., 2001). Primers specific for ribosomal genes in the Myxobolidae were developed by Andree et al. (1997). These primers amplify fragments of the 18S rRNA genes, and thus enabled RFLP or sequencing of a diverse range of *Myxobolus* species (Andree et al., 1998; 1999a,b; Eszterbauer et al., 2000; Kent et al., 2001).

The aim of the present paper was to investigate whether *M. aeglefini* from a gadid fish (cod) was identical to specimens described as *M. aeglefini* from plaice and dab. And further to investigate the 18S rRNA genes of three isolates of *M. aeglefini* isolated from cod, dab and plaice, respectively. Thus by using molecular methods (PCR and RFLP) a comparative analysis was conducted in order to detect differences between the isolates, which could indicate taxonomic differentiation.

Materials and Methods

Sampling

Cartilage samples were taken from infected cod (*Gadus morhua*), plaice (*Pleuronectes platessa*), and dab (*Limanda limanda*) caught in the Kattegat. Parasite infected tissue was then preserved in 96% ethanol. Single sporocysts (approx. 2-3 x10^6 myxospores) were freed from fish tissue under a dissection microscope (7-40x magnification) and subsequently placed in 0.5 ml microfuge tubes.

Myxobolus macrocapsularis and *M. pseudodispar* reference samples were taken from the gill of bream *Abramis brama* (L.) and from the muscle of roach *Rutilus rutilus* (L.) caught in Lake Balaton, Hungary. The plasmodia filled with mature spores were ruptured by a needle under dissection microscope (7-40x magnification) and the spore contents (approx. 1-2 x10^6 myxospores) were collected into microfuge tubes and preserved in 96% ethanol.

DNA extraction

Genomic DNA from myxospores (three isolates of putative *Myxobolus aeglefini* and isolates of *M. macrocapsularis* and *M. pseudodispar*) were extracted using DNA affinity columns from a DNeasy Tissue Kit (Qiagen). Briefly, myxospores were lysed by adding lysis-buffer containing Proteinase K (20 mg/ml) and subsequently incubating the sample at 55 ∞C overnight. Myxospore wall degradation was observed under microscope (400x magnification) before proceeding. The lysed sample was added to the DNA affinity column and subjected to a series of centrifugations at 7000x g after which the sampled DNA was eluted in 25 ml distilled water and stored at –20 ∞C before further processing.

DNA yield

The size and amount of DNA extracted from the samples were measured by agarose gel electrophoresis (2% agarose, 100V, 1 h) by comparing the result with a Low DNA Mass Ladder (Invitrogen). Only samples yielding DNA concentrations greater than 5 ng/ml were used for PCR.

PCR

The oligonucleotide primers MX3 and MX5, developed by Andree et al. (1997) specific for 18S rRNA genes in myxobolidae, were used. The sequence of the primers are as follow: Forward primer (MX5); 5´-CTGCGGACGGCTCAGTAAATCAGT-3´; and reverse primer (MX3); 5´-CCAGGACATCTTAGGGCATCACAGA-3´. The PCR reaction was conducted in PCR tubes with a total volume of 50 ml using Ready-To-Go PCR beads (Amersham Pharmacia Biotech). The reaction volume comprised minimum 25 ng in 5 ml of DNA sample template, distilled water (41 ml), Primer MX5 (1 mM), Primer MX3 (1 mM) and 1.5 U Taq DNA polymerase, 10mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl, 200 mM dNTP. PCR was conducted using a program of denaturation

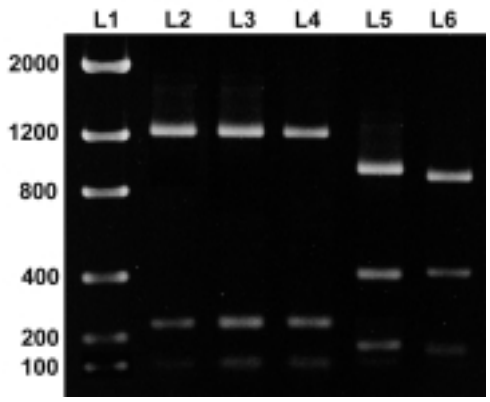

Figure 1. The approx. 1600 bp PCR fragment cut by the restriction enzyme *Hinf* I. L2 to L4 are *M. aeglefini* isolates from plaice, cod and dab respectively. L5 is *M. macrocapsularis* and L6 is *M. pseudodispar*. L1 is the marker.

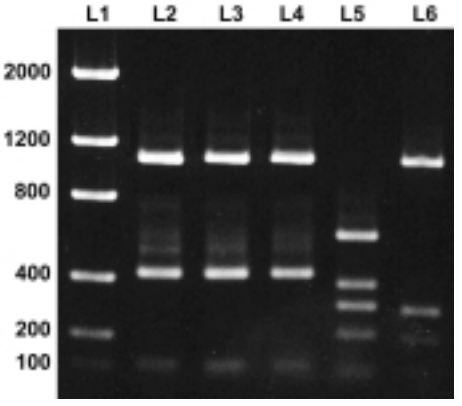

Figure 2. The approx. 1600 bp PCR fragment cut by the restriction enzyme *Msp* I. L2 to L4 are *M. aeglefini* isolates from plaice, cod and dab respectively. L5 is *M. macrocapsularis* and L6 is *M. pseudodispar*. L1 is the marker.

at 95 ∞C for 5 min followed by 30 cycles of 95 ∞C (1 min), 60 ∞C (2 min) and 72∞C (2 min) and an extended elongation period of 72 ∞C (10 min).

Gel purification

The PCR product (10 ml) was electrophoresed on a 2% agarose gel (150V, 2 h). The products (approx. 1600 bp) were cut and isolated from the gel and subsequently purified using a GFX Gel Band Purification Kit (Amersham Pharmacia Biotech) following the manufacturers manual. The purified DNA fragment was eluted in 50 ml of distilled water.

RFLP

The PCR products of the three isolates of *Myxobolus aeglefini* and the three reference species were subjected to a RFLP analysis using the restriction enzymes *Hinf* I and *Msp* I (New England Biolab) (Eszterbauer et al., 2000) and the enzymes *Hae* III (Gibco). Approximately 2 units of enzyme were added to 8 ml of PCR product including 1 ml of buffer (10x) and 0.8 ml distilled water and incubated at 37 ∞C for 2 h. The digested product was loaded on a 1.5% agarose gel for electrophoresis (150V, 2h).

Results and Discussion

The resulting product of *M. aeglefini* DNA after PCR and gel purification was a fragment of approx. 1600 bp in length. The reference species *M. macrocapsularis* and *M. pseudodispar* yielded similar fragments of approx. 1600 bp, which correspond well with the fragments found by Eszterbauer et al. (2000). Figures 1, 2 and 3 show the results of the enzymatic digestion by the restiction enzymes *Hinf* I, *Msp* I and *Hae* III, respectively. DNA fragment lengths were calculated by linear regression analysis for each digestion (Table 1). The comparative RFLP analysis of DNA from the three species of *M. aeglefini* showed no difference when using the restriction enzymes *Hinf* I, *Msp* I and *Hae* III (Table 1). The reliability of the procedure was confirmed by comparing with the RFLP from the species *M. macrocapsularis* and *M. pseudodispar* using the same enzymes as in the marine isolates (Table 1). The specific restriction enzymes were

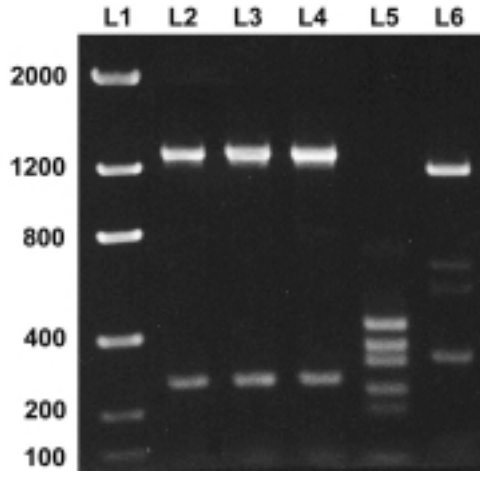

Figure 3. The approx. 1600 bp PCR fragment cut by the restriction enzyme *Hae* III. L2 to L4 are *M. aeglefini* isolates from plaice, cod and dab respectively. L5 is *M. macrocapsularis* and L6 is *M. pseudodispar*. L1 is the marker.

chosen in accordance with other authors, where *Hinf* I and *Msp* I were defined as enzymes for distinguishing the species *M. macrocapsularis* and *M. pseudodispar* (Eszterbauer et al., 2000). The enzyme *Hinf* I was also successfully used by Xiao & Desser (2000) to distinguish eighteen different Myxozoan parasites. However, Xiao & Desser (2000) used different primers in their experiments and the subsequent restriction digests are therefore not directly comparable to the restriction digests found in this paper. In addition, we used the enzyme *Hae* III and this enzyme successfully distinguished our reference species, but not the three *M. aeglefini* isolates. As indicated there is no justification to suggest that the three *M. aeglefini* isolates represent different species. In addition, the three host species inhabit the same marine habitat (demersal fish in Kattegat), which suggests that they all are exposed to infection with these myxozoans. If the isolates are all *M. aeglefini*, it could indicate relative low host specificity. However, this is not unique among myxozoans (Lom & Dykova, 1992). In addition, metazoan parasites from these hosts; e.g. *Hysterothylacium aduncum* and *Anisakis simplex* (Nematoda), *Echinorhynchus gadi*, *Pomphorhynchus laevis* (Acanthocephala), *Cryptocotyle lingua* (Digenea) do also successfully infect all three host species (own observations).

It could alternatively be suggested that marine myxozoans are more closely related than is the case for freshwater forms. However, future advances, such as sequencing of the approx. 1600 bp DNA fragment and subsequent RFLP, are necessary in order to obtain more conclusive evidence of the relationship within and between marine and freshwater myxozoans.

Sample	Species (host)	Approx. size of uncut product (bp)	Approx fragment size (bp)		
			Hinf I	*Msp I*	*Hae III*
1	*M. aeglefini* (plaice)	1600	1300/ 250/ 50	1150/ 450	1350/ 250
2	*M. aeglefini* (cod)	1600	1300/ 250/ 50	1150/ 450	1350/ 250
3	*M. aeglefini* (dab)	1600	1300/ 250/ 50	1150/ 450	1350/ 250
4	*M. macrocapsularis*	1600	950/450/150/50	650/450/350/150	500/425/375/250/150
5	*M. pseudodispar*	1600	1000/ 450/ 150	1150/ 300/ 200	1300/ 300

Table 1. Approximate sizes of fragments derived from restriction digests. Products were digested with the restriction enzymes of either *Hinf* I, *Msp* I or *Hae* III.

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