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ORIGINAL PAPER

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Molecular characterization and differentiation of opisthorchiid trematodes of the species *Opisthorchis felineus* (Rivolta, 1884) and *Metorchis bilis* (Braun, 1790) using polymerase chain reaction

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Abstract Adult specimens of the opisthorchiid liver fluke species *Opisthorchis felineus* and *Metorchis bilis* could be identified for the first time by molecular biological methods using species specific primers (OF and MB primers) in the polymerase chain reaction (PCR). The OF or MB primers were based on a part of the mitochondrial cytochrome *c* oxidase I gene. A specific product of approximately 200 bp could be amplified for *O. felineus* by means of the specific *O. felineus* primers. By contrast, the amplification of *M. bilis* DNA with MB primers produced a fragment of approximately 110 bp. A specificity of 100% could be demonstrated for both primer pairs. The sensitivities of the PCRs were 10 pg for the *O. felineus* DNA and 100 fg for the *M. bilis* DNA.

Introduction

Trematodes of the family Opisthorchiidae cause opisthorchiidosis in piscivorous mammals, birds and in humans. These liver flukes are biohelminths with an obligatory triheteroxenous aquatic life cycle including freshwater snails of the genus *Bithynia* as the first intermediate host and fish of the family Cyprinidae as thesecond intermediate host. Cases of human opisthorchiidosis only occur sporadically in Germany (Bernhard 1985; Sänger et al. 1991; Steiß et al. 2002). By contrast, cases of this disease constitute a major problem for human medicine in South-east Asia and areas of the former Soviet Union. The number of people suffering from opisthorchiidosis is estimated at 20 million worldwide (World Health Organization 1995).

In veterinary medicine, the importance of opisthorchiid liver flukes was underestimated for a long time. In the last 5 years, trematodes of the family Opisthorchiidae could be detected, at least for the region Berlin-Brandenburg, with high prevalences of up to 42% in the red fox (*Vulpes vulpes*) (Schuster et al. 1999, 2000, 2001). Whereas mainly opisthorchiid trematodes of the species *Metorchis bilis* could be isolated from the fox, the dissection of domestic cats mainly revealed specimens of *Opisthorchis felineus* (Hering-Hagenbeck and Schuster 1996; Schuster et al. 1997).

Up to now, the detection and differentiation of opisthorchiid liver fluke eggs in the faeces of the definitive hosts have been difficult due to the fact that the eggs are very small and cannot be assigned to a specific species using light microscopy. The aim of this study was, therefore, to establish a method for the differentiation and identification of opisthorchiid species using molecular biology.

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Materials and methods

To develop a polymerase chain reaction (PCR) for the detection of opisthorchiid liver fluke DNA, adult trematodes, which had been isolated from various definitive hosts, were used as the starting material for DNA extraction. The types of opisthorchiid liver flukes included in the experiments and their origins are listed in Table 1. All of the liver flukes used for DNA extraction had been preserved in 70% ethanol.

In order to test the PCR system for cross-reactivity with host DNA, additional DNA samples were taken from various intermediate and definitive hosts. Fresh tissue samples from uninfested red fox liver, from the muscles of ide (*Leuciscus idus*) bred in a parasite-free environment and from the intermediate host snails (*Bithynia leachi*, *Bithynia tentaculata*) were used in the experiment. In the case of the snails, care was taken to ensure that only head

Table 1 Species used and origin of test material

Species	Host	Origin
Opisthorchis felineus	Golden hamster (*Mesocricetus auratus*)	Experimental infection
	Muskrat (*Ondatra zibethica*)	Barnim district
	Domestic cat (*Felis silvestris* f. *catus*)	Steglitz, Berlin
	Red fox (*Vulpes vulpes*)	Berlin
	human (*Homo sapiens*)	Russia, western Siberia
Metorchis bilis	Golden hamster (*Mesocricetus auratus*)	Experimental infection
	Domestic cat (*F. s.* f. *catus*)	Experimental infection
	Red fox (*V. vulpes*)	Berlin
	Moor buzzard (*Circus aeruginosus*)	Russia

tissue was used for extraction as this is never attacked by the intermediate stages of opisthorchiid trematodes. For all tissue samples the amount used was 200 mg.

In order to examine whether various cestode species of carnivores react in the PCR with the primers listed below, DNA was extracted from one proglottid of *Joyeuxiella pasqualei*, *Taenia crassiceps*, *Taenia polyacantha*, *Taenia hydatigena* and *Mesocestoides* sp. All proglottids were preserved in 70% ethanol.

Threefold washing was undertaken with Coon's buffer prior to DNA extraction of the fixed trematodes and tapeworm proglottids. After washing, one adult liver fluke or one proglottid was extracted with the commercially available DNeasy Tissue Kit (Qiagen, Hilden, Germany) and stored at 4°C. The amount and purity of the extracted DNA could be determined by measuring absorption at 260 nm and 280 nm in the Gene Quant RNA/DNA calculator (Pharmacia Biotech, Freiburg, Germany).

As specific primer pairs were not available for the different opisthorchiid trematode species, with the exception of *Opisthorchis viverrini* (Wongratanacheewin et al. 2001), universal primers had to be selected initially for the PCR to detect opisthorchiid liver fluke DNA by means of which mitochondrial DNA could be amplified from platyhelminths. The chosen universal primers (Bowles and McManus 1994) bind complementary to the conserved region of a subunit of the mitochondrial cytochrome *c* oxidase I (COI) gene flanking a variable sequence of approximately 450 bp (Gasser et al. 1999) in various cestode species: JB3 (5′ TTTTTTGGGCATCCTGAGGTTTAT 3′) and JB4.5 (5′ TAAAGAAAGAACAT–AATGAAAATG 3′). These are referred to as COI-1 and COI-2, respectively.

After amplification of the mitochondrial DNA from *O. felineus* and *M. bilis* with the COI primers, the resulting 450 bp products were subjected to sequence analysis. Subsequently, specific primers for the amplification of the variable region of the COI gene of the two most frequent indigenous opisthorchiid trematode species (*O. felineus* and *M. bilis)* were constructed by means of the free available primer programme Oligos 1999-2002 version 9.4 (http://www.biocenter.helsinki.fi/bi/bare-1_html/oligos.htm). To specifically detect the mitochondrial DNA of *O. felineus*, primers of the following sequences were selected: OF-1 (5′ CCTATTTGGTTATGGTTTGG 3′) and OF-2 (5′ CGATCTCGAGTACCGGCAAG 3′). The specific amplification of *M. bilis* DNA was performed with the primers MB-1 (5′ GGGCTGGATTTGGGCACTGC 3′) and MB-2 (5′ ACGATCACGAGTACCAGCAAGC 3′).

Amplification of the partial COI gene was carried out in a final reaction volume of 50 µl containing 1× PCR buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 2.5 mM $MgCl_2$ and 1 U Ampli Taq Gold polymerase (PE Applied Biosystems Branchburg, N.J., USA), 200 µM of each dNTP (pH 7.5; Amersham Pharmacia Biotech), 45 pmol of each COI primer and 20 ng extracted trematode DNA from a single opisthorchiid liver fluke specimen as template. DNA samples isolated from red fox, ide, *B. tentaculata* and *B. leachi* as well as *J. pasqualei, T. crassiceps, T. polyacantha, T. hydatigena* und *Mesocestoides* sp. (each 20 ng) were subjected to the species-specific PCR as control templates.

In order to detect potential contamination, a PCR mixture with RNAase/DNAase-free water was regularly run in parallel as a negative control. The PCR reaction was performed in a TRIO-Thermoblock (Biometra, Germany) adjusted to the following cycling steps: activation of the Ampli Taq Gold polymerase and denaturation of the DNA at 95°C for 15 min, annealing at 50°C for 30 s and extension at 72°C for 2 min. Subsequently, the initial cycle was followed by 39 cycles with denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 2 min. The final extension was undertaken at 72°C for 10 min before the reaction mixture was slowly cooled down to 4°C.

The amplification of the conserved region of the partial COI gene of *O. felineus* and *M. bilis* using 45 pmol of each specific primer pair (OF and MB primers) was carried out in a 50 µl reaction volume as described above. To examine specificity, the DNA was isolated from ten specimens of both *O. felineus* and *M. bilis*, which had been isolated from various definitive hosts. In addition, DNA from four *O. felineus* was tested with MB primers and DNA of four *M. bilis* with OF primers in order to rule out cross-reactivitiy between primers and non-specific DNA. The primers were also tested with host DNA of the red fox, orfe, *B. leachi* and *B. tentaculata,* and the opisthorchiid liver fluke *Pseudamphistomum truncatum* and with the DNA from the different cestode species of carnivores already mentioned.

The analytical sensitivity of the OF and MB primers was checked by subsequent dilution of the DNA of both *O. felineus* and *M. bilis* with 1× PCR buffer II: 20 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg. Each dilution was tested with the respective specific primer pair.

The PCR was carried out as touchdown PCR according to the following thermal cycler programme: activation of the Ampli Taq Gold polymerase and denaturation of DNA at 95°C for 15 min, annealing at 69°C for 30 s and extension at 72°C for 2 min. This was completed by 39 cycles with denaturation at 94°C for 30 s, annealing at 69°C for 30 s, followed by another annealing step in which the initial temperature of 69°C was reduced within 30 s with a drop of 0.2°C/s to 63°C (touch down) and extension at 72°C for 2 min. As before, the final extension step was performed at 72°C for 10 min. Thereafter, the mixture was slowly cooled to 4°C.

After PCR, amplicons were subjected to submarine minigel electrophoresis in a 2% agarose gel stained with ethidium bromide (0.01%) at 75 V for 1 h.

Results

The amplification of the mitochondrial DNA from *O. felineus*, *M. bilis* and *P. truncatum* always yielded products of approximately 440 bp when using COI primers. Testing the DNA of the intermediate host snails (*B. leachi*), orfe and red fox with COI primers always proved to be negative. By contrast, from a total of five different cestode species, all parasites in carnivores, products with a length of approximately 440 bp could be obtained. Due to the cross-reactivity of the COI primers with the DNA of cestodes, the products of opisthorchiid liver fluke species were subjected to sequence analysis.

Table 2 Partial COI sequences of *Opisthorchis felineus* and *Metorchis bilis*. For *O. felineus*: sequence length: 444 bp. Sequences in bold: primer OF–1: 5′ CCTATTTGGTTATGG-TGGTTTGG 3′ (length = 23 bp), primer OF–2: 5′ CGATCTCGAGTAC-CGGCAAG 3′ (length = 20 bp). For M bilis: sequence length: 444 bp. Sequences in bold: primer MB–1: 5′ GGGCTGGATTTGG-GCACTGC 3′ (length = 20 bp), primer MB–2: 5′ ACGATC-ACGAGTACCAGCAAGC 3′ (length = 22 bp)

O pisthorchis felineus						
1	TTTTTTGGGC	ATCCTGAGGT	TTATGTGTTA	ATATTGCCGG	GGTTTGGAAT
51	GATTAGTCAT	GTTTGTACGA	CTCTAACAGG	GAAAGATTC**C**	**CTATTTGGTT**
101	**ATGGTGGTTT**	**GG**TGTTAGCC	ATGTTTGCTA	TAGTTTGTTT	GGGTAGTGTG
151	GTTTGAGCTC	ATCATATGTT	TACTGTAGGA	TTAGATTTAG	GGACTGCTAT
201	TTTTTTTAGT	TCGGTTACTA	TGATCATTGG	TGTACCTACA	GGGATAAAGG
251	TTTTTTCTTG	ATTATACATG	**CTTGCCGGTA**	**CTCGAGATCG**	TCTTTGGGAT
301	CCGATTATGT	GGTGGATAAT	CGGATTTATA	GTGCTTTTTA	CTATAGGTGG
351	GGTTACTGGG	ATAGTTCTTT	CTGCCTCTGT	GATTGATGCT	CTTTTTCATG
401	ATACATGATT	CGTTATTGCC	CATTTTCATT	ATGTTCTTTC	TTTA
Metorchis bilis					
1	TTTTTTGGGC	ATCCTGAGGT	TTATGTATTG	ATATTGCCGG	GGTTTGGAGT
51	TATTAGTCAT	ATTTGTACTA	CTCTAACTGG	GAAAGATTCT	TTATTCGGTT
101	ATGGGGGTTT	GGTGTTAGCT	GTTTGCTATA	GTTTGTTTGG	GTAGCGTGGT
151	TTGGGCGCAC	CACATGTTTA	CTGTT**GGGCT**	**GGATTTGGGC**	**ACTGC**TGTTT
201	TTTTTAGTTC	TGTAACTATG	ATGTTATAGG	TGTTCCTACT	GGTATAAAGG
251	TTTTTTCTTG	GTTGTATA**TG**	**CTTGCTGGTA**	**CTCGTGATCG**	**T**TTTTGAGAC
301	CCGATTATGT	GGTGGATAAT	AGGGTTTGTA	GTGCTTTTTA	CTATAGGGGG
351	GGTTACTGGT	ATAGTTTTAT	CTGCTTCTGT	GATTGATGCT	TTGTTTCATG
401	ATACTTGGTT	TGTTATTGCT	CATTTTCATT	ATGTTCTTTC	TTTA

The sequences of the mitochondrial partial COI gene of *O. felineus* and *M. bilis* had a length of 444 bp (Table 2).

Based on the partial COI sequence of *O. felineus*, suitable primers could be constructed which amplified part of the conserved region of the subunit of the COI gene between positions 90 and 290. Hence, the amplification of the mitochondrial DNA of *O. felineus* when using OF primers led to a product of 201 bp. This could be confirmed in ten different specimens of *O. felineus* which had been isolated from various definitive hosts (Fig. 1). There was no cross-reactivity when testing the OF primers with cestode DNA, intermediate host DNA or with DNA from the indigenous opisthorchiid liver fluke species *P. truncatum* and *M. bilis* (Figs. 2, 3, 4). The percentage of pairwise alignment identity between the amplified product of *O. felineus* (201 bp) and the partial COI sequence of *M. bilis* was 41.8% (84 bp). The constructed MB primers amplify a product of exactly 116 bp between positions 176 and 291 of the partial COI sequence. The suitability of the MB primers could be confirmed in ten *M. bilis* isolates from different hosts (Fig. 5). In addition, the MB primers were tested with DNA from different carnivore cestodes, from *P. truncatum*, the intermediate host species and with DNA from *O. felineus*. Weak cross-reactivity was observed with the DNA of *T. crassiceps*, *T. polyacantha, T. hydatigena* (Fig. 6), *L. idus* (Fig. 7) and DNA from two *O. felineus* specimens (Fig. 8). However, the amplified products were not in the range of the specific product of 116 bp for *M. bilis*. The percentage of the pairwise alignment identity between the amplified product of *M. bilis* (116 bp) and the partial COI sequence of *O. felineus* was 39.7% (46 bp).

When testing the sensitivity of the OF primers, DNA from *O. felineus* could be detected after dilution up to an amount of 10 pg (Fig. 9). *M. bilis* DNA could be amplified when using MB-specific primers up to a level of 100 fg (Fig. 10).

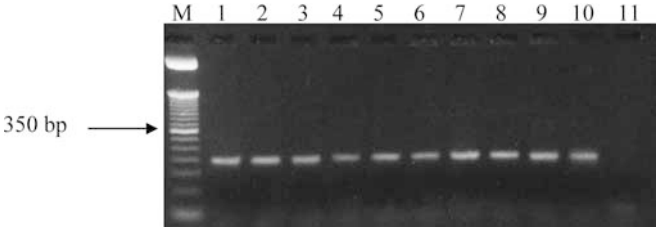

Fig. 1 OF amplificates of *Opisthorchis felineus* specimens from different definitive hosts. *Lane M*, 50 bp ladder; *lane 1*, *O. felineus* (golden hamster); *lane 2*, *O. felineus* (domestic cat); *lane 3*, *O. felineus* (red fox 1); *lane 4*, *O. felineus* (red fox 2); *lane 5*, *O. felineus* (red fox 3); *lane 6*, *O. felineus* (red fox 4); *lane 7*, *O. felineus* (red fox 5); *lane 8*, *O. felineus* (red fox 6); *lane 9*, *O. felineus* (muskrat); *lane 10*, *O. felineus* (human); *lane 11*, H_2O

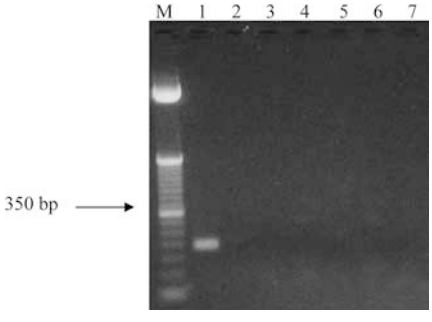

Fig. 2 Testing of OF primers with DNA from various cestodes of carnivores. *Lane M*, 50 bp ladder; *lane 1*, *Opisthorchis felineus* (positive control); *lane 2*, *Joyeuxiella pasqualei*; *lane 3*, *Taenia crassiceps*; *lane 4*, *Taenia polyacantha*; *lane 5*, *Taenia hydatigena*; *lane 6*, *Mesocestoides* sp.; *lane 7*, H_2O

Discussion

The testing of OF primers with the DNA from adult specimens of *O. felineus*, which had been isolated from ten different definitive hosts, produced a fragment of

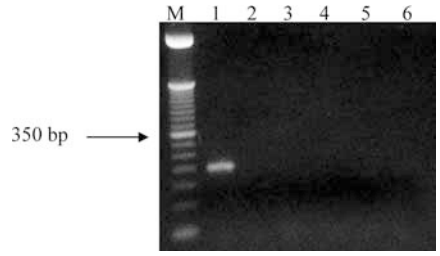

Fig. 3 Testing of OF primers with *Pseudamphistomum truncatum* DNA and host DNA. *Lane M*, 50 bp ladder; *lane 1*: *O. felineus* (positive control); *lane 2*, *Pseudamphistomum truncatum*; *lane 3*, *Bithynia leachi* (head tissue); *lane 4*, *Leuciscus idus* (muscle); *lane 5*, *Vulpes vulpes* (liver); *lane 6*: H_2O

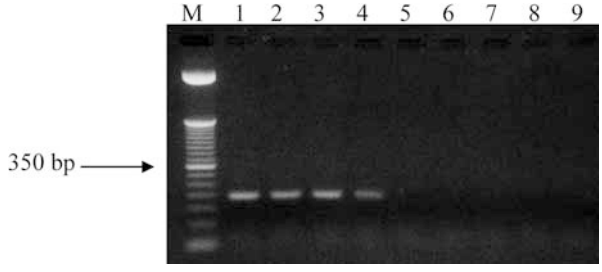

Fig. 4 Testing of OF primers with DNA from *Metorchis bilis*. *Lane M*, 50 bp ladder; *lane 1*, *O. felineus* (golden hamster); *lane 2*, *O. felineus* (domestic cat); *lane 3*, *O. felineus* (red fox 1); *lane 4*, *O. felineus* (red fox 2); *lane 5*, *M. bilis* (golden hamster); *lane 6*, *M. bilis* (domestic cat); *lane 7*, *M. bilis* (red fox 1); *lane 8*, *M. bilis* (red fox 2); *lane 9*, H_2O

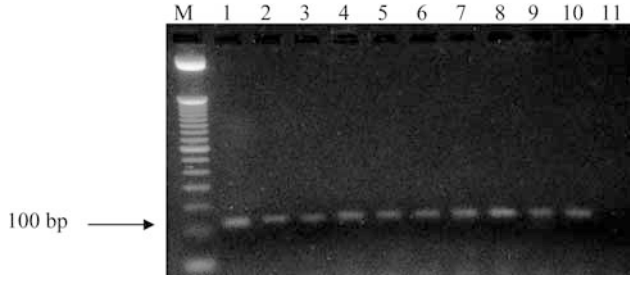

Fig. 5 MB amplificates of *M. bilis* specimens from different definitive hosts. *Lane M*, 50 bp ladder; *lane 1*, *M. bilis* (golden hamster); *lane 2*, *M. bilis* (domestic cat); *lane 3*, *M. bilis* (red fox 1); *lane 4*, *M. bilis* (red fox 2); *lane 5*, *M. bilis* (red fox 3); *lane 6*, *M. bilis* (red fox 4); *lane 7*, *M. bilis* (red fox 5); *lane 8*, *M. bilis* (red fox 6); *lane 9*, *M. bilis* (red fox 7); *lane 10*, *M. bilis* (moor buzzard); *lane 11*, H_2O

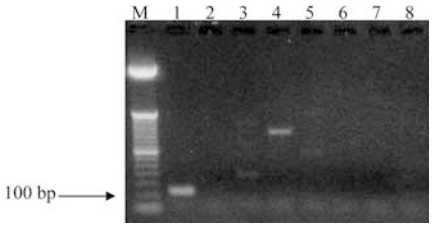

Fig. 6 Testing of MB primers with DNA from various cestodes of carnivores. *Lane M*, 50 bp ladder; *lane 1*, *M. bilis* (positive control); *lane 2*, *J. pasqualei*; *lane 3*, *T. crassiceps*; *lane 4*, *T. polyacantha*; *lane 5*, *T. hydatigena*; *lane 6*, *Mesocestoides* sp.; *lane 7*, *Hydatigera taeniiformis*; *lane 8*, H_2O

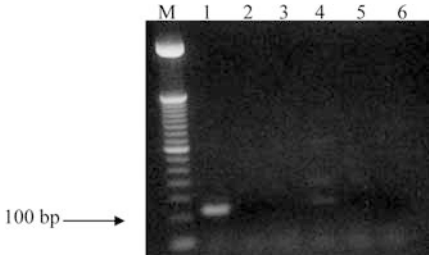

Fig. 7 Testing of MB primers with *P. truncatum* DNA and host DNA. *Lane M*, 50 bp ladder; *lane 1*, *M. bilis* (positive control); *lane 2*, *P. truncatum*; *lane 3*, *Bithynia tentaculata* (head tissue); *lane 4*, *Leuciscus idus* (muscle); *lane 5*, *Vulpes vulpes* (liver); *lane 6*, H_2O

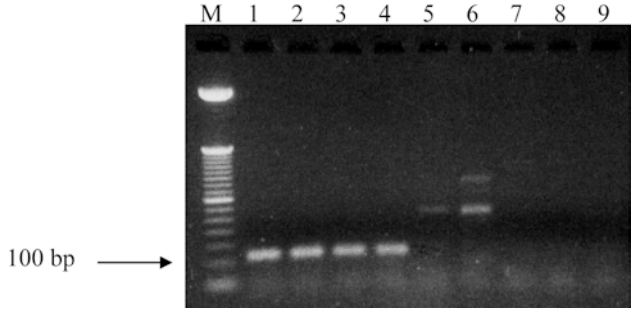

Fig. 8 Testing of MB primers with DNA from *O. felineus*. *Lane M*, 50 bp ladder; *lane 1*, *M. bilis* (golden hamster); *lane 2*, *M. bilis* (red fox 2); *lane 3*, *M. bilis* (red fox 3); *lane 4*, *M. bilis* (red fox 6); *lane 5*, *O. felineus* (red fox 1); *lane 6*, *O. felineus* (red fox 3); *lane 7*, *O. felineus* (red fox 4); *lane 8*, *O. felineus* (golden hamster); *lane 9*, H_2O

approximately 200 bp in each case. Moreover, according to the molecular characterization results using OF primers, the unclassified opisthorchiid trematodes isolated for the first time from the gallbladder of a muskrat (*Ondatra zibethica*) (R. Schuster personal communication) are in fact *O. felineus*. Furthermore, the DNA of *O. felineus* specimens, which came from a human being in western Siberia, tested positive in the PCR with the OF primers, indicating that these specimens belong to the same species as the trematodes isolated from indigenous definitive hosts. Based on geographical distribution, no difference could be determined in species classification. Since no *O. felineus* were detected in Germany, within its present borders, prior to World War II, the correlation between the OF amplificates from western Siberia with indigenous isolates suggests that these species might have been introduced by infected soldiers of the Red Army or by imported cats from Russia.

The OF primers used in the PCR did not react with the DNA of various cestodes or with the DNA of *P. truncatum* or *M. bilis*. The DNA of the different host species also regularly proved to be negative. The

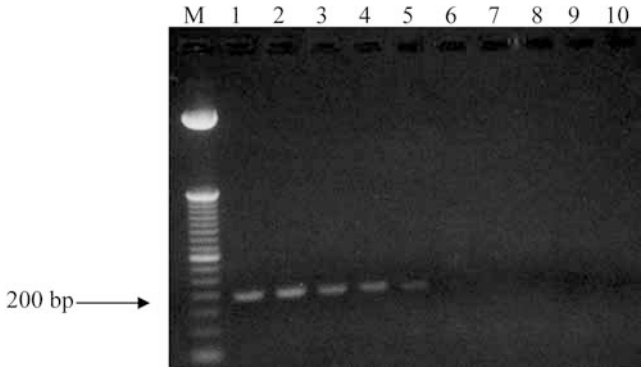

Fig. 9 Testing of the analytical sensitivity of OF primers with *O. felineus*–DNA. *Lane M* 50 bp ladder, *lane 1* 20 ng, *lane 2* 10 ng, *lane 3* 1 ng, *lane 4* 100 pg, *lane 5* 10 pg, *lane 6* 1 pg, *lane 7* 100 fg, *lane 8* 10 fg, *lane 9* 1 fg, *lane 10* H_2O

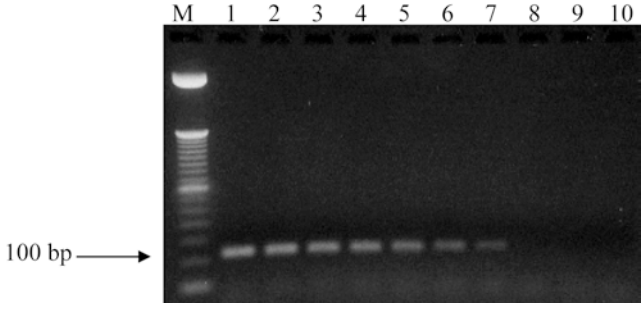

Fig. 10 Testing of analytical sensitivity of MB primers with *M. bilis*–DNA. *Lane M* 50 bp ladder, *lane 1* 20 ng, *lane 2* 10 ng, *lane 3* 1 ng, *lane 4* 100 pg, *lane 5* 10 pg, *lane 6* 1 pg, *lane 7* 100 fg, *lane 8* 10 fg, *lane 9* 1 fg, *lane 10* H_2O

primers are therefore 100% specific for the detection of *O. felineus* DNA. When examining the analytical sensitivity of these primers, the DNA of *O. felineus* could still be detected in an amount as low as 10 pg.

The specificity and sensitivity of the MB primers constructed for the detection of *M. bilis* DNA could be tested in the same way as described for the OF primers. They reacted with the DNA from *M. bilis* specimens which had been obtained from different definitive hosts. In all cases, there was a fragment of approximately 110 bp. Interestingly, the primers also showed a positive reaction of the same length with the DNA of an unclassified *Metorchis* isolate, which came from a moor buzzard (*Circus aeruginosus*) from Russia. It is not yet clear whether this is in fact *M. bilis* or another *Metorchis* species, as no tests could be undertaken within the framework of the described examinations for cross-reactivity with other *Metorchis* species. In morphological terms, the specimens from the moor buzzard could not, however, be distinguished from *M. bilis*, which strongly suggest a *Metorchis* species. When testing the MB primers with DNA from *L. idus*, the cestodes species *T. crassiceps*, *T. polyacantha, T. hydatigena*, and the trematode *O. felineus*, some unspecific products were visible, which were, however, not in the range of the approximately 110 bp product for *M. bilis*. The reason for the occurrence of non-specific strands may be explained by the fact that foreign DNA shows partially complementary base sequences for the specific primers of *M. bilis*. At certain temperatures, the primers used may then bond as a mismatch and amplify. In this way, several unspecific products of different lengths may occur. A mismatch of this kind of MB primer with DNA from *O. felineus* is shown in Fig. 8 (e.g. lane 6). Nevertheless, it can be assumed that MB primers have 100% specificity since the length of the non-specific products is not in the range of the specific amplificate for *M. bilis*. The analytical sensitivity of the MB primers is two powers of magnitude higher than for OF primers with a volume of 100 fg *M. bilis* DNA.

The differentiation of the two opisthorchiid liver fluke species examined is clearly possible using the PCR methods presented and can serve as the basis for further studies.

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