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ORIGINAL PAPER

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Use of ITS rDNA for discriminating of larval stages of two microphallid (Digenea) species using *Hydrobia ulvae* (Pennant, 1777) and *Corophium volutator* (Pallas, 1766) as intermediate hosts

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Abstract Digenean trematodes encompass several species with little morphological differentiation in the larval stages and, as a result, uncertainty prevails regarding species identification. The microphallid trematode *Maritrema subdolum* occurs widespread geographically in mud snail and crustacean hosts in European marine shallow-water ecosystems. Larval stages of this and other congeneric species are, however, difficult to separate morphologically. To verify the species status and to secure identification of two co-occurring microphallids, *M. subdolum* and microphallid sp. no. 15 (Deblock, 1980), we examined the nucleotide sequences of the internal transcribed spacer regions (ITS1, ITS2). From fragments consisting of both ITS regions and the 5.8S gene (nearly 1,200 bp), a sequence divergence of 2.9% between the two types was recorded. In accordance with the morphological traits of the cercariae (stylet shape, length), the results support the view that the two types actually represent different species. Species-specific primers were prepared for each species. They proved to be efficient diagnostic tools for identifying single larval-stage individuals. Using these primers, infections in host organisms were also verified without performing a dissection of the host individuals.

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Introduction

The description of DNA sequences through the application of PCR and sequencing techniques is increasingly being applied as a supplement to traditional morphological approaches for the identification of trematodes (Nadler 1990; Jousson and Bartoli 2000). Knowledge of the nucleotide sequences of DNA markers, such as the ribosomal internal transcribed spacer regions (ITS1, ITS2), enables a distinction between closely related genotypes (e.g. Luton et al. 1992; Morgan and Blair 1995). Furthermore, in principle diagnostic primers can be prepared from sequence data (Porter and Collins 1991) to identify parasite individuals in any form and stage of its life cycle.

We explored DNA techniques in a study of microphallid trematodes in intermediate host populations from marine shallow-water ecosystems. The mud snail *Hydrobia ulvae* (Pennant, 1777), which is a widespread and ecologically important gastropod in shallow-water lagoons and estuaries along the North Sea coast and Atlantic shorelines of Europe, is intermediate host to numerous digenean species. We are currently focusing on microphallid species utilizing *H. ulvae* and *Corophium volutator* (Pallas, 1766) as first and second intermediate host, respectively, and shorebirds as final host. Two of these trematodes [*Maritrema subdolum* (Jaegerskjold, 1909) and *Microphallus claviformis* (Brandes, 1888)] are reported to influence the population dynamics of their amphipod hosts (Lauckner 1987; Jensen and Mouritsen 1992; Mouritsen and Jensen 1997; Jensen et al. 1998; Meissner and Bick 1997, 1999; Meissner 2001). Considering that closely related microphallids may have quite different host effects (Combes 1995), a proper identification of sibling species is required for ecological studies. Among the microphallids utilizing *H. ulvae*, a few types exist in which uncertainty prevails regarding their species status. One of these, whose cercariae are morphologically very similar to those

belonging to *M. subdolum* has been named microphallid sp. no. 15 by Deblock (1980). Its life-cycle stages in its second intermediate host and final host have not yet been described.

This paper presents ITS sequence data for the two above-mentioned microphallids (from cercaria of *M. subdolum* and cercaria of microphallid sp. no. 15) collected from Danish and French shallow-water localities in order to verify their species status. Based on sequences obtained from larvae, species-specific primers have been prepared and used for species diagnosis of a number of microphallid life-cycle stages.

Materials and methods

Collection and storing

Microphallid larvae were obtained from naturally infected invertebrate hosts. Mud snails (*H. ulvae*) were collected from intertidal localities in the Danish Wadden Sea (Højer and Skallingen, 1996–1998) and from the south-west coast of France (Bellevue, Ile d' Oléron and Fier d'Ars, Ile de Ré, 1996–1997). Of the infected snail stock, 44 specimens were from the French sites and 23 were from the Danish sites. They were stored in laboratory tanks supplied with running seawater and provided with a natural substratum rich in epipelic diatoms. Mud shrimps (*C. volutator*) were collected at Højer, Denmark (autumn 1999). Before dissection and the isolation of metacercariae, the mud shrimps were kept in aquaria at 15 °C. In addition, samples of infected host specimens were stored in 80% ethanol at 4 °C prior to DNA analysis.

Preparation of life-cycle stages

Cercariae clones

Infected *H. ulvae*specimens were incubated individually in Petri dishes at 25 °C under an artificial light source for 20 h. Emerged clones of cercariae (50–300) were harvested using a micropipette. They were identified by stylet size and shape, according to Deblock (1980). A total stock of 67 infected snails, producing at least one unique clone of cercariae each was used for analysing microphallid DNA.

Single specimens of cercariae and sporocysts

Following the initial DNA identification procedure (PCR, sequencing, alignment), 11 snails were selected for species diagnosis using ITS primers. From each group of cercariae shed by snails, a single specimen was isolated. After being harvested for cercariae, snails were fed to stimulate further parasite production. Two snails, infected with microphallid sp. no. 15 and *M. subdolum*, respectively, were dissected; and nine microphallid sp. no. 15 and ten *M. subdolum* sporocysts were sampled from their gonads. To eliminate the possibility of double infections and to examine whether microphallid DNA could be isolated directly from snail hosts, cercariae and snail tissue remnants were also isolated for further diagnostic analysis.

Metacercariae

Infected specimens of *C. volutator* were dissected to isolate microphallid cysts, easily recognized and sampled from the abdomen of infected amphipods. The cysts obtained were separated according to size and shape.

Corophium

Specimens of *C. volutator* were examined for parasites using diagnostic primers. Before DNA extraction, *C. volutator*specimens were homogenized with liquid nitrogen.

DNA extraction

DNA from larval stages of the parasites and from *C. volutator* specimens was isolated by the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). Cercarial pellets were lysed in a mixture of 150 µl CTAB buffer and 2 µl proteinase K and incubated for 1 h at 60 °C. DNA was extracted by standard precipitation techniques.

DNA amplification and sequencing

In the microphallid genome, the 18S–28S intergenic region of rDNA was amplified by PCR using the primers BDA (5′-GTCGTAACAAGGTTTCCGTA-3′; Bowles and McManus 1993) and ITS4 (5′-CGTATAGTTATTCGCCTCCT-3′; Baldwin 1993) and annealing to highly conserved sites in the 18S and 28S genes, respectively. Due to the length of ITS1, this region was occasionally amplified with the primers BDA and ITS2JH (5′-GCTGCGCTCTTCATCGACAC-3′; modified after Baldwin 1993), the latter being complementary to a sequence in the 5.8S gene. Sequencing of the DNA fragments from the two parasites types enabled us to identify and prepare species-specific primers. Thus, a species diagnosis was performed with two internal ITS1 primers: M.s-ITS2r (5′-AACGAAGCGACAAAGCCGAAT-3′) specific for *M. subdolum* and Mic.15-ITS1f (5′-AGTTCTGCCCAGTCTCAAATA-3′) specific for microphallid sp. no. 15, in combination with the BDA and ITS4 primers, respectively.

PCR was carried out in 20-µl reaction volumes with mixtures containing 10 pmol each primer, 0.2 m *M* dNTP and 1.5 units DyNAzymeTM II DNA polymerase (Finnzymes Oy) with the included buffer (manufacturer's

recipe) and 1–3 μl genomic DNA. The cycling conditions were as follows: 3 min at 94 °C, followed by 39 cycles of 30 s at 93 °C, 40 s at 55 °C and 40 s at 72 °C. In the last cycle, the extension step was prolonged to 7 min at 72 °C. The same protocol was used in the diagnostic amplification set-ups, except for the annealing temperature, which was adjusted to 60 °C and 58 °C, respectively (Table 1).

PCR products were analysed on 2% agarose gels (SeaKem) and purified with the QIAquick gel extraction kit (KeboLab). Fragments were sequenced with the Thermo sequenase fluorescence-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech), using Cy5 end-labelled primers, and then run on an ALFexpress DNA sequencer (Amersham Pharmacia Biotech). Subsequent sequences were edited in a sequence-editing programme (D.G. Gilbert, ver. 0.6) and alignments were performed on the BCM search launcher interface, provided by the Human Genome Sequencing Center.

Results

DNA extracted from each clone of the cercariae shed by the 67 isolated microphallid-infected mud snails was amplified in both directions with ITS-flanking primers. In all cases, the PCR procedure provided a single fragment nearly 1,200 bp long. Sequencing of this fragment containing ITS regions, the 5.8S gene and small pieces of the 18S and 28S gene-coding regions resulted in two different sequences, one with a length of 1,160 bp and the other with a length of 1,164 bp. This was consistent with the two types of microphallid cercaria (*M. subdolum*, microphallid sp. no. 15) identified among the cercariae shed by the infected snails. The alignment of sequences from the two types shows ITS1 as the more variable spacer, containing 28 variant sites, whereas the ITS2 only has 6 variant sites (Fig. 1). The overall sequence divergence is 2.9% (including the rDNA coding regions) between the two morphotypes (Fig. 1, Table 1).

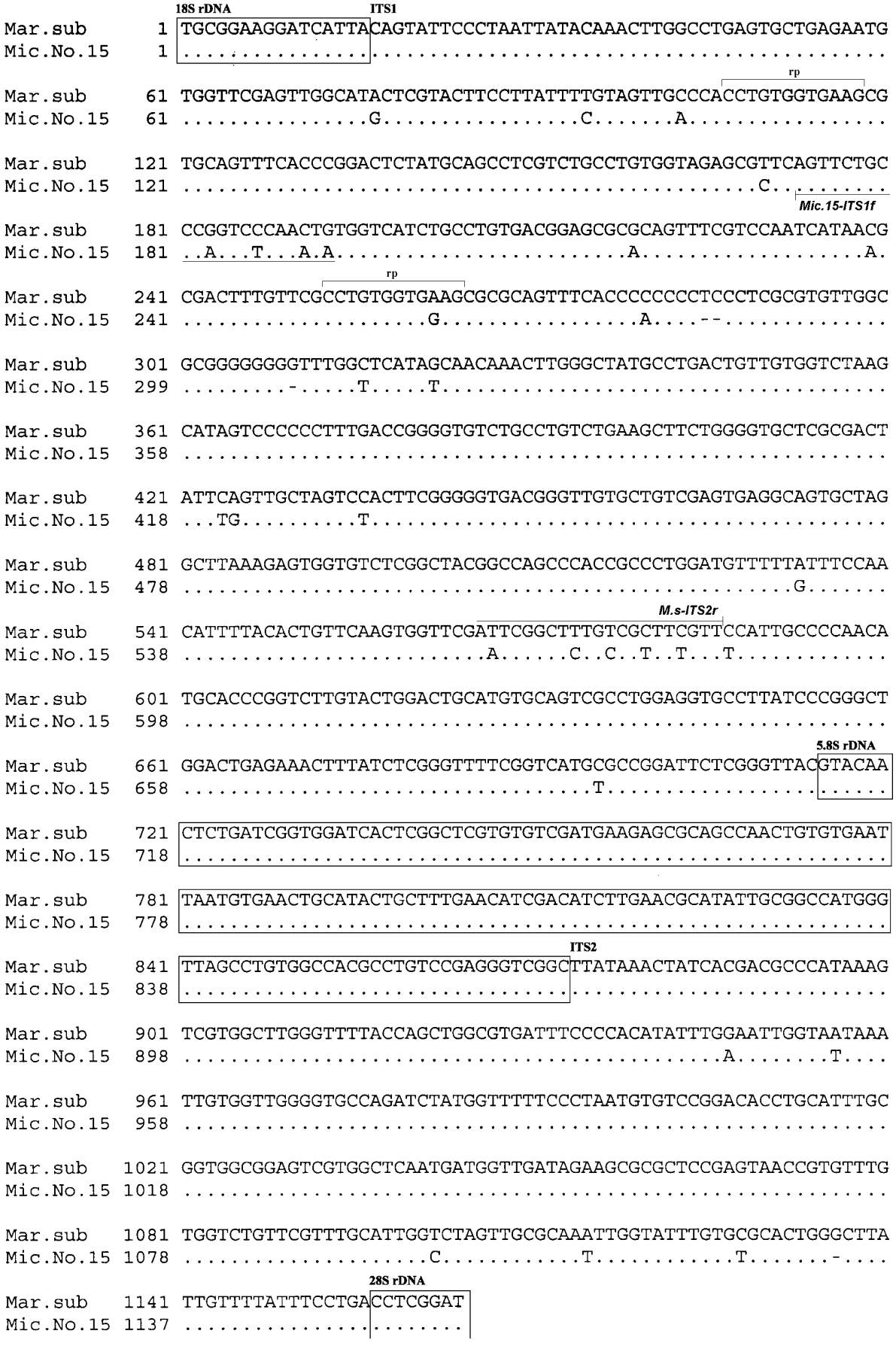
In five of the 30 *M. subdolum* clones, a transition in the ITS1 region (position 582) was observed. None of the observed sequence variants could be related to the geographical origin of the specimens. In both species, the 12-bp sequence CCTGTGGTGAAG was repeated twice in the first half of the ITS1 region (Fig. 1). The two trematode species occurred with nearly equal frequencies among the 67 microphallid-infected snails (30 had *M. subdolum*, 37 had microphallid sp. no. 15), also indicating their equal prevalence in snail populations from the sampling sites in France and Denmark.

Species-specific internal ITS primers applicable for identifying larval stages otherwise indistinguishable by morphological criteria (such as immature and mature metacercariae) were designed from the sequence data (Table 1). A consistent species diagnosis for all isolated larval stages was obtained using these primers. As an example, the band patterns of DNA extracted from 11 unique clones of cercariae amplified with universal BDA and ITS4 (1,200 bp) primers (Fig. 2a) and the bands produced by the amplification of DNA extracted from individual cercariae in the same clones with the primer specific for microphallid sp. no. 15 (Mic.15-ITS1f) were in accordance with species identification using stylet size as criteria (Fig. 2b). The same diagnostic primer was also applied to amplify DNA extracted from sporocysts (nine specimens) isolated from a snail infected with microphallid sp. no. 15. Amplification with BDA and M.s-ITS2r primers did not produce any bands of the relevant length, whereas amplification with Mic.15-ITS1f and ITS4 produced bands in all cases. The results indicate that only microphallid sp. no. 15 had infected the examined snail. A similar analysis was performed with a *M. subdolum*-specific primer (M.s-ITS2r) on sporocysts isolated from a *M. subdolum*-infected snail

Fig. 1 Alignment of two different sequences consisting of the ITS regions and the 5.8S gene in the ribosomal DNA of two microphallid specimens identified as *M. subdolum* (*Mar.sub*) and microphallid sp. no. 15 (*Mic.No.15*), according to Deblock (1980). Similarity of nucleotides in the two sequences is indicated by a *dot*. The length of the sequences (expressed as the number of basepairs) is indicated at the *left*. *Boxes* represent rDNA genes (5.8S, small fragments of 18S and 28S). Starting points for ITS1, ITS2 and 5.8S rDNA are indicated *above* the sequences. A small repeat sequence at the 3′ end of ITS1 is indicated by *rp* and annealing sites of the applied species-specific internal ITS primers used (M.s-ITS2r, Mic.15-ITS1f) are indicated by *arrows and names*

Table 1 Summary of sequence variation in internal transcribed spacer (ITS) regions and 5.8S gene in the ribosomal DNA between two microphallid species. The species-specific primers used in combination with BDA and ITS4 primers as diagnostic tools are also indicated. The annealing temperature is that at which half of the potential binding sites are thought to have primer bound to them

Character	*Maritrema subdolum*	Microphallid sp. no. 15
Percentage sequence variation between the two species		
ITS1	3.9%	
ITS2	2.1%	
Total, including 5.8S	2.9%	
Other characters		
Diagnostic primer (21mer)	M.s-ITS2r	Mic.15-ITS1f
Oligo sequence	5′-AACGAAGCGACAAAGCCGAAT-3′	5′-AGTTCTGCCCAGTCTCAAATA-3′
Annealing temperature	60°C	58°C

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                 18S rDNA                ITS1
Mar.sub       1 TGCGGAAGGATCATTACAGTATTCCCTAATTATACAAACTTGGCCTGAGTGCTGAGAATG
Mic.No.15     1 ..................................................................

                                                                        rp
Mar.sub      61 TGGTTCGAGTTGGCATACTCGTACTTCCTTATTTTGTAGTTGCCCACCTGTGGTGAAGCG
Mic.No.15    61 ................G.................C.......A.....................

Mar.sub     121 TGCAGTTTCACCCGGACTCTATGCAGCCTCGTCTGCCTGTGGTAGAGCGTTCAGTTCTGC
Mic.No.15   121 ..................................................C.........
                                                                  Mic.15-ITS1f
Mar.sub     181 CCGGTCCCAACTGTGGTCATCTGCCTGTGACGGAGCGCGCAGTTTCGTCCAATCATAACG
Mic.No.15   181 ..A...T...A.A.........................A...................A.

                        rp
Mar.sub     241 CGACTTTGTTCGCCTGTGGTGAAGCGCGCAGTTTCACCCCCCCCTCCCTCGCGTGTTGGC
Mic.No.15   241 ....................G.................A....--..............

Mar.sub     301 GCGGGGGGGGTTTGGCTCATAGCAACAAACTTGGGCTATGCCTGACTGTTGTGGTCTAAG
Mic.No.15   299 .........-.....T.....T..................................

Mar.sub     361 CATAGTCCCCCCTTTGACCGGGGTGTCTGCCTGTCTGAAGCTTCTGGGGTGCTCGCGACT
Mic.No.15   358 ..................................................................

Mar.sub     421 ATTCAGTTGCTAGTCCACTTCGGGGGTGACGGGTTGTGCTGTCGAGTGAGGCAGTGCTAG
Mic.No.15   418 ...TG..........T.....................................................

Mar.sub     481 GCTTAAAGAGTGGTGTCTCGGCTACGGCCAGCCCACCGCCCTGGATGTTTTTATTTCCAA
Mic.No.15   478 ..................................................G.......

                                                        M.s-ITS2r
Mar.sub     541 CATTTTACACTGTTCAAGTGGTTCGATTCGGCTTTGTCGCTTCGTTCCATTGCCCCAACA
Mic.No.15   538 ........................A......C..C..T..T...T.............

Mar.sub     601 TGCACCCGGTCTTGTACTGGACTGCATGTGCAGTCGCCTGGAGGTGCCTTATCCCGGGCT
Mic.No.15   598 ..................................................................

                                                                    5.8S rDNA
Mar.sub     661 GGACTGAGAAACTTTATCTCGGGTTTTCGGTCATGCGCCGGATTCTCGGGTTACGTACAA
Mic.No.15   658 ....................................T.......................

Mar.sub     721 CTCTGATCGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGCGCAGCCAACTGTGTGAAT
Mic.No.15   718 ..................................................................

Mar.sub     781 TAATGTGAACTGCATACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCCATGGG
Mic.No.15   778 ..................................................................

                                                                ITS2
Mar.sub     841 TTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCACGACGCCCATAAAG
Mic.No.15   838 ............................................................

Mar.sub     901 TCGTGGCTTGGGTTTTACCAGCTGGCGTGATTTCCCCACATATTTGGAATTGGTAATAAA
Mic.No.15   898 ..............................................A........T....

Mar.sub     961 TTGTGGTTGGGGTGCCAGATCTATGGTTTTTCCCTAATGTGTCCGGACACCTGCATTTGC
Mic.No.15   958 ..................................................................

Mar.sub    1021 GGTGGCGGAGTCGTGGCTCAATGATGGTTGATAGAAGCGCGCTCCGAGTAACCGTGTTTG
Mic.No.15  1018 ..................................................................

Mar.sub    1081 TGGTCTGTTCGTTTGCATTGGTCTAGTTGCGCAAATTGGTATTTGTGCGCACTGGGCTTA
Mic.No.15  1078 ......................C.............T.............T.......-....

                                    28S rDNA
Mar.sub    1141 TTGTTTTATTTCCTGACCTCGGAT
Mic.No.15  1137 ........................
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(Fig. 3). As indicated, the primers only caught the target DNA. It was also possible to use this species-specific primer on groups of microphallid cysts sampled from infected *C. volutator* specimens supposed to include *M. subdolum* specimens (Fig. 3). The present analyses, together with experimental infections in *C. volutator* with the two microphallid species (unpublished data), indicate that both species produce spherical or slightly ovoid cysts that cannot be identified on the basis of morphometric criteria. Maximum sizes recorded for the spherical and ovoid types are 190 μm and 180–220 μm, respectively.

Finally, DNA extracted from infected Danish *C. volutator* individuals was amplified with a *M. subdolum*-specific primer (M.s-ITS2r) and all examined individuals were positive for *M. subdolum* (Fig. 3). So, even when the target DNA exhibits an insignificant fraction of the total DNA concentration, the species-specific primers still function.

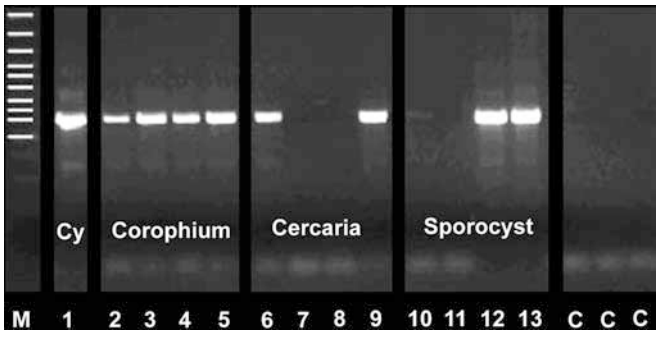

Fig. 3 Bands represent PCR products of DNA extracted from different microphallid larval stages and amplified with specific ITS primers (M.s-ITS2r). *Lane 1* A group of spherical metacercariae (160–180 μm) collected from Danish specimens of *C. volutator*, *lanes 2–5* whole specimens of *C. volutator* infected with microphallids, *lanes 6–9* individual cercariae (*lanes 6, 9* identified as *M. subdolum*, *lanes 7, 8* identified as microphallid sp. no. 15, based on stylet size), *lanes 10–13* individual sporocysts sampled from snails shedding cercaria of either microphallid sp. no. 15 (*lanes 10, 11*) or *M. subdolum* (*lanes 12, 13*)

Discussion

The applicability of the ITS regions as a diagnostic measures

In general, the ITS regions contain a proper level of variation for species separation within the digeneans (Luton et al. 1992; Morgan and Blair 1995; McManus and Bowles 1996). Due to its conserved nature, the ITS2 region is a sensitive and reliable diagnostic marker at the species or genus level (Adlard et al. 1993; Kane and Rollinson 1994; Blair et al. 1997a, 1997b). In comparison, the ITS1 region is far more variable and therefore useful for investigating intra-specific patterns of variation. For instance, using ITS1 sequences, Van Herwerden et al. (1998, 1999) obtained a better resolution among members of the cryptic species complexes *Schistosoma japonicum* and *Paragonimus westermani* and found high degrees of both intra- and inter-individual variation, including a varying number of internal repeats. In the present study of microphallid DNA, we have not found such repeats among a large sample of ITS1 sequences from locations separated by more than 1,000 km and only a negligible amount of intra-specific variation was detected (unpublished sequence data from two other microphallids related to *M. claviformis* reveals ITS1 repeats, but no intra-specific variation). The same pattern was observed in the genus *Echinostoma* (Morgan and Blair 1995), where morphologically indistinguishable sibling species did not show any intra-specific differences and exhibited sequence variation (1.1–3.7%) at the same level as that seen among the microphallids reported here.

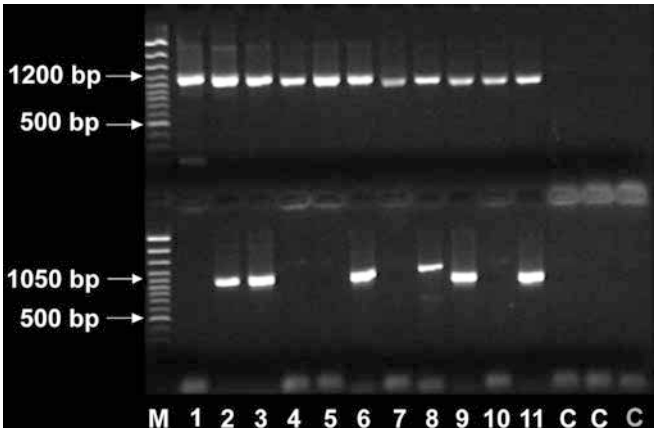

Fig. 2 The upper panel shows PCR products of DNA extracted from 11 unique clones of microphallid cercariae and amplified with the universal rDNA primers BDA and ITS4. The bands (*lanes 1–11*) correspond to the1,200-bp size marker. Five of the clones (*lanes 1, 4, 5, 7, 10*) were identified as *M. subdolum* according to stylet length, whereas the remaining clones (*lanes 2, 3, 6, 8, 9, 11*) were identified as microphallid sp. no. 15. The lower panel shows PCR products of DNA from the same cercaria-clones but amplified with primers Mic.15-ITS1f and ITS4, a primer-combination specific for microphallid sp. no. 15. In accordance with this, bands were only produced from the cercarial clones identified as microphallid sp. no. 15 specimens. Band lengths of around 1,050 bp were also in accordance with the species identification. Species identification was verified through sequencing of all bands. *M* Ladder with 100-bp steps, *C* Control samples without DNA

Thus, studies of trematode DNA have discovered very different degrees of ITS1 variation. This phenomenon is probably linked to the way concerted evolution processes act in the homogenisation of multiple ITS copies in the rDNA tandem array (Kane et al. 1996; Van Herwerden et al. 1998, 1999). Like in the echinostomes, it seems that the two microphallid species belong to a genus/family where the homogenizing mechanisms within the parasite genome keep the intra-specific variation in ITS regions very low (Dover 1982; Hillis and Dixon 1991).

The morphology of larval stages of microphallid sp. no. 15 was described by Deblock (1980). Its status as a species is based on a few features of the sporocysts (colour) and the cercariae (size, shape) that differ from the typical appearance of these stages in *M. subdolum*

(Deblock 1980). The present ITS sequence data provide additional evidence that microphallid sp. no. 15 and *M. subdolum* actually represent two species and that the shape and size of the stylets are useful species diagnostics. However, the limited sequence variation of only 2.9% between the two species clearly indicates a close relatedness.

The consistent sequence patterns we found in individuals from different subpopulations enabled us to prepare species-specific primers. The identification of trematode species on the basis of ITS-specific primers is reliable, as long as intra-specific variation in the spacer regions is nearly absent. However, even if we have two congruent data sets, which clearly distinguish the two species from each other, precautions should still be taken. We are aware of the possibility that there could be other undescribed microphallid variants in related parasite–host systems showing a nucleotide sequence identical to one of the primers prepared here. As an example, some ITS sequences from DNA extracted from metacercariae hosted by the isopod *Cyathura carinata* are very similar to the sequences reported here (Schulenburg et al. 1999). Therefore, one must test whether a diagnostic primer can distinguish between its target species and a type differing in only two bases at the primer-binding site. Further evidence is required before the general applicability of our primers can be verified.

Host and patterns of distribution

According to our own experimental data (Damsgaard 2002), both species can infect *C. volutator*.*M. subdolum* has been recorded from several second intermediate host species, including amphipods, isopods and decapods (Reimer 1963; Rebecq 1964; Deblock 1980; Kostadinova and Gibson 1994; Zander et al. 2002), and the species has been found in a range of different water birds (Reimer 1963; Kostadinova and Gibson 1994). However, both regarding host spectra and geographical distribution, it has to be recognized that *M. subdolum* and microphallid sp. no 15 cercariae may have been confused in recordings before Deblock's (1980) description; and, even since then, investigators may have overlooked microphallid sp. no. 15 in routine studies because of its morphological similarity to *M. subdolum*. Particularly regarding metacercariae and adults, the diagnostic primers presented may help clarifying morphological differences between adults occurring in birds.

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