RFLP analysis of PCR-amplified small subunit ribosomal DNA of three fish microsporidian species

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SUMMARY

The phylogenetic relationships of the microsporidian species *Microgemma caulleryi*, *Pleistophora finisterrensis* and *Tetramicra brevifilum* were investigated on the basis of restriction fragment length polymorphism (RFLP) analysis of PCR-amplified small-subunit rDNA (SSUrDNA). Using PCR primers specific for microsporidian SSUrDNA, a single product was obtained from each species, and heteroduplex analysis indicated a high degree of sequence homology among the 3 products. In RFLP analysis of the PCR-amplified SSUrDNA, the enzymes *Alu*I and *Dde*I gave restriction patterns that differed among all 3 species. Phylogenetic analysis using restriction patterns as differential characters indicated that *Microgemma caulleryi* and *Tetramicra brevifilum* are more closely related to each other than to *Pleistophora finisterrensis*.

Key words: microsporidia, riboprinting, phylogeny, small subunit ribosomal RNA, polymerase chain reaction.

INTRODUCTION

Currently, taxonomy and species classification of marine microsporidians is based on type of host, mode of transmission and ultrastructural features, including the morphology of the spores (size, nuclear arrangement, number of turns of the polar tube, and life-cycle characteristics) (Weiss & Vossbrinck, 1998). However, morphological markers may not be sufficient for systematic analysis of microsporidia, and probably need to be supported by molecular characterization (Moser *et al*. 1998). Much of the molecular work on microsporidia has concentrated on the genes coding for ribosomal RNA (rRNA), which occur as tandemly repeated units with numerous copies in the genome (Gatehouse & Malone, 1998). There have been numerous studies of the rRNA-coding genes of microsporidians pathogenic in man (Vossbrinck *et al*. 1993; Zhu *et al*. 1994; Visvesvara *et al*. 1994; Baker *et al*. 1995; da Silva *et al*. 1996; Ombrouck *et al*. 1997; Raynauld *et al*. 1998), but comparatively few studies of microsporidians that infect fish (though see Kent *et al*. 1996; Docker *et al*. 1997; Pomport-Castillon, Romestand & De Jonckheere, 1997; Nilsen, Endresen & Hordvik, 1998).

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In the present study we investigated procedures for the species-level identification of the fish microsporidians *Microgemma caulleryi*, *Pleistophora finisterrensis* and *Tetramicra brevifilum*, based on restriction fragment length polymophism (RFLP) analysis of PCR-amplified small-subunit ribosomal DNA (SSUrDNA).

MATERIALS AND METHODS

Microsporidian spores

Spores of *Microgemma caulleryi* (Leiro *et al*. 1999*a*) were isolated from the liver of naturally infected greater sand-eels *Hyperoplus lanceolatus*, spores of *Tetramicra brevifilum* (Matthews & Matthews, 1980; Estévez *et al*. 1992) from muscle of naturally infected *Scophthalmus maximus*, and spores of *Pleistophora finisterrensis* (Leiro *et al*. 1996*a*) from muscle of naturally infected blue whiting *Micromesistius poutassou*, in all cases as described previously (Estévez *et al*. 1992). Spores were then dried in a Speed-Vac (Savant Instruments, USA) and stored at room temperature until use.

Genomic DNA extraction and purification

Genomic DNA was extracted from microsporidian spores as previously described (Leiro *et al*. 1999*b*). Briefly, the spores were washed by centrifugation in 1 mM Tris–HCl containing 10 mM EDTA, pH 7·5.

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An equal volume of glass balls (diameter 425–600 μm) was then added to the pellet, together with 300 μl of lysis buffer (10 mM Tris–HCl, pH 7·8, containing 100 mM EDTA, 2 mg/ml proteinase K and 200 mM dithiothreitol), and the mixture was incubated for 1 h at 37 °C with shaking, then for 15 min with vortexing (to rupture spores). Two hundred μl of lysis solution (10 mM Tris–HCl, pH 7·8, containing 100 mM EDTA, 2 mg/ml proteinase K, 20 mM dithiothreitol and 0·8 % SDS) were then added, and the mixture was incubated overnight at 37 °C with shaking. DNA was then extracted with phenol–chloroform–isoamyl alcohol (PCIA) and precipitated with ethanol (Sambrook, Fritsch & Maniatis, 1989). RNA was degraded by digestion with 100 mg/ml RNase for 1 h at 37 °C. DNA concentrations were measured using an automated spectrophotometer (GeneQuant II, Amersham Pharmacia Biotech).

Polymerase chain reaction (*PCR*) *and analysis of PCR-amplified DNA*

Small subunit ribosomal DNA (SSUrDNA) of the microsporidian species was amplified using forward primer 5′-GGTTGATTCTGCCTGACGT-3′ (Baker *et al*. 1994) and reverse primer 5′-GACGGGCGGTGTGTACAAAG-3′ (Pomport-Castillon *et al*. 1997). PCR reaction mixtures (100 μl) contained reaction buffer (10 mM Tris–HCl, 50 mM KCl, 1·5 mM $MgCl_2$, pH 9·0), 0·2 mM of each deoxynucleoside triphosphate (Amersham-PharmaciaBiotech), 0·4 μM of each primer, 0·05 units/μl of r*Taq* DNA polymerase (Amersham-PharmaciaBiotech) and 50 ng of genomic microsporidian DNA as template. The reactions were run in an automatic Thermal Cycler GeneAmp PCR System 2400 (Perkin–Elmer), initially at 95 °C for 5 min, then for 30 cycles at 94 °C for 1 min, 55 °C for 1·5 min and 72 °C for 2 min. After completion of the 30 cycles, a 10-min extension phase at 72 °C was performed. PCR products (25 μl aliquots) were separated on a 1 % agarose gel in Tris–borate–EDTA (TBE buffer; 45 mM Tris base, 45 mM boric acid and 1 mM EDTA, pH 8·0), stained with 0·5 μg/ml of ethidium bromide, and photographed with a Sony digital camera under a variable-intensity 312 UV transilluminator (Spectroline, USA). Image analysis was done with the aid of the program Adobe Photoshop version 4.0 (Adobe Systems, Inc.).

Heteroduplex analysis

PCR-amplified products were assayed for the generation of homoduplexes or heteroduplexes by the method described by Soto & Sukumar (1992) and Didier *et al*. (1996*a*). Aliquots (12·5 μl) of PCR products from the different microsporidian species were mixed together, heated to 95 °C during 15 min and then cooled to 25 °C to allow random and complete duplex formation. The duplexes formed were separated by electrophoresis in 1 % agarose, with ethidium bromide staining.

Restriction fragment length polymorphism (*RFLP*) *analysis*

Aliquots (25 μl) of PCR products were digested with 10 units of restriction endonuclease (Boehringer–Mannheim, Germany) for 3 h at 37 °C. The reactions were stopped by addition of 10 volumes of a buffer containing 95 % formamide, 25 mM EDTA, pH 8·0, 0·05 % bromophenol blue and 0·05 % xylene cyanole. The restriction digests were electrophoresed through 3 % NuSieve GTG and 1 % SeaKem GTG agarose (FMC Bioproducts, USA) in TBE buffer for 2 h at 5 V/cm, then stained with 1 μg/ml ethidium bromide for 15 min at room temperature, then photographed with a digital camera under UV illumination.

Phylogenetic analysis

Phylogenetic trees were constructed using the Dollop and Dolpenny Polymorphism Parsimony Program (version 3.5c) contained in the PHYLIP package (Felsenstein, 1995). In this analysis each fragment is considered as a different character (Pomport-Castillon *et al*. 1997).

RESULTS

For 3 marine microsporidians, *Microgemma caulleryi* (a parasite of the greater sand eel *Hyperoplus lanceolatus*), *Pleistophora finisterrensis* (a parasite of the blue whiting *Micromesistius poutassou*), and *Tetramicra brevifilum* (a parasite of the turbot *Scophthalmus maximus*), PCR amplification of small-subunit ribosomal DNA (SSUrDNA) gave a single product from each species about 1340 base-pairs long (Fig. 1). Heteroduplex analysis revealed a high degree of homology among the 3 products. In this analysis, the PCR products from 2 species were mixed together, heated to separate the DNA strands, and cooled to allow reannealing. If complementary strands have identical sequences, the homoduplexes will migrate to the same positions as the original products. In theory, if even some mismatch exists between complementary strands, heteroduplexes form: these migrate much more slowly than the homoduplexes due to conformational changes. In all 3 analyses (Fig. 2) we observed only homoduplexes.

Digestion of the PCR products with the restriction endonucleases *Alu*I, *Ban*II, *Dde*I and *Sca*I generated different RFLP patterns for the 3 species studied

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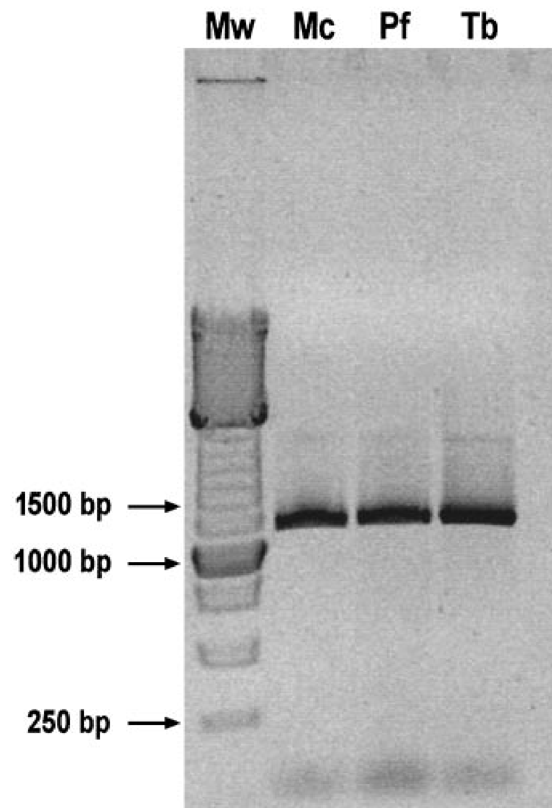

Fig. 1. Electrophoretic analysis (1 % agarose gel) of PCR-amplified products of microsporidian SSUrDNA from *Microgemma caulleryi* (Mc), *Pleistophora finisterrensis* (Pf) and *Tetramicra brevifilum* (Tb) stained with ethidium bromide. Mw, Molecular weight markers (250 bp ladder).

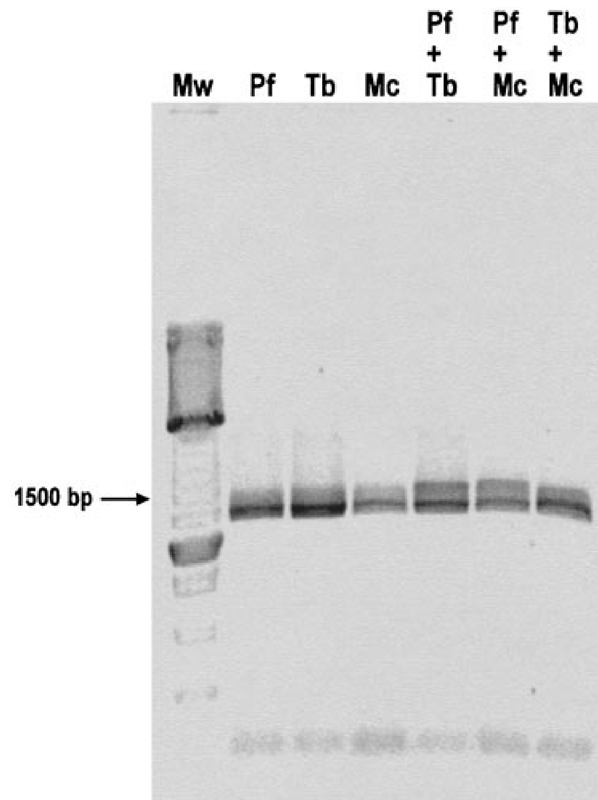

Fig. 2. Double-stranded DNA heteroduplex mobility shift analysis for comparing the PCR SSUrDNA products of the fish microsporidians *Microgemma caulleryi* (Mc). *Pleistophora finisterrensis* (Pf) and *Tetramicra brevifilum* (Tb). Aliquots of PCR-amplified SSUrDNA were mixed as shown, denatured, and allowed to reanneal. The production of homoduplexes was confirmed by electrophoresis in 1 % agarose gel stained with ethidium bromide. Mw, Molecular weight markers (250 bp ladder).

(Fig. 3). *Hae*III and *Mva*I gave identical patterns for *M. caulleryi* and *T. brevifilum* (Fig. 3). *Rsa*I gave identical patterns for *M. caulleryi* and *P. finisterrensis* (Fig. 3). *Pst*I did not cut the SSUrDNA of either *M. caulleryi* or *T. brevifilum* (Fig. 3). *Hin*dII cut the SSUrDNA of all 3 species at the same single site (Fig. 3). *Sal*I (Fig. 3), *Hin*dIII, *Eco*RI, *Eco*RV, *Nae*I and *Pvu*II (results not shown) did not cleave the SSUrDNA of any of the species studied.

Analysis of restriction patterns using the Dollop parsimony method and the Penny algorithm for Dollop or polymorphism parsimony (branch-and-bound to find all most parsimonious trees) a single most parsimonious tree showing the phylogenetic relationship between the 3 species (Fig. 4).

DISCUSSION

Classification of microsporidians has generally been based primarily on histological and ultrastructural studies (Sprague, Becnel & Hazard, 1992). In recent years, however, more sensitive and specific methods have begun to be used, including SDS-PAGE, immunoblotting, DNA sequencing, random amplified polymorphic DNA (RAPD) analysis, and PCR-RFLP analysis (Vossbrinck *et al*. 1993; Leiro *et al*. 1994; Fedorko, Nelson & Cartwright, 1995; Hartskeerl *et al*. 1995; Didier *et al*. 1995, 1996*a*, *b*; Docker *et al*. 1997; Pomport-Castillon *et al*. 1997; Mathis *et al*. 1997; Nilsen *et al*. 1998; Koudela *et al*. 1998; Gatehouse & Malone, 1998; Moser *et al*. 1998). The primary targets for PCR amplification are the rRNA-coding genes, which were found by Vossbrinck & Woese (1986) and Vossbrinck *et al*. (1993) to have prokaryote properties. In the present study, we amplified SSUrDNA from 3 species of microsporidian (*M. caulleryi*, *P. finisterrensis* and *T. brevifilum*), using a pair of primers developed by Baker *et al*. (1994) and Pomport-Castillon *et al*. (1997). In all cases we obtained a single fragment of about 1340 base-pairs in length. Sequencing studies of microsporidians have indicated that the length of the entire SSUrDNA gene ranges from 1252 base-pairs in *Nosema bombycis* to 1352 base-pairs in *Ictyosporidium* sp. (Baker *et al*. 1995).

Recently, the use of double-stranded DNA heteroduplex mobility-shift analyses ('heteroduplex analyses') has been proposed as an alternative to sequencing of the rRNA-coding genes for identification of microsporidian species (Didier *et al*. 1993,

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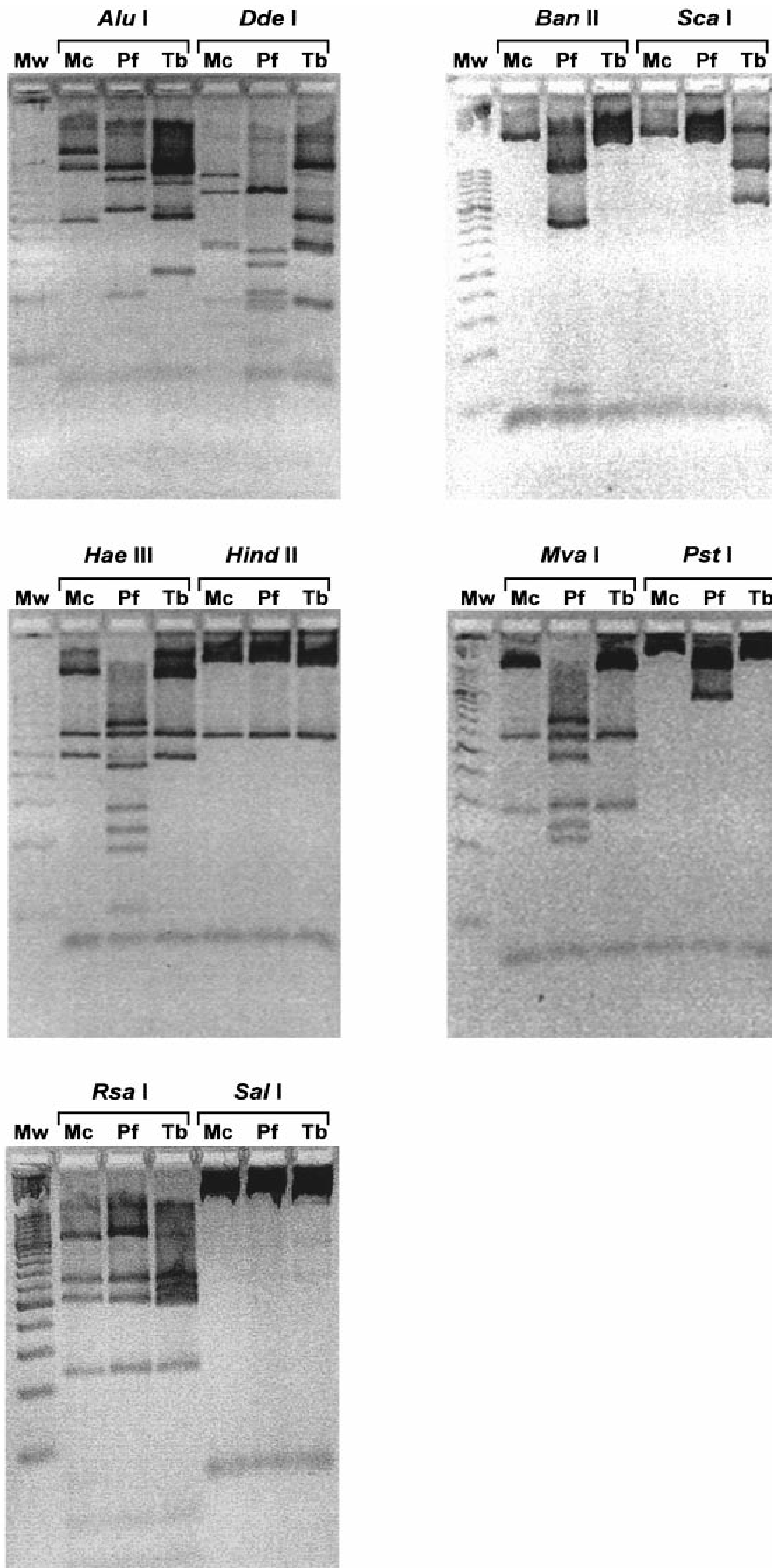

Fig. 3. RFLP analysis of PCR-amplified SSUrDNA of the microsporidians *Microgemma caulleryi* (Mc), *Pleistophora finisterrensis* (Pf) and *Tetramicra brevifilum* (Tb). The PCR-amplified SSUrDNA genes were cleaved with 10 restriction enzymes and analysed by agarose gel electrophoresis with ethidium bromide staining. Mw, Molecular weight markers (50 bp ladder).

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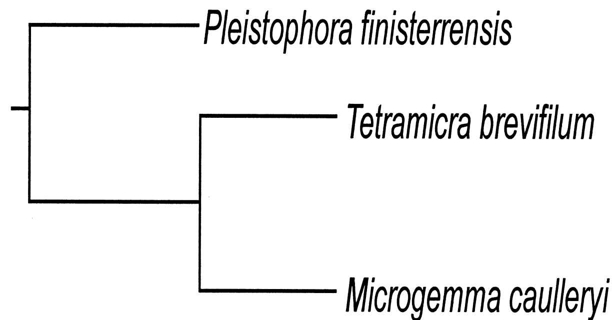

Fig. 4. Phylogenetic tree inferred from parsimony analysis of restriction patterns SSUrDNA of the 3 microsporidian species, as performed using the Dollop and Dolpenny program of the PHYLIP package. Bootstrap values were all 100% or very close to 100%.

1996*a*, *b*). In the present study, no heteroduplexes were detected when PCR-amplified SSUrDNA of the 3 species studied were incubated pairwise together, indicating scant variability in the nucleotide sequence of this gene. Similarly, heteroduplexes were not detected in a previous study of PCR-amplified SSUrDNA of microsporidians (Vossbrinck *et al*. 1993), indicating that the SSUrDNA gene is relatively invariable. In view of these previous findings, Didier *et al*. (1995) argued that differences among microsporidian species probably reside in the hypervariable intergenic spacer region.

DNA sequencing provides a basis for detailed and accurate analysis of the phylogenetic relationships among taxa, but it is expensive and time-consuming. RFLP analysis constitutes a simpler and more rapid alternative, which has been widely used for the identification of microsporidian species (Vossbrinck *et al*. 1993; Fedorko *et al*. 1995; Hartskeerl *et al*. 1995; Didier *et al*. 1995, 1996*a*, *b*; Pomport-Castillon *et al*. 1997; Raynaud *et al*. 1998; Koudela *et al*. 1998; Moser *et al*. 1998). The 'riboprints' of *M. caulleryi* (i.e. the restriction-fragment patterns obtained after digestion of PCR-amplified SSUrDNA with endonucleases, in this case *Alu*I, *Hae*III and *Rsa*I) coincide closely with those obtained for *Microgemma ovoidea* using the same PCR primers (Amigó *et al*. 1996; Pomport-Castillon *et al*. 1996). This provides further molecular-level support for our view that *M. caulleryi* – a microsporidian from the liver of *H. lanceolatus* that was initially assigned to the genus *Glugea* (Van Den Berghe, 1940) – indeed belongs to the genus *Microgemma* (Leiro *et al*. 1999*a*).

The branching pattern of the most parsimonious tree indicates a close phylogenetic relationship between *M. caulleryi* and *T. brevifilum*. This result is in accordance with the marked similarities observed between *Tetramicra* and *Microgemma*, both as regards microstructure (Ralphs & Matthews, 1986) and surface antigens (Leiro *et al*. 1996*b*). A previous study has suggested that *P. finisterrensis* is closely related to species of the genus *Glugea* (Nilsen *et al*. 1998), while our results (both those of the present study, and those of a previous study of surface antigens; Leiro *et al*. 1994) indicate that it is phylogenetically distant from both *M. caulleryi* and *T. brevifilum*.

In conclusion, analysis of restriction digests of PCR-amplified SSUrDNA from 3 microsporidians of marine fishes (*M. caulleryi*, *P. finisterrensis* and *T. brevifilum*) indicates that 2 endonucleases (*Alu*I and *Dde*I) are potentially of value for identification of these species. However, this needs to be confirmed in further studies of other microsporidian species of marine fishes. Our results also indicate a close phylogenetic relationship between *M. caulleryi* and *T. brevifilum*.

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