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Detection of *Dermocystidium anguillae* in imported elvers of American eel *Anguilla rostrata* in China

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Abstract: In recent years, an emerging dermocystidiosis caused by *Dermocystidium anguillae* Spangenberg, 1975 has been found to pose a threat to the culture of American eel, *Anguilla rostrata* (Lesueur), as well as Chinese perch, *Siniperca chuatsi* (Basilewsky), in China. *Dermocystidium anguillae* was originally described from European eel, *Anguilla anguilla* (Linnaeus), and it is thus important to identify the possible source of this pathogen. In the present study, we compared *D. anguillae* from European eels cultured in China with those from American eels. Molecular analysis showed that the SSU rDNA of *D. anguillae* infecting European eels was identical to that of *D. anguillae* infecting American eels, suggesting their conspecificity. To investigate the source of *D. anguillae* causing dermocystidiosis in American eels cultured in China, a specific PCR assay for the detection of *D. anguillae* was developed with high sensitivity (10^{-6} ng/µl of *D. anguillae* genomic DNA). Using the present molecular detection method, the water and sediment of culture ponds, fish feed and American eel elvers imported from America were screened for the presence of *D. anguillae*. No amplicons were detected from the water, sediment and fish feed samples. However, positive amplicons were found in American eel elvers, indicating that *D. anguillae* has been introduced from American eel elvers to China. It is suggested that American eel elvers imported from America should be examined for the presence of *D. anguillae* before their exportation abroad to prevent the spread of this pathogen.

Keywords: Mesomycetozoea, dermocystidiosis, Anguillidae, species identification, diagnosis

The American eel, *Anguilla rostrata* (Lesueur), is a catadromous and economically important fish, which is widely distributed ranging from southern Greenland to the Gulf of Mexico and Caribbean Sea (Benchetrit and McCleave 2016, Triyanto et al. 2020). The life cycle of American eels is complex, comprising several different developmental stages (Arai 2020, Cresci 2020). After hatching in the Sargasso Sea, leptocephali of American eels migrate with oceanic currents to coastal or inland water; during the migration, leptocephali metamorphose into glass eels. Thereafter, glass eels enter inland water and become yellow eels; approximately 5–25 years later, yellow eels metamorphose into silver eels and start downstream migration back to spawning area (Vøllestad 1992, Pujolar 2013, Monteiro et al. 2020). As a cultured fish, American eels have been introduced into East Asia and Europe, such as China, Germany, Denmark and the Netherlands (Han et al. 2002, Frankowski et al. 2009, Bao 2016).

In China, due to the shortage of elvers of Japanese eel, *Anguilla japonica* Temminck et Schlegel, and European eel, *Anguilla anguilla* (Linnaeus) (Ginneken and Maes 2006), American eels have become to be a highly demanded cultured fish (Ye et al. 2019). In recent years, an emerging dermocystidiosis caused by *Dermocystidium anguillae* Spangenberg, 1975 has been found to pose a threat to the culture of American eels as well as Chinese perch, *Siniperca chuatsi* (Basilewsky), in China (Liu et al. 2021). *Dermocystidium anguillae* was originally described from European eels (Spangenberg 1975) and it is important to identify the possible source of this pathogen. Before the introduction of American eels, *D. anguillae* had not been reported in China (Chen and Xie 1960, Xiao and Chen 1993, Zhang and Wang 2005). In addition, American eel elvers cultured in China were imported from America. It is reasonable to suspect that *D. anguillae* was introduced to China by imported American eel elvers.

In order to verify the above hypothesis, we compared *D. anguillae* from European eels cultured in China with those from American eels. In addition, a specific PCR assay was developed to detect the presence of *D. anguillae* in the water and sediment of culture ponds, fish feed and American eel elvers imported from America.

MATERIALS AND METHODS

Identification of *Dermocystidium anguillae* from gills of European eels

Fish collection. Thirty-two European eels, 0.3–4.6 (1.6 ± 1.0) g in weight and 7.8–15.3 (11.7 ± 1.7) cm in length, were obtained from a fish farm in Fuzhou City, Fujian Province, China

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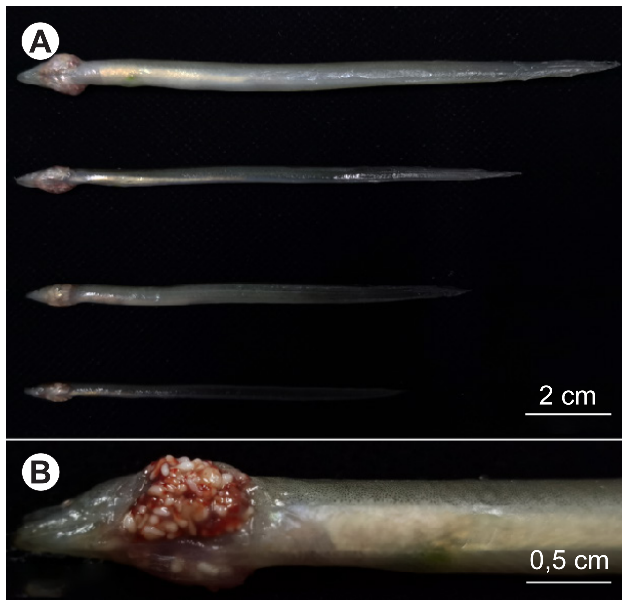

Fig. 1. European eel *Anguilla anguilla* (Linnaeus) infected with *Dermocystidium anguillae* Spangenberg, 1975. **A** – infected European eels showing operculum protrusion; **B** – a large number of cysts in gills of European eels.

in November 2020. All collected fish were transported live to the laboratory of Aquatic Animal Parasitology in School of Marine Science and Engineering, Qingdao Agricultural University, and held in aquarium. The fish were anesthetised with an overdose of tricaine methanesulfonate (MS222, SIGMA; 100 mg/l) prior to the dissection.

Morphological examination. Infected gills were removed and washed by 0.65% normal saline, then the cysts were isolated from gills, some of which were immediately fixed in molecular-grade absolute alcohol. The fresh cysts were ruptured on slides to release the spores which were examined and photographed with a ZEISS Lab.A1 microscope equipped with a ZEISS Axiocam 105 color camera (ZEISS, Oberkochen, Baden-Württemberg, Germany). Morphological and morphometric analysis of the fresh cysts and spores were performed according to Liu et al. (2021). All the fish specimens used in the present study were treated in accordance with the guidelines of the Laboratory Animal Administration Law of China, with the permit number SD2007695 approved by the Ethics Committee of the Laboratory Animal Administration of Shandong Province, China.

DNA extraction, amplification, and sequencing. Genomic DNA was extracted from the ethanol-fixed cysts using Universal Genomic DNA Kit (CWBio, Beijing, China) following the manufacturer's protocol. The SSU rDNA was amplified following the procedure of Liu et al. (2021). The PCR products were separated using a 1.0% agarose gel and sequenced in both directions with an ABIPRISM® 3730XL DNA sequencer (Applied Biosystems Inc., Foster, USA). A contiguous DNA sequence was assembled and deposited in GenBank.

Development of PCR assay for the detection of *Dermocystidium anguillae.*

DNA extraction. Genomic DNA of *D. anguillae* (from gills of American eels in Liu et al. 2021), American eels and Chinese perch were extracted as described in the above section.

Primer design. The SSU rDNA of *D. anguillae* was chosen as the PCR assay target. Primer set (DaF: TTCGCTTCTCGAAAGCGGC, DaR: TTACCCATACCTTCCGGTACAGGTG) specific for *D. anguillae* was designed based on the alignment of SSU rDNA sequences of *D. anguillae* (MT644942–MT644948), American eel (FM946071) and Chinese perch (AY452490) using Primer premier 5.0 software (Premier), expecting to produce a DNA fragment of 836 bp.

PCR assay conditions. The annealing temperature of the primer set was optimised (data not given). After optimisation, the

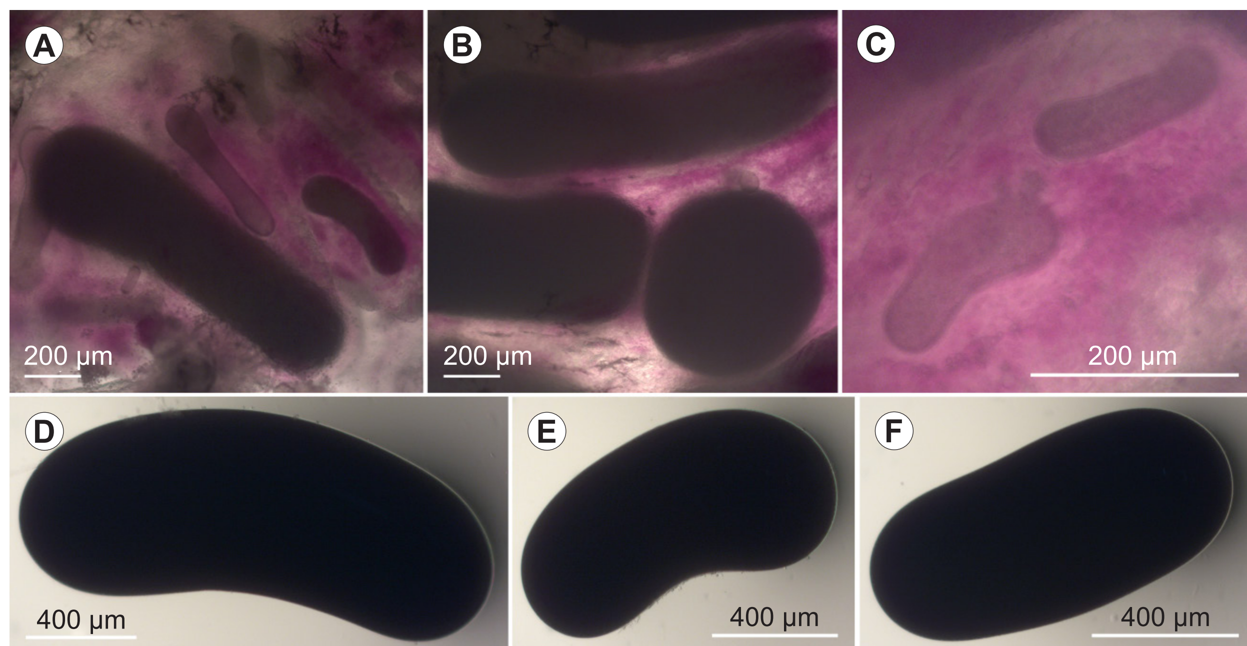

Fig. 2. Cysts of *Dermocystidium anguillae* Spangenberg, 1975 from gills of European eel *Anguilla anguilla* (Linnaeus).

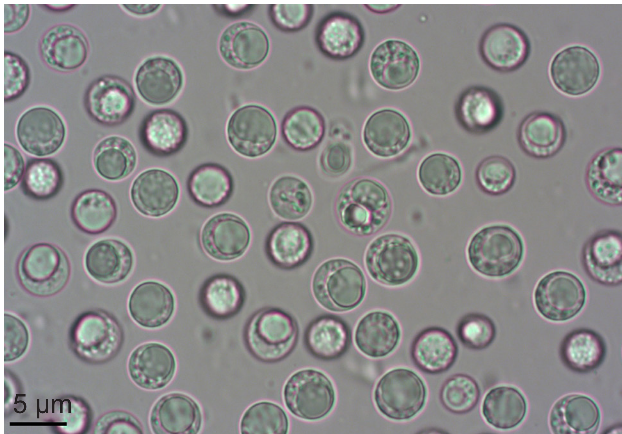

Fig. 3. Spores of *Dermocystidium anguillae* Spangenberg, 1975.

PCR reaction was performed with a total volume of 20 µl containing 0.5 µl (10 µM) of each primer, 1 µl (1 ng/µl) genomic DNA, 10 µl 2×Taq Plus PCR Master Mix (RuiBiotech, Beijing, China) and 8 µl distilled water. The PCR protocol consisted of 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 1 min and final extension at 72°C for 5 min. PCR products were electrophoresed on a 1% agarose gel.

PCR assay specificity. Specificity of the present PCR assay was tested with genomic DNA of *D. anguillae*, American eels and Chinese perch. Genomic DNA of American eels and Chinese perch were used as negative controls. An aliquot (5 µl) of each amplified product was electrophoresed on 1% agarose gel.

PCR assay sensitivity. Sensitivity of the present PCR assay was estimated using a mixture of 100 ng DNA of each host (American eel, Chinese perch) and serially diluted *D. anguillae* DNA (from 1 to 10^{-8} ng/µl). PCR products were electrophoresed on 1% agarose gel.

Molecular detection of *Dermocystidium anguillae*

Detection of *D. anguillae* in the water of culture ponds. Water samples were collected from 15 culture ponds (nine American eel culture ponds and six European eel culture ponds) in Fuzhou City, Fujian Province, China in November 2020, where dermocystidiosis caused by *D. anguillae* occurred in recent years. Three sampling points were selected for each pond and 200 ml of water was taken from each sampling point. A total of 600 ml of water was passed serially through 15 µm and 0.2 µm membrane filter, with filter membranes collected and fixed in molecular-grade absolute ethanol. Given that the 0.2 µm filter membrane was clogged frequently, one filter membrane was used for filtering 100 ml of water. Genomic DNA was extracted from filter membranes using EZNA water DNA kit (Omega, Norcross, Georgia, America) or Universal Genomic DNA Kit (CWBio, China) according to the manufacturer' s protocol. Molecular detection of *D. anguillae* in the water of culture ponds was performed using the PCR assay developed in the present study.

Detection of *D. anguillae* in the sediment of culture ponds. Sediment samples were collected from the same ponds of water samples in the above section. A total of 50 g of sediment was collected from two sampling points in each pond and stored in 50 ml tubes, which were transported to laboratory at low temperature and stored in -20°C. Genomic DNA was extracted from the sediment samples using soil Genomic DNA kit (CWBio, China) according to the manufacturer' s protocol. Molecular detection of *D. anguillae* in the sediment of culture ponds was performed using the PCR assay developed in the present study.

Detection of *D. anguillae* in the fish feed. Fish feed comprising a naidid (*Limnodrilus* sp.) as starter feed for elvers and artificial feed for commercial fish of American eels were obtained in Fuzhou City, Fujian Province, China in November 2020, and fixed in molecular-grade absolute ethanol. Genomic DNA was extracted from fish feed using Universal Genomic DNA Kit (CWBio, China) following the manufacturer' s protocol. Molecular detection of *D. anguillae* in the fish feed was performed using the PCR assay developed in the present study.

Detection of *D. anguillae* in the American eel elvers imported from America. Sixty-six American eel elvers 0.06–0.20 (0.13 ± 0.03) g in weight and 4.8–6.5 (5.4 ± 0.4) cm in length imported from America were obtained in November 2020 (28 elvers) and April 2021 (38 elvers), and fixed in molecular-grade absolute ethanol. Genomic DNA was extracted from the head of elvers using Universal Genomic DNA Kit (CWBio, China) following the manufacturer' s protocol. Molecular detection of *D. anguillae* in the American eel elvers was performed using the PCR assay developed in the present study.

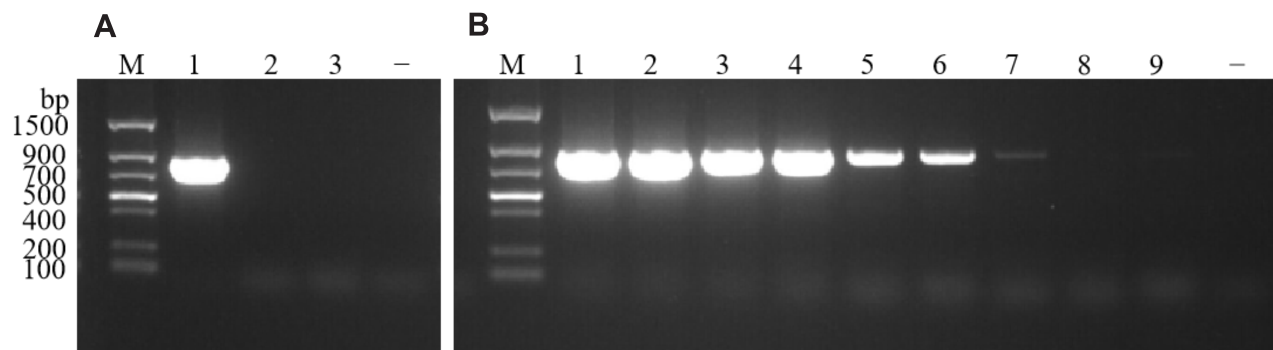

Fig. 4. Specificity and sensitivity analysis of the present primer set. **A** – specificity analysis. Lane 1: *Dermocystidium anguillae* Spangenberg, 1975; 2: *Anguilla rostrata* (Lesueur); 3: *Siniperca chuatsi* (Basilewsky); **B** – sensitivity analysis. Lanes 1–9: 10-fold dilution series of *D. anguillae* DNA ranging from 1 ng/µl to 10^{-8} ng/µl; 100 ng DNA of each host (American eel, Chinese perch) was added into each reaction. Lane M: DL1500™ Molecular marker (TRANS); lane –: negative control.

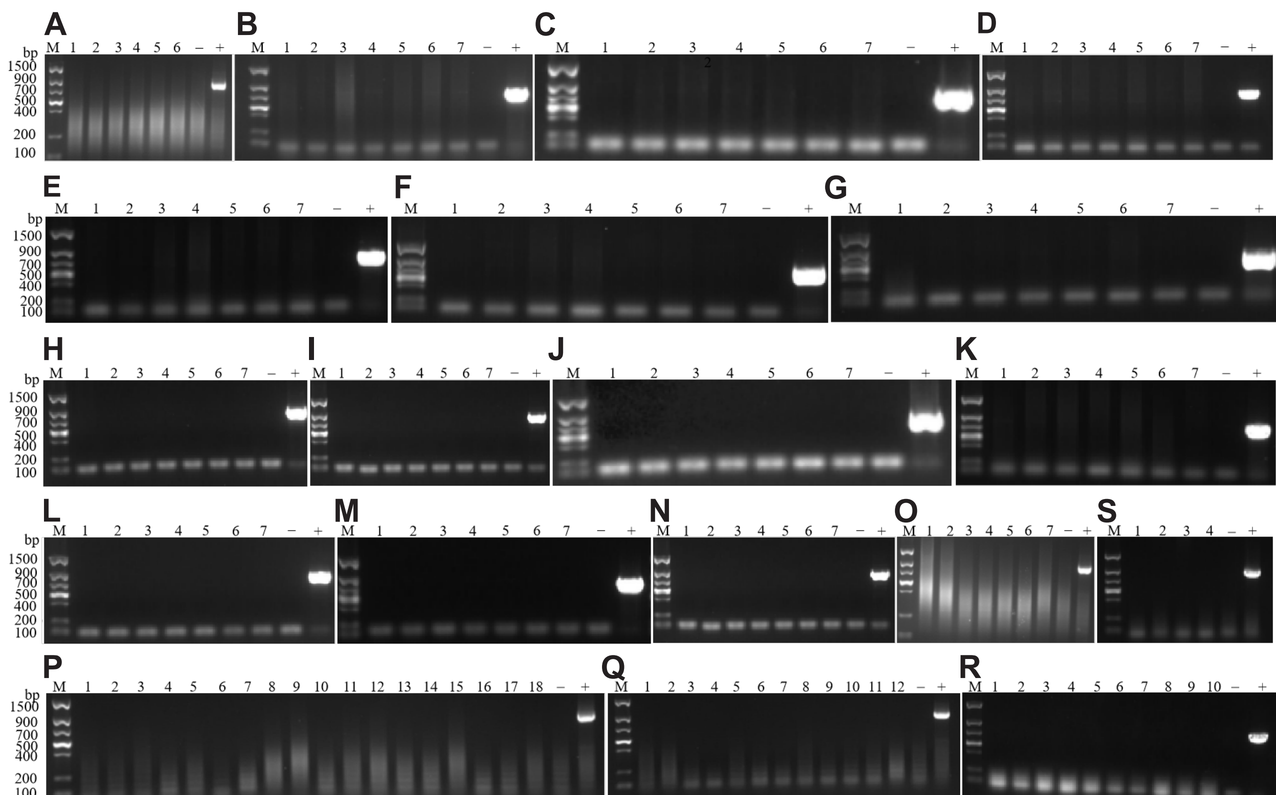

Fig. 5. Detection of *Dermocystidium anguillae* Spangenberg, 1975 in the water and sediment of culture ponds and fish feed. **A**–**O** – detection of *D. anguillae* in the water of culture ponds (**A**–**I** – nine American eels culture ponds; **J**–**O** – six European eels culture ponds). Lane 1: 15 µm filter membrane; lanes 2–7: 0.2 µm filter membranes (in Fig. 5A, lanes 2–6: 0.2 µm filter membranes. One of the 0.2 µm filter membrane was destroyed during DNA extraction). **P**, **Q** – detection of *D. anguillae* in the sediment of culture ponds (P – American eels culture ponds; Q – European eels culture ponds; in P, lanes 1–18: sediment samples; in Q, lanes 1–12: sediment samples). **R**, **S** – detection of *D. anguillae* in the fish feed (in R, lanes 1–10: starter feed; in S, lanes 1–4: artificial feed). Lane M: DL1500™ Molecular marker (TRANS); lane –: negative control; lane +: positive control.

RESULTS

Identification of *Dermocystidium anguillae* from gills of European eels

Morphological description. The infected European eels showed conspicuous operculum protrusion (Fig. 1A). After removing the operculum, innumerable whitish, round or slightly bent longish cysts (189 –2,100 µm in length) were observed in gills (Figs. 1B, 2). The cysts contained a large number of spherical spores (Fig. 3), measuring 3.9–5.2 (4.5 ± 0.3) µm in diameter, majority of which was dominated by a large refractile body with 2.1–4.0 (3.1 ± 0.3) µm in diameter.

Sequence analysis. A SSU rDNA sequence 1,691 bp long was obtained in the present study (MZ414189). Sequence analysis showed that the newly obtained sequence of *D. anguillae* infecting gills of European eels was identical to that of *D. anguillae* from gills of American eels (100% similarity with MT644944).

Development of PCR assay for the detection of *Dermocystidium anguillae*

Specificity of the present PCR assay was verified by that a single band with expected length was amplified from the target species, while no amplicons were found from non-target species (Fig. 4A). According to the result of agarose gel electrophoresis, the present PCR assay could detect 10^{-6} ng/µl of genomic DNA of *D. anguillae* (Fig. 4B).

Molecular detection of *Dermocystidium anguillae*

No amplicons were found from the water samples (Fig. 5A–O), in the sediment of culture ponds (Fig. 5P–Q), and in the starter feed (Fig. 5R) and the artificial feed (Fig. 5S). Positive amplicons were found in all 28 elver samples obtained in November 2020 (Fig. 6A) and no amplicons were found in any of 38 obtained in April 2021 (Fig. 6B). Two positive amplicons were sequenced and the obtained sequences were identical to the target fragment (data not given).

DISCUSSION

Dermocystidium anguillae was first described from gills of European eels in Germany (Spangenberg 1975). Then, dermocystidiosis of European eels caused by *D. anguillae* was reported frequently in Europe (Wootten and McVicar 1982, Molnár and Sövényi 1984). In the present study, a *Dermocystidium* species infecting gills of European eels was isolated in China. When comparing cysts and spores of the present species with those of all described species

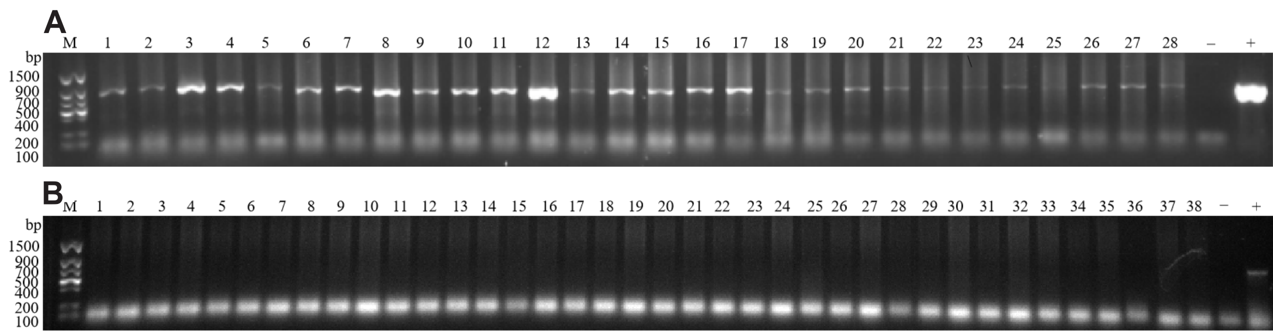

Fig. 6. Detection of *Dermocystidium anguillae* Spangenberg, 1975 in American eel elvers imported from America (**A** – lanes 1–28: elver samples obtained in November 2020; **B** – lanes 1–38: elver samples obtained in April 2021). Lane M: DL1500™ Molecular marker (TRANS); lane -: negative control; lane +: positive control.

of *Dermocystidium* Pérez, 1908, the present species was most similar to *D. anguillae*. Although mean sizes were not identical, ranges of dimensions overlap (Table 1). In addition, molecular analysis showed that the SSU rDNA of the present species was identical to that of *D. anguillae* infecting gills of American eels. In summary, based on the above biological, morphological and molecular data, the present species of *Dermocystidium* isolated from gills of European eels is identified as *D. anguillae.*

According to Liu et al. (2021), *Dermocystidium fennicum* Pekkarinen, Lom, Murphy, Ragan et Dyková, 2003 infecting gills, fins and skin of common perch, *Perca fluvi-*

Table 1. Comparison of *Dermocystidium anguillae* Spangenberg, 1975 infecting gills of European eel *Anguilla anguilla* (Linnaeus) from China with described *Dermocystidium* species. Dashes (-): data not given.

Species	Shape of cyst	Size of cyst (mm)	Size of spore (μm)	Size of refractile body (μm)	Host	Site of infection	Reference
D. anguillae Spangenberg, 1975	longish, round	0.19–2.10	4.5 ± 0.3 (3.9–5.2)	3.1 ± 0.3 (2.1–4.0)	*Anguilla anguilla*	gills	Present study
D. anguillae	longish	0.7–2.8	5.8 ± 0.6 (4.6–7.3)	3.7 ± 0.5 (3.0–5.8)	*Anguilla rostrata*	gills	Liu et al. (2021)
D. anguillae	dumb-ell-shaped	> 0.8–1.0 × 3.8	7.2 (4.8–8.5)	5.4 (2.4–7.3)	*Anguilla anguilla*	gills	Spangenberg (1975)
D. arabica Hassan, Osman et Mahmoud, 2014	longate	> 0.1	8.2 (6.4–10.3)	-	*Johinus maculatus*, *Lethrinus nebulosus*, *Lutjanus ehrenbergi*, *Lutjanus malabaricus*, *Cephalopholis hemistiktos*, *Epinephelus polyphekadion*	gills, muscle, viscera	Hassan et al. (2014)
D. branchialis Léger, 1914	round	0.2–0.6	7.0–8.0	-	*Salmo trutta fario*	gills	Léger (1914)
D. cottae Gundrizer, 1980	oviform	1.2–3.0	-	-	*Cottus kneri*	skin	Gundrizer (1980)
D. cuticulare Scheer, 1956	elongate	3	5.9	-	*Gasterosteus aculeatus*, *Pungitius pungitius*	skin	Scheer (1956)
D. cyprini Červinka, Vítovec et Lom, 1974	ovoid	0.6–2.0	4–5 (fresh) 1.3–3 (stained)	1–2 (stained)	*Cyprinus carpio*	gills	Červinka et al. (1974)
D. erschowi Garkavi, Denisov et Afanasjev, 1980	elongate	0.16–2.0	14.0–16.0	-	*Cyprinus carpio*	skin	Garkavi et al. (1980)
D. gasterostei Elkan, 1962	longish	2.0–3.0 × 0.2–0.3	3.0–5.0	-	*Gasterosteus aculeatus*, *Pungitius pungitius*	skin	Elkan (1962)
D. granulosum Sterba et Naumann, 1970	longish	2.0–3.0 × 0.8	6.5–8.5	3.5–5.0	*Tetraodon palembangensis*	skin, gills	Sterba and Naumann (1970)
D. kamilovi Allamuratov, 1965	oval	0.6–1.3 × 0.2–1.3	-	-	*Cyprinus carpio*	gills	Allamuratov (1965)
D. kobiacevi Allamuratov, 1965	oval	0.2	-	-	*Cyprinus carpio*	skin	Allamuratov (1965)
D. koi Hoshina et Sahara, 1950	elongate	0.04–0.30	6–14	4.5–10.0	*Cyprinus carpio*	skin, muscle	Hoshina and Sahara (1950)
D. kwantungensis Chen et Ma, 1960	longish	6.5–8.4 × 0.1–0.2	8.5 (6.5–10.3)	5.8 (2.9–7.4)	*Ophiocephalus maculatus*	gills	Chen and Xie (1960)
D. percae Reichenbach-Klinke, 1950	elongate	0.1–2.0 × 0.04–0.18	6–7.75	-	*Perca fluviatilis*	skin	Reichenbach-Klinke (1950)
D. salmonis Davis, 1947	round	1	7–8	-	*Salmo salar*, *Oncorhynchus mykiss*, *Oncorhynchus tshawytscha*	skin, gills	Davis (1947)
D. sinensis Xiao et Chen, 1993	-	-	13.8 (11.6–16.2)	9.5 (8.0–11.0)	*Carassius auratus gibelio*	skin	Xiao and Chen (1993)
D. vejdovskyi Jirovec, 1939	round	1.2	3.5–4.5 × 3–4	-	*Coregonus lavaretus*, *Esox lucius*	skin, gills	Jírovec (1939)

atilis Linnaeus, was identified as synonymous with *D. anguillae* by molecular analysis, although the round and dumbbell-shaped cysts of *D. fennicum* were distinct from the elongate cysts of *D. anguillae*. In the present study, both round and elongate cysts of *D. anguillae* from European eels were observed, indicating that the cyst shape of *D. anguillae* may be variable.

Owing to the high specificity and sensitivity, a number of molecular detection methods were developed to detect parasite infections (Grossel et al. 2005, Li et al. 2017). In order to investigate the source of *D. anguillae* infecting American eels cultured in China, a specific PCR assay for the detection of *D. anguillae* was developed in the present study. To evaluate the sensitivity of the present PCR assay, 100 ng DNA of each host (American eel, Chinese perch) was added into each reaction. Sensitivity analysis showed that the present PCR assay could detect 10^{-6} ng/µl of the *D. anguillae* genomic DNA. In summary, the present PCR assay was an effective tool to detect *D. anguillae*.

Before a study by Liu et al. (2021), *D. anguillae* had not been reported in China. Given that American eel elvers cultured in China were imported from America, we suspect that *D. anguillae* was introduced to China by imported American eel elvers. In addition, the possibility that American eels were infected by *D. anguillae* in cultures in China could not be excluded. To investigate the source of *D. anguillae* causing dermocystidiosis of American eels cultured in China, we tried to detect *D. anguillae* in the water and sediment of culture ponds, fish feed, and American eel elvers imported from America using the PCR assay developed in this study. Whereas no amplicons were obtained from samples of water, sediment and fish feed, positive amplicons were found in American eel elvers imported from America. This indicates that *D. anguillae* has been introduced to China by imported American eel elvers. Currently, several *Dermocystidium* species have been reported to be introduced between countries along with their aquatic hosts (Shamsi et al. 2020).

Besides China, American eels have also been introduced into Germany, Denmark and the Netherlands (Han et al. 2002, Frankowski et al. 2009, Bao 2016). To prevent the spread of *D. anguillae* worldwide, it is necessary to examine American eels for the presence of this pathogen before their export from America. A hypothesis that European eel elvers had brought *D. anguillae* from their sea sites was suggested by Wootten and McVicar (1982) and Molnár and Sövényi (1984). To verify this hypothesis, it is necessary to detect *D. anguillae* in European eel elvers in the future.

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