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Development of a multiplex PCR method for the simultaneous detection of four myxosporeans infecting gibel carp *Carassius auratus gibelio*

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ABSTRACT: Gibel carp *Carassius auratus gibelio* (Bloch), a commercially important freshwater-cultured fish in China, is threatened by myxosporeans, particularly *Thelohanellus wuhanensis*, *Myxobolus honghuensis*, *M. wulii* and *M. turpisrotundus*. Here, we developed a multiplex PCR assay for simultaneous detection of these 4 myxosporeans. The specific primers for each species were designed based on the 28S rDNA gene of *T. wuhanensis*, the ITS–5.8S rDNA of *M. honghuensis* and *M. wulii*, and the 18S rDNA gene of *M. turpisrotundus.* Specificity testing confirmed that the 4 primer sets have no cross-reactivity with other related myxosporean species tested. Detection limits of the multiplex PCR assay were 0.2, 0.3, 3.1 and 3.8 spores for *T. wuhanensis, M. honghuensis*, *M. wulii* and *M. turpisrotundus*, respectively. Following screening of 104 field samples, the analytical sensitivity of the present multiplex PCR assay was found to be similar to the sensitivity obtained by the singleplex PCR assays and was higher than that of microscopic examination. Moreover, Kappa analysis showed a strong agreement between the results of the singleplex and multiplex PCR assays, indicating that the developed multiplex PCR assay was an efficient approach for the diagnosis of the 4 myxosporeans infecting gibel carp.

KEY WORDS: Multiplex PCR · Molecular detection · Myxosporeans · Gibel carp · rDNA

INTRODUCTION

Gibel carp *Carassius auratus gibelio* (Bloch) is one of the most commercially important farmed freshwater fish in China. It has been cultured for more than 30 yr, with an annual production of more than 2.5 million tons in 2014 (Gui & Zhou 2010, Wang et al. 2011, Fishery Bureau 2014). With increasing densities in ponds, parasitic infections have been reported to cause mass morbidity and mortality of gibel carp, and myxosporean infections are emerging as a major issue (Zhang 2010a, Liu et al. 2012, Yuan et al. 2015).

To date, more than 50 myxosporean species have been reported to infect different organs and tissues of gibel carp (Chen & Ma 1998, Liu et al. 2012, 2014a,b, Yuan et al. 2015). Although most of the species do not cause severe diseases, some have been reported as pathogens resulting in mass mortality or significant economic losses (Liu et al. 2012, Yuan et al. 2015). Of these, the best known species are *Myxobolus honghuensis* Liu, Whipps, Gu, Zeng, Huang, 2012, which infects the pharynx of the host, causing anorexia, slow swimming, tissue necrosis and inflammation (Liu et al. 2012); *Myxobolus wulii* Landsberg & Lom, 1991, which infects the hepatopancreas, resulting in swollen abdomen, anorexia, lethargic behaviour and chronic mortality (Zhang et al. 2010a); *Myxobolus turpisrotundus* Zhang, Wang, Li, Gong, 2010, which produces numerous plasmodia in multiple organs including body surface, gill, kidney and intestine (Y. Liu et al. 2010, Zhang et al. 2010b); and *Thelohanellus wuhanensis* Xiao & Chen, 1993, which

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forms plasmodia in the skin of the host and is responsible for chronic mortality and poor growth (Liu et al. 2014b). Minimizing the severe negative impact of myxosporidiosis on commercially important gibel carp is considered a priority in China. Early and accurate detection of myxosporeans could help fish farmers to carry out prompt drug therapy and minimize the likelihood of a myxosporidiosis outbreak (Jia et al. 2015). Therefore, a simple and efficient assay for detection of myxosporean pathogens is needed.

Microscopic examination of squash preparations or histological sections is the most direct and commonly used method to detect and distinguish myxosporean parasites (St-Hilaire et al. 1997, Kelley et al. 2004). However, this approach can be time- and labor-consuming, and detection and identification is mainly based on the mature spore stages, with the early parasite stages likely to be neglected (Grabner et al. 2012, Mahony et al. 2015). Immunological methods based on antibodies or lectins (Knaus & El-Matbouli 2005, Estensoro et al. 2013) have also been developed. Nevertheless, these methods are generally restricted by cross-reactivity between different species (Lu et al. 2002, Estensoro et al. 2014, Jia et al. 2016). In recent years, PCR-based molecular detection and identification with higher sensitivity, specificity and rapidity has been utilised widely in myxosporean examination (Qureshi et al. 2002, Cavender et al. 2004, Clark 2006, Grabner et al. 2012, Jeon et al. 2014). Given the multiple copies and moderate intraspecific variation of regions within rDNA, it is commonly used as a molecular target for myxosporean species (Whipps et al. 2004, Bartošová et al. 2009, Atkinson & Bartholomew 2010, Piazzon et al. 2012). In the present study, we developed a multiplex PCR method targeting rDNA to simultaneously detect *M. honghuensis*, *M. wulii*, *M. turpisrotundus* and *T. wuhanensis* in infected gibel carp.

MATERIALS AND METHODS

Parasite DNA samples

Myxosporeans were obtained from infected gibel carp caught from fish farms in Hubei Province, PR China, during October 2011 and June 2015. Plasmodia of each species were washed 3 times in phosphate buffered saline (PBS, pH 7.4, 1 M) and spores were isolated by discontinuous gradient centrifugation using Percoll as described by Chase et al. (2001). Mature spore samples were pooled and used to extract parasite DNA using a phenol-chloroform protocol (Eszterbauer et al. 2001). Genomic DNA of *Thelohanellus testudineus*, *T. kitauei*, *Myxobolus nielii*, *M. shantungensis*, *M. koi* and uninfected gibel carp were used as negative controls in the present study. In addition, 10 and 100 spores of the 4 targeted species were counted by haemocytometer and were used to extract genomic DNA to calculate the approximate amount of genomic DNA per spore (Baldwin & Myklebust 2002). All genomic DNA was stored at −20°C. The concentration of the DNA samples was obtained in triplicate by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

Field samples

A total of 104 gibel carp (3.0 to 12.4 cm in length) were collected from Honghu Lake and Dongxi Lake in Hubei province, China in July 2015. Wet mount preparations from skin, gill, pharynx and hepatopancreas tissues of each fish were made for morphological detection of parasites. Approximately equal amounts of skin, gill, pharynx and hepatopancreas tissues were mixed (approximately 50 mg total weight fish^{-1}) from which genomic DNA was extracted using a phenol-chloroform protocol (Eszterbauer et al. 2001). The extracted DNA was stored at −20°C until further analysis.

Primer design

The 18S rDNA gene sequences of *Myxobolus turpisrotundus*, 28S rDNA gene sequences of *Thelohanellus wuhanensis* and ITS–5.8S rDNA sequences of *Myxobolus wulii* and *M. honghuensis* were chosen as the PCR assay targets. Specific primer sets with similar annealing temperatures were designed using Primer premier 5.0 software (Premier). The nucleotide sequences and target regions of the primers are shown in Table 1. Primer positions were chosen so as to produce different sized amplification products for each target parasite species. The specificity of 4 primer sets was evaluated *in silico* by using the BLASTN algorithm from the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Singleplex and multiplex PCR

Each singleplex PCR reaction was performed with a final volume of 25 µl containing 1× ExTaq buffer

Table 1. Primer sets for the detection of myxosporeans by singleplex and multiplex PCR

Target parasite	Primer name	Sequence (5′ to 3′)	Product size (bp)
Myxobolus turpisrotundus	Mt18S F	ACC AGA TAT TTC GAG GAG TCG TTG	894
	Mt18S R	TTC CAC AAC GTT GCT ATA TAG CCA	
M. wulii	MwITS F	GCC GTA CAT GAA TTG AGG CTT GAC	590
	MwITS R	TGC TCA CAA CGT TAA CTC GAA CC	
M. honghuensis	MhITS F	ACG ACC AAT ATA TGA AAT TGT TTC TCG A	447
	MhITS R	TTC ATT CGT AAA AAT GAC CTC TAC TTT ATA C	
Thelohanellus wuhanensis	Tw28S F	TTC ATT CGT AAA AAT GAC CTC TAC TTT ATA C	245
	Tw28S R	TCG AAA CGC CAA AAC TAC GTC A	

(Mg^{2+} free), 1.5 mM $MgCl_2$, 100 µM dNTP mixture, 1 U ExTaq DNA polymerase (TaKaRa), 0.2 µM of each primer and 100 ng DNA template. An EDC-810 DNA engine (EastWin Bio.) was used to control the cycling conditions: 94°C for 30 s, 57°C for 30 s and 72°C for 60 s for 35 cycles, with an initial denaturation at 95°C for 5 min and a terminal extension at 72°C for 7 min. PCR products were electrophoresed on a 1.0% agarose gel and stained with GelRed (Bio-Rad).

For the multiplex PCR, the working concentrations of 4 primer sets were optimized by a serial tests. The concentrations of Mg^{2+}, dNTP mixture and *Taq* DNA polymerase were then determined by an orthogonal test (L_9) (R. J. Liu et al. 2010) (see Figs. S1–S3 in the Supplement at www.int-res.com/articles/suppl/d124p031_supp.pdf). After optimization, the multiplex PCR was performed with a final volume of 25 µl reaction mixture containing 3.0 mM $MgCl_2$, 400 µM dNTP mixture, 1 U of ExTaq DNA polymerase, 0.32 µM of each Mt18S primer, 0.16 µM of each MwITS primer, 0.24 µM of each MhITS primer, 0.16 µM of each Tw28S primer and 100 ng DNA template. The PCR reaction was carried out following the cycling conditions 94°C for 30 s, 58°C for 30 s and 72°C for 70 s for 35 cycles, with an initial denaturation at 95°C for 5 min and a terminal extension at 72°C for 7 min. PCR products were electrophoresed on a 2% agarose gel.

Analytical specificity

The analytical specificity (lack of cross-reactivity) of the singleplex PCR methods was tested with DNA isolated from the non-target myxosporean species *M. nielii*, *T. testudineus*, *T. kitauei*, *M. shantungensis*, *M. koi* and the uninfected gibel carp. The analytical specificity (lack of cross-reactivity) of the multiplex PCR method was tested with the above DNA and combinations of equal amounts of DNA (100 ng) from the 4 target parasite species (see Fig. 2 for species combinations).

Analytical sensitivity

The DNA samples extracted from target myxosporean spores were diluted to 10 ng $µl^{-1}$ and then serially diluted 10-fold in distilled water. Analytical sensitivity (detection limit) of the singleplex PCR assays was determined using the diluted DNA samples (from single target species) in the presence of host DNA (100 ng) to evaluate their detection limits. For the multiplex PCR assay, analytical sensitivity was determined using a mixture of host DNA (100 ng) and differing amounts of DNA from different myxosporean species as follows: a 10-fold higher parasite DNA concentration (from 10 ng to 10^{-3} ng) from a single species in reactions with 10 times lower DNA concentration of the other 3 species (see Fig. 4). The analytical sensitivity with respect to DNA amounts detected was converted to number of spores detected based on data obtained previously for DNA content of known number spores.

Evaluation of field samples

Detection of myxosporean infections was carried out by morphological examination, singleplex and multiplex PCR assays. For morphological detection, wet-mount preparations of different tissues, as described previously, were examined under a light microscope and the positive infections were identified when target mature spores were observed. For molecular detection, genomic DNA extracted from pooled tissues from individual wild gibel carp (n = 104) was used as template in the singleplex and multiplex PCR assays to evaluate their efficiency at

parasite detection and identification. Detection results obtained by these 3 methods were compared with each other. Unfortunately, though morphological examination of each fish was performed and results recorded, the fish samples were not identified individually, so the results could not be associated with corresponding PCR results. Therefore, only the agreement between the results of singleplex and multiplex PCR assays was estimated by Kappa analysis.

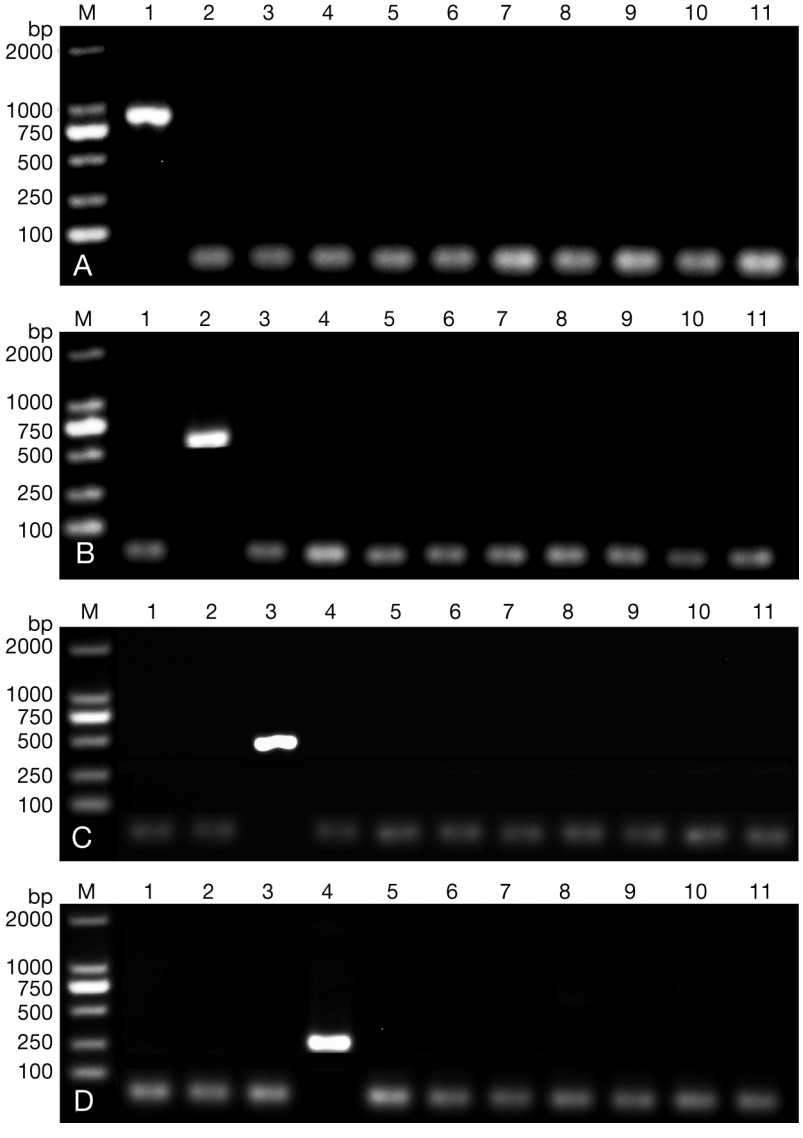

Fig. 1. Analytical specificity tests of the primer sets (A) Mt18S, (B) MwITS, (C) MhITS and (D) Tw28S. Lane M: DL2000™ Molecular marker (TaKaRa); Lane 1: *Myxobolus turpisrotundus*; 2: *M. wulii*; 3: *M. honghuensis*; 4: *Thelohanellus wuhanensis*; 5: *M. nielii*; 6: *T. testudineus*; 7: *T. kitauei*; 8: *M. shantungensis*; 9: *M. koi*; 10: uninfected gibel carp DNA; 11: water. Bands in A–D show the 894, 590, 447 and 245 base pair sequences generated from Mt18S, MwITS, MhITS and Tw28S primer sets, respectively

Cloning and sequencing

Products of singleplex and multiplex PCR amplifications with expected product size were purified using the High Pure PCR Product Purification Kit (Omega Bio-Tek). The PCR products were cloned into the pMD 19-T Vector (TaKaRa). Three positive clones of each product size from each PCR method were sequenced in both directions using the ABI PRISM® 3739 DNA sequencer (Applied Biosystems). Forward and reverse sequence segments were aligned. A standard nucleotide–nucleotide BLAST search was conducted to query the sequences obtained for the PCR products.

RESULTS

Analytical specificity

PCR amplification products of expected size were generated by the singleplex PCR assays for their respective target species only (*Myxobolus turpisrotundus*: Fig. 1A, Lane 1; *M. wulii*: Fig. 1B, Lane 2; *M. honghuensis*: Fig. 1C, Lane 3; and *Thelohanellus wuhanensis*: Fig. 1D, Lane 4), whereas no fragments were generated from non-target species nor from host DNA. The analytical specificity of the PCR assays in a multiplex (the 4 primer pairs) format was also tested and was found not to amplify non-target species (Fig. 2, Lanes 15–21). Additionally, the multiplex PCR was also tested using different combinations of target species (Fig. 2, Lanes 1–14). Only PCR amplification products of expected size for those target species present in the reaction were obtained, demonstrating that the primer pairs did not cross-react to generate non-specific products.

Analytical sensitivity

The DNA amounts of known number of spores for 4 target myxosporeans were calculated as follows: 2.63 ng (100 spores) and 0.26 ng (10 spores) for *M. turpisrotundus*, 3.18 ng (100 spores)

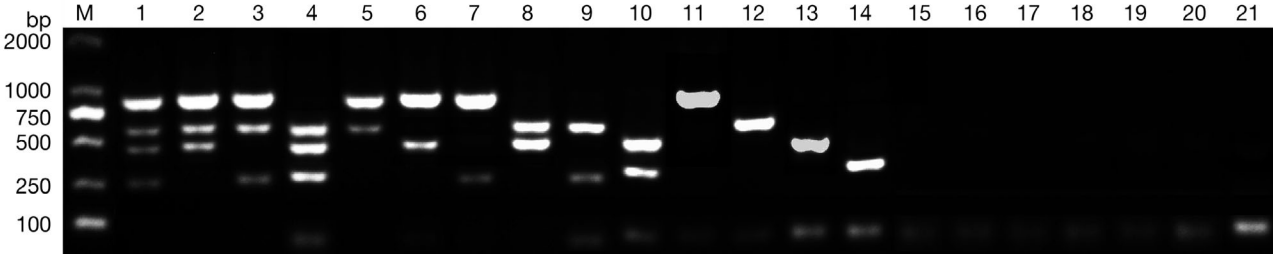

Fig. 2. Analytical specificity test of 4 primer sets in multiplex PCR with the DNA from 4 target myxosporeans: (a) *Myxobolus turpisrotundus*, (b) *M. wulii*, (c) *M. honghuensis* and (d) *Thelohanellus wuhanensis* as positive controls and other species as negative controls. Lane M: DL2000™ Molecular marker (TaKaRa); Lanes 1–10: mixed DNA; Lane 1: (a) + (b) + (c) + (d); 2: (a) + (b) + (c); 3: (a) + (b) + (d); 4: (b) + (c) + (d); 5: (a) + (b); 6: (a) + (c); 7: (a) + (d); 8: (b) + (c); 9: (b) + (d); 10: (c) + (d); 11: (a); 12: (b); 13: (c); 14: (d); 15: *M. nielii*; 16: *T. testudineus*; 17: *T. kitauei*; 18: *M. shantungensis*; 19: *M. koi*; 20: uninfected gibel carp DNA; 21: water

and 0.32 ng (10 spores) for *M. wulii*, 2.94 ng (100 spores) and 0.30 ng (10 spores) for *M. honghuensis* and 4.34 ng (100 spores) and 0.42 ng (10 spores) for *T. wuhanensis*. Singleplex PCR could detect 10^{-3} ng of DNA (0.04 of a spore) from *M. turpisrotundus* (Fig. 3A), 10^{-4} ng DNA (0.003 of a spore) from *M. wulii* (Fig. 3B), 10^{-3} ng DNA (0.03 of a spore) from *M. honghuensis* (Fig. 3C) and 10^{-4} ng DNA (0.002 of a spore) from *T. wuhanensis* (Fig. 3D). Multiplex PCR, in the presence of DNA from multiple target species, was able to detect 0.1 ng DNA (3.8 spores) of *M. turpisrotundus* (Fig. 4A), 0.1 ng DNA (3.1 spores) of *M. wulii* (Fig. 4B), 0.01 ng DNA (0.3 of a spore) of *M. honghuensis* (Fig. 4C) and 0.01 ng DNA (0.2 of a spore) of *T. wuhanensis* (Fig. 4D).

Sequence analysis

Positive PCR products amplified from the standard parasite DNA of the 4 target myxosporeans were sequenced to confirm the specificity of Mt18S, MwITS, MhITS and Tw28S primer sets. When compared with reference sequences, the sequences of amplified fragments were identical to rDNA sequence of the relevant target species. In addition, 3 positive products of

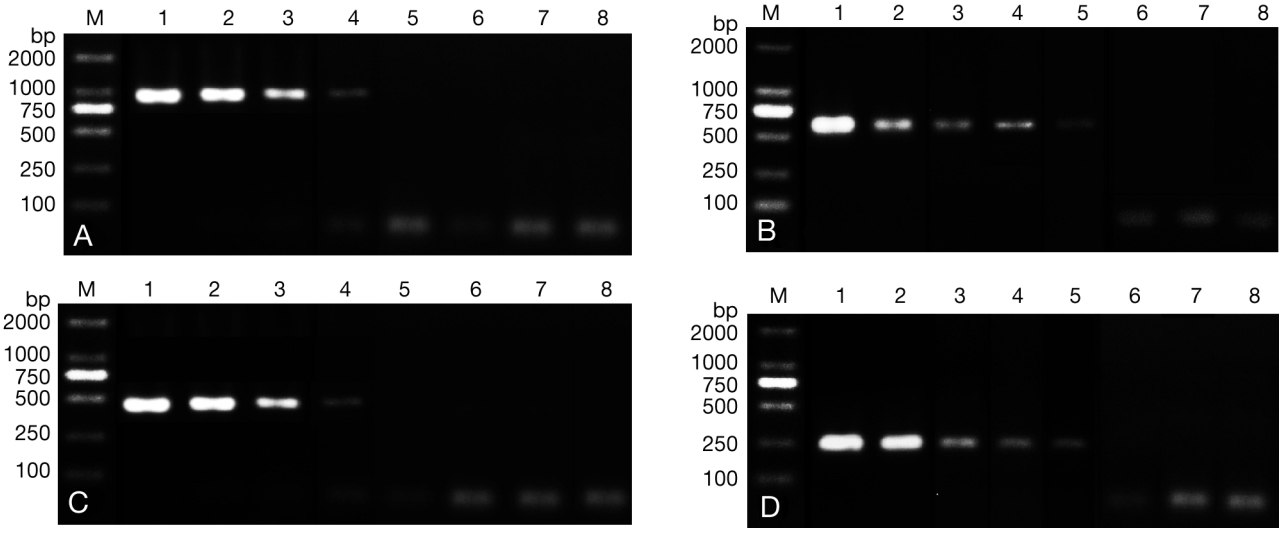

Fig. 3. Analytical sensitivity tests of single PCR with mixture of host DNA and serial dilution of parasite DNA from (A) *Myxobolus turpisrotundus*, (B) *M. wulii*, (C) *M. honghuensis* and (D) *Thelohanellus wuhanensis*. Lane M: DL2000™ Molecular marker (TaKaRa); Lanes 1–7: 10-fold dilution series of DNA sample ranging from 1 ng to 10^{-6} ng; 100 ng host DNA was added into each reaction; Lane 8: water. Faint products can be observed in Lane 4 for (A) *M. turpisrotundus* and (C) *M. honghuensis*, and Lane 5 for (B) *M. wulii* and (D) *T. wuhanensis*

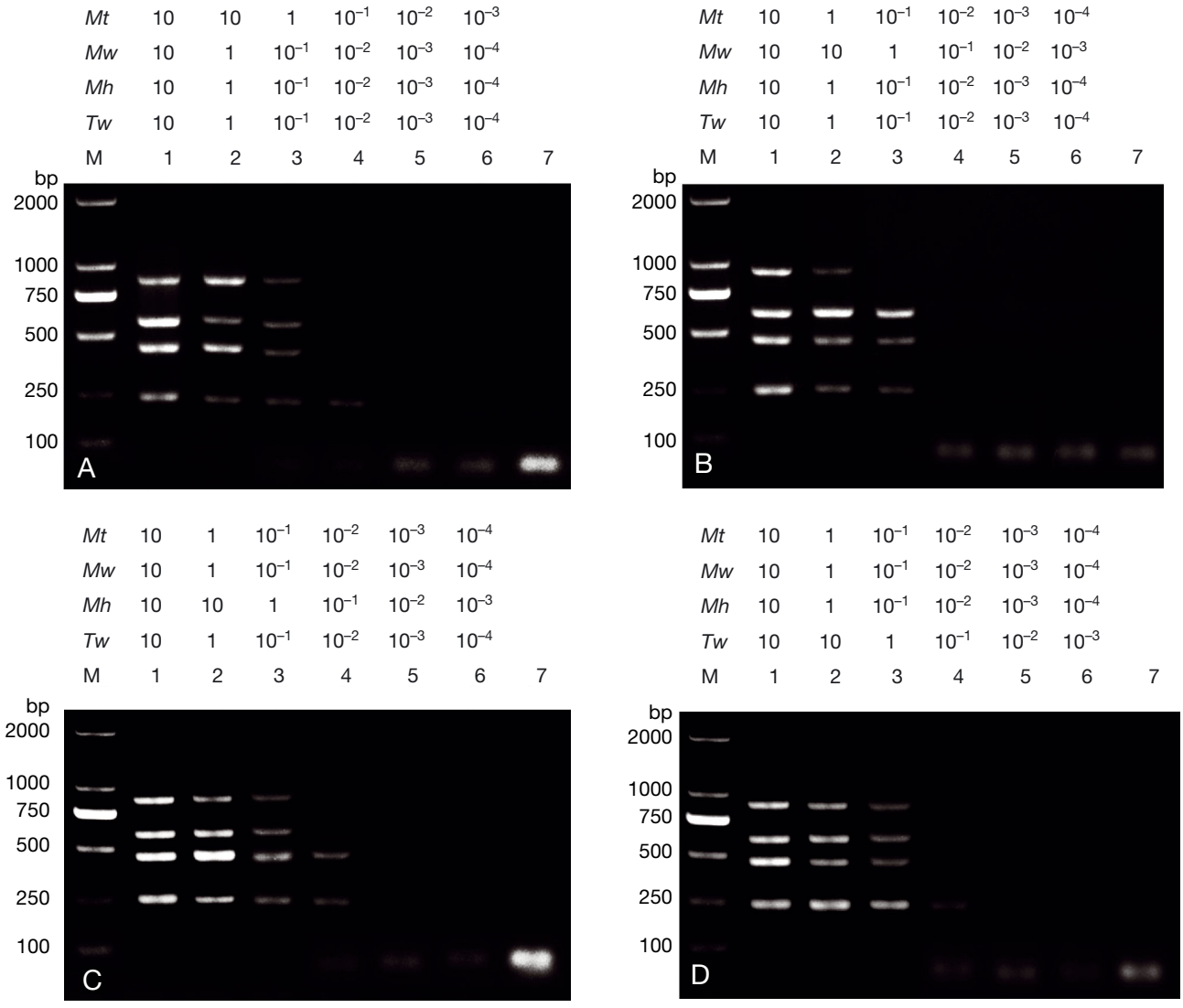

Fig. 4. Analytical sensitivity tests of multiplex PCR with mixtures of host DNA and different amounts of DNA from 4 myxosporean species as follows: a 10-fold higher parasite DNA concentration from a single species — (A) *M. turpisrotundus*, (B) *M. wulii*, (C) *M. honghuensis* and (D) *T. wuhanensis* — in reactions with 10 times lower DNA concentration of the other 3 species (from 10 ng to 10^{-4} ng). Lane M: DL2000™ Molecular marker (TaKaRa); Lanes 1^{-6}: mixed DNA from 4 myxosporeans (*Mt*: *M. turpisrotundus*; *Mw*: *M. wulii*; *Mh*: *M. honghuensis*; *Tw*: *T. wuhanensis*); 100 ng host DNA was added into each reaction; Lane 7: water

each species in each method from field samples were sequenced and compared with each target sequence, which showed the identities ranged from 98.7 to 99.5%.

Detection of field samples

The results of the PCR assays and microscopic examination of target myxosporeans in 104 field samples are provided in Table 2 and Table S1 in the Supplement at www.int-res.com/articles/suppl/d124p031_supp.pdf. The positive samples of single infection accounted for 45.2% (47/104) by singleplex PCR method, 48.1% (50/104) by multiplex PCR method and 34.6% (36/104) by microscopic examination. With regard to co-infections, dual infection accounted for 30.8% (32/104), 22.1% (23/104) and 7.7% (8/104) using singleplex PCR, multiplex PCR and microscopic examination, respectively. Triple infection was determined to be 5.8% (6/104), 2.9% (3/104) and 0% (0/104) by singleplex PCR, multiplex PCR and microscopic examination, respectively. Kappa analysis (*k*-value = 0.739) indicated strong agreement between the singleplex and multiplex PCR assays.

Table 2. Results of 104 samples of gibel carp *Carassius auratus gibelio* tissues for myxosporean infection detected by 2 PCR assays and microscopic examination. *Mt*: *Myxobolus turpisrotundus*; *Mw*: *M. wulii*; *Mh*: *M. honghuensis*; *Tw*: *Thelohanellus wuhanensis*

Myxosporean infections	Proportion of positive samples (%, and number out of 104)		
	Singleplex PCR	Multiplex PCR	Microscope
Mt	3.8 (4)	2.9 (3)	0
Mw	20.2 (21)	19.2 (20)	12.5 (13)
Mh	16.3 (17)	20.2 (21)	13.5 (14)
Tw	4.8 (5)	5.8 (6)	8.7 (9)
Mt + *Mw*	1.0 (1)	0	0
Mt + *Mh*	8.7 (9)	5.8 (6)	0
Mt + *Tw*	1.9 (2)	0 (0)	0
Mw + *Mh*	15.4 (16)	11.5 (12)	4.8 (5)
Mw + *Tw*	0	0	0
Mh + *Tw*	3.8 (4)	4.8 (5)	2.9 (3)
Mt + *Mw* + *Mh*	3.8 (4)	1.9 (2)	0
Mt + *Mw* + *Tw*	0	0	0
Mt + *Mh* + *Tw*	0	0	0
Mw + *Mh* + *Tw*	1.9 (2)	1.0 (1)	0
Mt + *Mw* + *Mh* + *Tw*	0	0	0

DISCUSSION

Molecular detection methods with high specificity and sensitivity have been developed and widely applied in the examination of myxosporean infections (Skirpstunas et al. 2006, Meng & Li-Chan 2007, Fytilis et al. 2013). Although some methods such as nested PCR or *in situ* hybridization can achieve higher sensitivity and accurate species identification (Kelley et al. 2004, Markussen et al. 2015), they involve complex procedures and are time- and labor-consuming for routine diagnosis. In addition, most of the developed methods are aimed at detecting single myxosporean pathogen species (Baldwin & Myklebust 2002, Clark 2006, Mahony et al. 2015). However, various myxosporean pathogens have been reported to simultaneously infect the same host (Grabner et al. 2012, Woo et al. 2014, Ye et al. 2014). In the present study, we developed a multiplex PCR assay for simultaneous detection of 4 myxosporean pathogens of gibel carp.

Ribosomal DNA (including 18S rDNA, ITS–5.8S rDNA, 28S rDNA), as an effective molecular marker, has been used widely in the detection of myxosporeans (Whipps et al. 2004, Grossel et al. 2005, Grabner et al. 2012, Piazzon et al. 2012). Chai et al. (2014) developed a nested PCR test targeting the 18S rDNA gene to detect *Thelohanellus wuhanensis*, but no PCR assays have been previously developed for *Myxobolus honghuensis*, *M. wulii* and *M. turpisrotundus*. In order to get a size-distinguishable PCR product, species-specific primer sites amplifying a region of 245 bp were found in the 28S rDNA gene of *T. wuhanensis* and this was selected as a targeted sequence for molecular detection. In the case of *M. honghuensis* and *M. wulii*, with high similarities between the species in their 18S and 28S rDNA gene sequences, the ITS rDNA, with higher intraspecific mutation rates, was selected to prevent cross-reactivity of the primers (Cunningham 1997, Whipps et al. 2004, Woo et al. 2014). After sequence analysis, 2 variable regions of the ITS–5.8S rDNA gene sequence, giving 447 and 590 bp products, were chosen as targeted sequences for molecular detection of *M. honghuensis* and *M. wulii*, respectively. To clearly distinguish 4 target bands from amplified products in multiplex PCR, a region of the 18S rDNA gene of *M. turpisrotundus* giving a PCR product of 894 bp was chosen. Analytical specificity of each primer set was initially confirmed by the singleplex PCR using host and several myxosporean DNA samples, including the common *Myxobolus* and *Thelohanellus* species of cultured fish in China. No amplified product from non-target species was observed. For the multiplex PCR, optimization of reaction conditions needed to be resolved to improve its overall efficiency. Annealing temperature and the concentration of the 4 primer sets and reaction reagents including Mg^{2+}, dNTPs and *Taq* DNA polymerase are important influencing factors. After optimization (see Figs. S1–S3 in the Supplement), the multiplex PCR was able to specifically detect relative myxosporeans from both single and mixed parasite DNA preparations (Fig. 2). In the analytical sensitivity tests, 100 ng host DNA was added into each reaction since the presence of host DNA was inevitable during practical application of the tests. As shown in the results of analytical sensitivity tests, the detection limit of the multiplex PCR was lower than the singleplex PCR assays, when multiple parasites were simultaneously detected. This is expected, since in a multiplex PCR reaction for detection of multiple targets, all primer pairs will compete for limited reaction reagents, resulting in sub-optimal amplification efficiencies (Bilgiç et al. 2013, Moustacas et al. 2013).

When applied to screening of the 104 field samples, the multiplex PCR and the 4 singleplex PCR methods were proven to be more effective for detection of the 4 target myxosporeans compared to microscopic examinations. During the microscopical detection, although the early stages of myxosporeans could be

observed, only samples infected by mature spores were recorded as positive, as the early stages of myxosporeans were difficult to distinguish morphologically to species level. The detection results of the multiplex PCR assay were similar to those of the singleplex PCR assays in general, although the prevalence of *M. turpisrotundus* was determined to be 10.6% (11/104) by the multiplex PCR, lower than the 19.2% (20/104) prevalence determined by the singleplex PCR. Kappa analysis (*k*-value = 0.739) in the present study indicated that there was strong agreement between the results of the singleplex and multiplex PCR assays for the myxosporean infections per fish (Silcocks 1983).

The result of co-infection detection, showing approximately one-third of the 104 samples to be infected with 2 or more myxosporeans, highlights the advantage of developing a multiplex PCR method to detect myxosporean pathogens of gibel carp. The multiplex PCR developed in the present study provides a practical method for detection of myxosporeans when applied to a large number of samples. Early detection of myxosporean infections could enable fish farmers to carry out timely treatments, thus limiting myxosporean disease impacts.

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