PCR detection of *Tetramicra brevifilum* (Microspora) infection in turbot (*Scophthalmus maximus* L.) musculature

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SUMMARY

This study investigated the spatial distribution of *Tetramicra brevifilum* spores in the musculature of infected turbot *Scophthalmus maximus*, with the aim of identifying the most appropriate body locations for diagnostic assays. A PCR protocol optimized for the detection of *T. brevifilum* spores in turbot muscle is also described. In fish showing low- and moderate-intensity infection, the spatial distribution of spores was best fitted by a negative binomial distribution, indicating a clumped spatial pattern; the negative binomial coefficient k was lower for fish with low-intensity infection, indicating a more markedly clumped pattern in these fish. In fish with high-intensity infection, the spatial distribution of spores was best fitted by the Poisson distribution, indicating a random pattern. In both low- and moderate-intensity infection, spores were present at highest density in the musculature adjoining the dorsal fins. Samples for PCR were therefore obtained from this location. PCR amplification was of the small subunit ribosomal DNA (SSUrDNA), using a pair of species-specific primers that amplify the 1250 bp product. The PCR protocol developed showed better sensitivity than microscopical techniques (detection rate by microscopy 25%, versus 42% by PCR), suggesting that it may be useful for routine screening for *Tetramicra brevifilum* infection in cultured turbot.

Key words: *Tetramicra brevifilum*, spatial distribution, turbot, small subunit ribosomal DNA, PCR.

INTRODUCTION

Tetramicra brevifilum Matthews and Matthews, 1980 is the only microsporidian known to cause severe disease in turbot, *Scophthalmus maximus* L., causing major losses particularly in fry and juveniles (Estévez *et al*. 1992). It infects the connective tissues of the body musculature (Matthews & Matthews, 1980), and may also infect the liver and intestine (Estévez *et al*. 1992). Although trials have shown that some fish-infecting microsporidians (e.g. *Loma salmonae*) can be effectively controlled with drugs such as fumagillin (Kent & Dawe, 1994), no drugs are currently commercially available for the treatment of infections by microsporidians, including *T. brevifilum*. Thus, at present, the only effective strategy for controlling a microsporidian infection in a fish farm is to detect and isolate the affected fish as quickly as possible; it is also important to identify the source of infection, which is even more difficult (Docker *et al*. 1997*a*).

For early detection of *T. brevifilum* infection, it is first important to determine the best body locations for the assay, and secondly to develop rapid and sensitive methods of diagnosis that are capable of detecting all developmental stages: the traditional diagnostic method (microscopical examination: see Dyková, 1995) is ineffective for detecting *T. brevifilum* during the early stages of development. To achieve these aims, in the present study, we used a statistical approach to investigate the spatial distribution of *T. brevifilum* in the turbot musculature, with the aim of identifying areas with typically high spore density. We also optimized a PCR protocol for diagnosis. PCR has proved highly effective for the detection of other fish microsporidians (Barlough *et al*. 1995; Kent *et al*. 1996; Docker *et al*. 1997*a*, *b*; Georgiadis, Gardner & Hedrick, 1998; Bell *et al*. 1999; Gresoviac *et al*. 2000). In these assays, amplification is of a region of the gene that codes for the small subunit of rRNA (i.e. of the SSUrDNA that codes for SSUrRNA) of the parasite (Leiro *et al*. 2000), using species-specific primers.

MATERIALS AND METHODS

Fish

Microsporidian-infected juvenile turbot weighing 41–51 g (mean 45 g) were obtained from a fish farm in northern Galicia (Spain).

Isolation and counting microsporidian spores in turbot musculature

Spores of *Tetramicra brevifilum* were purified as previously described (Estévez *et al*. 1992). The

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musculature was divided into fifty 64 mm² quadrats, excluding the craneal area and the fins. Each quadrat was dissected out with a scalpel, weighed and sonicated in a Branson Sonifier (Branson Ultrasonic, USA) in 1 ml of 0·1 M NaCl until its complete disintegration. The spores contained in the homogenate were quantified (20 μl aliquots) in a haemocytometer, and the results then expressed as number of spores per mg of muscle.

DNA extraction and purification

Muscle samples were obtained as for isolation of spores, then placed in 0·3 ml of lysis buffer containing 10 mM Tris, pH 7·6, 100 mM EDTA, pH 8·0, 2 mg/ml of proteinase K and 20 mM dithiothreitol. The tissue was disaggregated using a Polytron (Kinematica AG, Switzerland) at full speed for 1 min. The same volume of glass beads (diameter 425–600 μm) was then added, and the mixture was vortexed for 1 min. After removal of the beads, 0·2 ml of lysis buffer containing 0·8 % SDS was added to the vortexed mixture which was incubated overnight at 37 °C with shaking. DNA was then extracted with phenol–chloroform–isoamyl alcohol (PCIA) and precipitated with ethanol. DNA concentrations were measured using an automated spectrophotometer (GeneQuant II, AmershamPharmacia-Biotech).

PCR amplification, cloning and sequencing of T. brevifilum *SSUrDNA*

Initially, SSUrDNA of *T. brevifilum* was amplified as described previously (Leiro *et al*. 2000), using a pair of arbitrary primers (Pomport-Castillon, Romestand & De Jonckheere, 1997). The PCR product obtained was purified by Microcon-PCR (Millipore, USA) and cloned in the pGEM^{R}-T Easy system (Promega, USA) using the kit supplied by the manufacturer. After ligating the PCR fragment, the $DH_{5\alpha}$ cells were transformed by electroporation in a GENE PULSER II (Bio-Rad, USA) using Gene Pulser®/*E. coli* Pulser™ cuvettes, with a 0·1 cm gap at a voltage of 1·8 kV, capacitance of 25 μF, and resistance of 200 Ω. Transformed cells were selected on the basis of antibiotic sensitivity and α-complementation, by culture on LB agar plates containing 100 μg/ml ampicillin, with 50 μl of a stock solution of 20 mg/ml of 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) and 20 μl of a 0·5 M solution of isopropylthio-β-D-galactoside (IPTG) spread over the surface: white colonies were amplified in LB overnight at 37 °C and plasmid DNA was purified using the Plasmid Maxi kit (Qiagen, Germany) following the manufacturer's instructions. To confirm the presence and size of the cloned fragment, we amplified it by PCR using primers SP6 and T7, which flank the region of insertion of plasmid pGEM-T Easy, under the following thermocycling conditions: 94 °C for 5 min, then 30 cycles each of 30 sec of denaturation at 94 °C, 30 sec of annealing at 42 °C, and 2 min of polymerization at 72 °C, with an additional 7 min at 72 °C at the end of the reaction. The PCR-amplified products were analysed by agarose gel electrophoresis to verify the presence of a single band of the correct size. The SSUrDNA fragment cloned in the pGEM-T Easy vector was sequenced in complementary directions using the Dye terminator cycle sequencing kit (CEQ™ DTCS Kit, Beckman Coulter, USA) and loaded into the automatic CEQ™ 2000 DNA Analysis System (Beckman Coulter, USA). Nucleotide sequences are deposited in GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA) with accession number AF364303.

Primer selection and PCR conditions

Tetramicra brevifilum-specific PCR primers were designed in view of the nucleotide sequence obtained, avoiding secondary structure and self-complementary sequences, using the 'Design Primers' program available online at the *Saccharomyces* Genome Database site (http://genome-www.stanford.edu/Saccharomyces). The forward primer used was 5′-TTGATTCTGCCTGACCT-GGA-3′ (F-SP-SSU), and the reverse primer 5′-GGCGGTGTGTACAAAGAACA-3′ (R-SP-SSU). These primers amplify a 1250-bp fragment. The other PCR components were as described (Leiro *et al*. 2000). The template was 50 ng of genomic DNA, obtained from muscle samples as indicated in the previous section. Thermal cycling was as follows: 94 °C for 5 min, then 30 cycles at 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 2 min, and finally extension for 7 min. The PCR-amplified products were analysed on a 1 % agarose gel in TBE buffer, then stained with ethidium bromide and photographed with a digital camera (Leiro *et al*. 1999).

Statistical analysis

All statistical analyses were performed with the freeware program Krebs/Win (version 0.9), available online from ftp://gausse.biology.ualberta.ca/pub/jbrzusto/krebs. The fits of theoretical frequency distributions (Poisson, binomial, negative binomial) to the observed distributions (see Fig. 2) were assessed by chi-square goodness-of-fit tests. In addition, we calculated 3 indices of dispersion (see Krebs, 1989): the index of dispersion I (i.e. variance-to-mean ratio), the Morisita index of dispersion I_D, and the standardized Morisita index of dispersion I_P.

RESULTS

Spatial distribution of T. brevifilum *xenomas*

Fig. 1 shows a graphic representation of the density (spores/mg) of *T. brevifilum* xenomas in each

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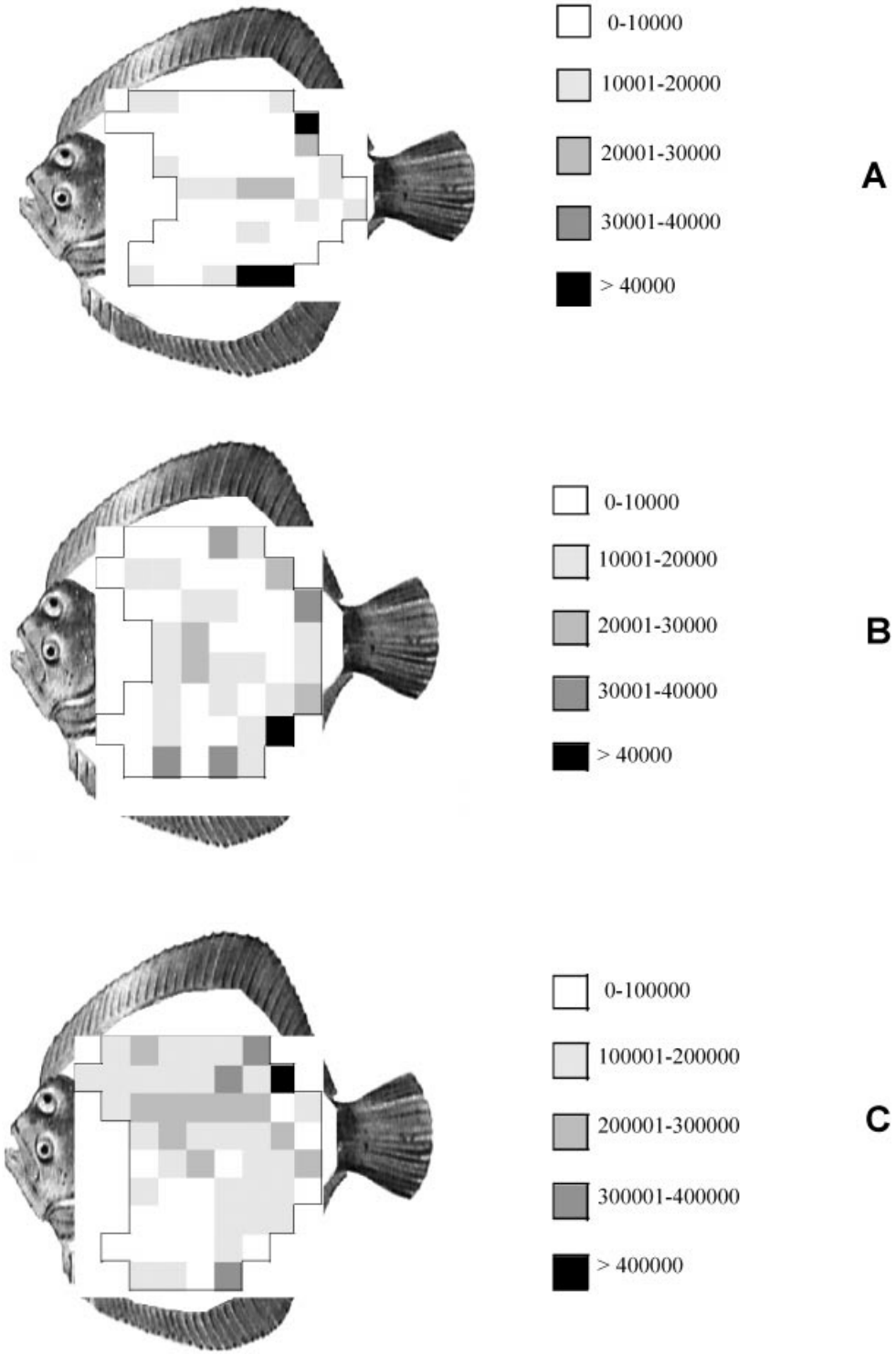

Fig. 1. Schematic diagrams showing spatial patterns of *Tetramicra brevifilum* infection intensity (spores per mg of muscle) in quadrats of the musculature of turbot with mild, moderate and severe infection (A, B and C respectively).

quadrant of each of the 3 fish type studied (A, mildly infected; B, moderately infected; C, severely infected; assessments of infection intensity on the basis of microscopical examination). As can be seen, in the mildly and moderately infected fish, xenomas were present at higher density in the musculature at the base of the dorsal fins, whereas in severely infected fish, the xenomas were homogeneously distributed.

To characterize the observed spatial patterns (as clumped, random, or regular: see Fowler, Cohen & Jarvis, 1998), we used several alternative procedures.

First, we used chi-square tests to investigate the fit of the observed frequency distributions to the Poisson distribution (indicating a random spatial distribution), the binomial distribution (indicating a regular distribution), and the negative binomial distribution (indicating a clumped distribution)

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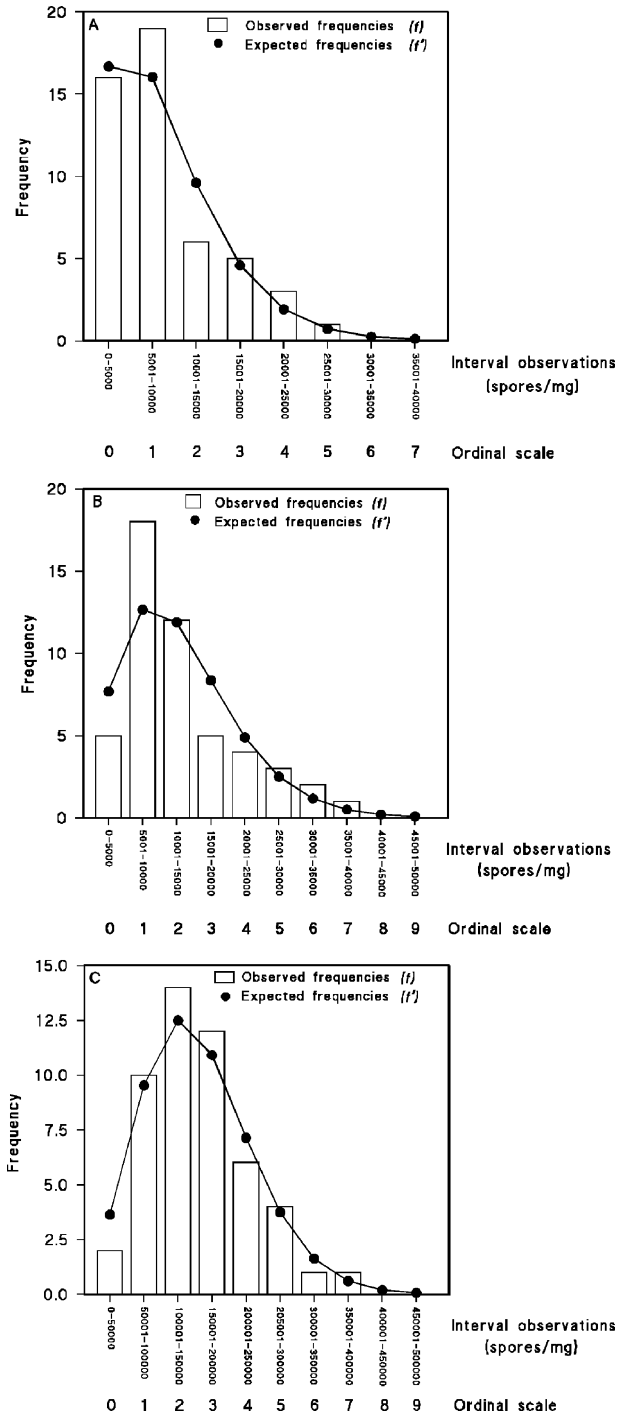

Fig. 2. Frequency distributions of *Tetramicra brevifilum* infection intensities (number of spores per mg of muscle). The results are expressed on an interval scale, converted to an ordinal scale for the statistical analysis. The histograms shows observed frequencies in each fish (mild infection, A; moderate infection, B; severe infection, C; see Fig. 1).

(Krebs, 1989) (Fig. 2). The chi-square values obtained (Table 1) indicate that the data for lightly and moderately infected fish were best fitted by a negative binomial distribution, indicating a clumped pattern, while the data for the severely infected fish were best fitted by a Poisson distribution, indicating a random pattern. The value for the negative

Table 1. Statistical descriptors of the spatial distribution of *Tetramicra brevifilum* spores in infected turbot (Mild infection, A; moderate infection, B; severe infection, C; results of chi-square tests to assess fit to candidate theoretical distributions, negative binomial coefficient (k), and indices of dispersion (index of dispersion, I; Morisita index of dispersion, I_D; standardized Morisita index of dispersion, I_P).)

Samples	Mean ($\mu = \bar{x}$)	Variance (S^2)	Statistical distributions				Indices of dispersion			
			Distribution	χ^2 Goodness-of-fit test	D.F.	Probability	I	k	I_D	I_P
Sample A	1·260	1·670	Negative binomial	3·060	7	0·691	1·320	4·061	1·254	0·372
			Poisson	5·443	6	0·488				
			Binomial	22·715	5	0·000				
Sample B	2·140	2·900	Negative binomial	6·196	9	0·517	1·350	7·185	1·164	0·409
			Poisson	9·747	8	0·283				
			Binomial	181·016	7	0·000				
Sample C	2·620	2·280	Negative binomial	589·498	7	0·000	0·871		0·951	−0·182
			Poisson	2·024	9	0·980				
			Binomial	21·363	7	0·003				

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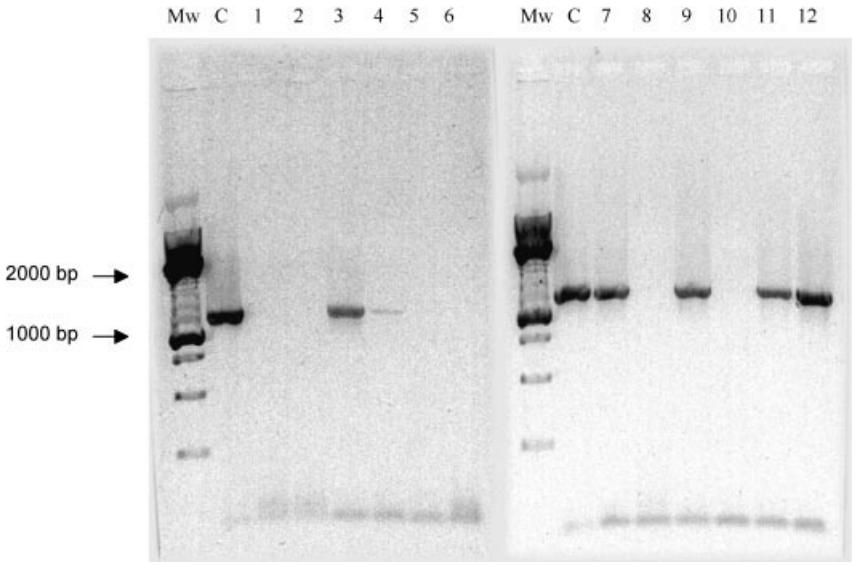

Fig. 3. Ethidium bromide-stained agarose gel electrophoresis of PCR-amplified products of SSUrRNA gene of *Tetramicra brevifilum*, from 12 samples of DNA isolated from the musculature adjoining the dorsal fins of a turbot showing mild *T. brevifilum* infection (as determined by microscopical examination). Lane C shows the positive control (DNA extracted from *T. brevifilum* spores) and molecular weight markers (250 bp ladder).

binomial coefficient k was higher for the mildly infected fish than for the moderately infected fish, indicating more marked clumping in the former (see Krebs, 1989).

Secondly, we calculated the standardized Morisita index of dispersion (I_P) (Smith-Gill, 1975; Krebs, 1989); this is one of the best measures of dispersion because it is independent of population density and sample size (Myers, 1978). I_P was greater than 0 for the mildly and moderately infected fish (0·372 and 0·409 respectively), and less than 0 (−0·182) for the severely infected fish. I_P values greater than 0·5 indicate a clumped pattern with 95% confidence, while I_P values less than −0·5 indicate a regular pattern with 95% confidence. The values obtained for the mildly and moderately infected fish are therefore close to statistical significance for clumping, while the value obtained for the severely infected fish is far from statistical significance, and thus consistent with the random distribution indicated by the good fit of the Poisson distribution. Similar results were obtained with the index of dispersion (I) and the Morisita index of dispersion (I_D) (see Krebs, 1989) (Table 1).

PCR analysis

Finally, we analysed genomic DNA obtained from 12 muscle quadrats located adjacent to the dorsal fins of the mildly infected fish, using PCR to amplify the SSUrDNA, with a pair of specific primers that amplify a 1250 bp fragment (Fig. 3). Clear amplification was obtained for 6 of the 12 samples; 4 of these samples were from quadrats in which we had detected spores by microscopy, while the other 2 (samples 4 and 11) were from quadrats in which we had not detected spores in microscopical examination. Spores were not detected by microscopy in any of the 7 quadrats in which amplification was not obtained.

DISCUSSION

The results of the present study suggest that colonization of the turbot musculature by *T. brevifilum* starts mainly in the muscle adjoining the dorsal fins, probably because this is a very active musculature with an abundant blood supply. Once the infection has established, xenomas break down to release spores to the connective tissues. The released spores characteristically cluster into small groups (Matthews & Matthews, 1980). Finally, the parasite invades the connective tissue via the musculature, as a result of (a) direct infection by sporoplasms, (b) invasion of meronts derived from an initial phase of multiplication elsewhere within the host (Matthews & Matthews, 1980) or (c) xenoma rupture and *in situ* germination of spores, which re-invade neighbouring cells and create new foci of infection. According to this latter hypothesis, microsporidians progress from foci grouped in the peripheral muscle to the central musculature, with random dispersion. However, some authors have queried whether xenoma spores are capable of extruding and initiating a new infection (Lom & Dyková, 1992).

Until relatively recently, the diagnosis of infections of turbot by *T. brevifilum* was based almost exclusively on histological and ultrastructural studies (Matthews & Matthews, 1980; Estévez *et al.* 1992; Figueras *et al.* 1992). Currently, however, several highly sensitive molecular diagnostic systems are available (Leiro *et al.* 1999, 2000, 2001). One of

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the principal advantages of PCR by comparison with microscopical detection is its capacity for detecting infections early (Andree, MacConnell & Hedrick, 1998). Furthermore, the use of PCR based on amplification of small-subunit ribosomal DNA (SSUrDNA, i.e the DNA coding for the SSUrRNA) offers a significant increase in assay sensitivity, in view of the multiple copies of this gene in the spore genomic DNA (Long & Dawid, 1980): in studies with *Loma salmonae*, the limit of detection was as low as 40 spores per g of gill tissue (Docker *et al*. 1997*b*). Recently, PCR products of the SSUrRNA gene have been used as probes for the diagnosis of microsporidian infections in fish on the basis of *in situ* hybridization techniques (Sánchez, Speare & Markham, 1999; Lee *et al*. 2000; Leiro *et al*. 2001). In the present study, we used PCR to amplify the SSUrRNA gene in muscle of turbot in which microscopical examination had indicated mild *T. brevifilum* infection, using a pair of primers specifically designed relative to the nucleotide sequence of that gene of *T. brevifilum*. The samples were obtained from the musculature adjoining the dorsal fins. Microscopical examination confirmed the presence of spores in 4 of the 12 samples (detection rate 33 %), while PCR amplification gave a positive result in 6 of the samples (detection rate 50 %). All 4 samples judged positive by microscopy were likewise positive by PCR, while only 2 of the 8 samples negative by microscopy were positive by PCR. An increase in detection rate with respect to microscopical examination has similarly been observed in previous studies using PCR for the detection of microsporidians: for example 45 % using nested PCR versus 30 % on the basis of Uvitex staining (Bell *et al*. 1999).

In conclusion, the colonization of the turbot musculature by *T. brevifilum* starts in marginal regions close to the dorsal fins, and as the infection progresses the parasites disperse randomly throughout the body from these initial foci. The PCR protocol described in the present study gives a better detection rate than microscopical examination, suggesting that it may be useful for routine screening for *T. brevifilum* infection in farmed turbot. PCR also has other advantages, including detection of all developmental stages, and a lower risk of misidentification than microscopy.

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