

アサリに寄生するPerkinsus olseniとP. honshuensisのPCR-RFLPによる識別法の開発

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Development of a PCR-RFLP Method for Differentiation of *Perkinsus olseni* and *P. honshuensis* in the Manila Clam *Ruditapes philippinarum*

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ABSTRACT—We developed a method using PCR-RFLP to differentiate *Perkinsus olseni* and *P. honshuensis*, the latter of which was recently discovered as a new species in Mie, Japan, in the Manila clam *Ruditapes philippinarum*. In normal gill samples spiked with cultured trophozoites of the parasites, the minimum infection levels that could be detected by this method were 100 cells per 10 mg sample for *P. olseni* and one cell for *P. honshuensis*. Using this method, we found that clams from the western Seto Inland Sea were infected with both species of the parasites. This is the first report of *P. honshuensis* from areas outside the type locality of this species.

Key words: *Perkinsus olseni*, *Perkinsus honshuensis*, *Ruditapes philippinarum*, PCR-RFLP

Perkinsus olseni is a protozoan parasite that infects clams, abalones and several other bivalves, and gastropods¹⁾. Infection of the Manila clam *Ruditapes philippinarum* with *P. olseni* has been reported in the Far East, including Japan, Korea, China, and south-western Europe. The majority of Manila clam populations in Japan are infected with *P. olseni*²⁾. Furthermore, *P. olseni* is thought to have caused the decline in Manila clam production in Japan and Korea in the 1980s^{3,4)}. Recently, a new species of parasite, *P. honshuensis*, was described in Manila clams in Mie Prefecture⁵⁾. These two parasite species are morphologically similar. Thus, species identification is confounded as it requires establishment of clonal strains and sequencing of several genomic regions of clones, including internal transcribed spacer regions (ITS). To investigate the distribution of these parasites, a fast and

convenient method for identification is needed. Furthermore, we had already launched a study on *Perkinsus* infection in Manila clams in the western Seto Inland Sea when *P. honshuensis* was first described. Thus, it was an urgent need to reveal whether the clam populations we investigated were infected with *P. olseni* only or co-infected with the both species.

Abollo *et al.* (2006) developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method to differentiate *Perkinsus chesapeaki*, *P. marinus*, *P. olseni* and *P. mediterraneus*⁶⁾. In this study, we developed a PCR-RFLP method to differentiate *P. olseni* and *P. honshuensis*. We then tested infection of the parasites in Manila clams from several localities in Japan.

Materials and Methods

We obtained clonal strains of *P. olseni* and *P. honshuensis* from the American Type Culture Collection (ATCC # PRA 181 and PRA 177, respectively). The trophozoite cells of them were subcultured using a routine method in *Perkinsus* broth medium (ATCC medium 1886) according to the instruction by American Type Culture Collection.

We obtained Manila clams that were infected with *P. olseni* and/or *P. honshuensis* from three localities for PCR-RFLP analysis developed in this study. Two of the localities, a clam farm in Hiroshima Prefecture and a tidal flat in Oita Prefecture, are located in the western Seto Inland Sea. The third locality is a tidal flat located in Yatsushiro Bay, Kumamoto Prefecture. Based on our preliminary examinations, the clams from the western Seto Inland Sea were heavily infected with *Perkinsus* (prevalence 100%, geometric mean intensity of infection > 10⁶ cells/g gill weight), while those from Yatsushiro Bay were very lightly infected (prevalence 44%, geometric mean intensity of infection ~10⁴ cells/g gill weight). The numbers of clams applied for the analysis were 3, 4 and 3 for the clam farm in Hiroshima, the tidal flat in Oita and the tidal flat in Kumamoto, respectively. We also obtained uninfected clams from Akkeshi, Hokkaido. We confirmed the absence of infection in clams from this locality during a preliminary examination using Ray's fluid thioglycolate medium method and *Perkinsus* genus-specific PCR¹⁾.

We extracted DNA from cultured trophozoites and sample clams using a QIAmp DNA Mini Kit (Qiagen, USA), following the manufacturer's protocol. First, we centrifuged (300 × *g*, 5 min) 10-day-old cultures of trophozoites of the two *Perkinsus* strains and extracted DNA from each pellet (~10⁵ cells/pellet). As trophozoites of the two *Perkinsus* species frequently formed massive clusters in the medium, the cell density was measured as follows: 10 μL of the pellet was resuspended in 90 μL of PBS and passed through a 25G hypodermic

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needle to break up the cell clusters. The suspension was then diluted 100 times with 100% ethanol. Following this, we mounted 0.5 or 1 μ L of the suspension onto a slide and stained the trophozoite nuclei with DNA-binding dye (Hoechst 33342) diluted in SlowFade Gold antifade reagent (Invitrogen, USA). The cells were then counted under a fluorescence microscope. Second, we extracted DNA from normal (= uninfected) gill samples (10 mg each) spiked with $1-1 \times 10^9$ cultured trophozoites. Last, we extracted DNA from 10 mg of the gills of cultured or wild Manila clams (shell length 7–18 mm) obtained from the two localities in the western Seto Inland Sea and one locality in Yatsushiro Bay.

For PCR amplification, we used following *Perkinsus* specific primers: PerkITS-85 and PerkITS-750⁷⁾. We compared the nucleotide sequences of the ITS region of congeneric species registered in GenBank (*P. olseni*, DQ370464–DQ370472 and DQ516703–DQ516715; *P. honshuensis*, DQ516697–DQ516702). Based on this comparison, we concluded that *P. olseni* and *P. honshuensis* could be differentiated by RFLP analysis using the restriction enzyme *Dde* I.

We performed PCR amplification in a total volume of 20 μ L containing 16 μ L of the DNA extract, dNTP Mixture (0.2 mM per nucleotide, TaKaRa, Japan), 0.75 μ M of each primer, and 0.025 units/ μ L TaKaRa Ex TaqTM (Hot start version; TaKaRa). The extract of cultured cells was diluted, and the amounts of DNA were adjusted to 10^{-8} – 10 ng for each PCR. The thermal conditions were 94°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. This was followed by a single step at 72°C for 10 min.

For RFLP analyses, 5 μ L of the PCR products were digested with 5–7 units of a restriction enzyme (*Hinf* I, *Rsa* I, or *Dde* I: Toyobo, Japan) in buffers provided from the manufacturer in a final volume of 10 μ L for 4 h at 37°C. Following digestion the fragments were loaded onto a 1.5% agarose gel, stained with SYBR safe DNA gel stain (Invitrogen) and visualized with Safe Imager (Invitrogen). The fragments obtained from cultured trophozoites were purified using a DNA purification kit (QIAquick PCR purification kit, Qiagen) before being loaded on the gel, while those from the gill samples spiked with trophozoites and from the collected clams were loaded without purification.

Results

RFLP band patterns after *Rsa* I and *Hinf* I digestions that were deduced from nucleotide sequences were very similar between *P. olseni* and *P. honshuensis*. Expected fragment lengths after *Rsa* I digestion were 406, 193 and 74 bp for *P. olseni* and 413, 194 and 59 bp for *P. honshuensis*. Those after *Hinf* I were 363 and 159–151 pb (a combined band) for *P. olseni* and 371 and 150–145 (a combined band) for *P. honshuensis*.

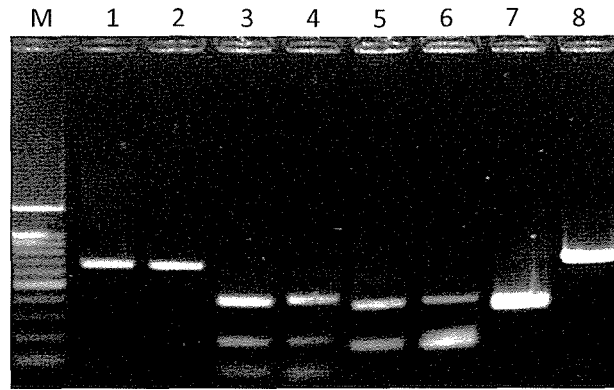


Fig. 1. PCR-RFLP electrophoretic patterns of *P. olseni* and *P. honshuensis* trophozoites digested with *Rsa* I, *Hinf* I, and *Dde* I. M, 100 bp marker; 1, undigested *P. olseni*; 2, undigested *P. honshuensis*; 3, *P. olseni* with *Rsa* I; 4, *P. honshuensis* with *Rsa* I; 5, *P. olseni* with *Hinf* I; 6, *P. honshuensis* with *Hinf* I; 7, *P. olseni* with *Dde* I; 8, *P. honshuensis* with *Dde* I.

Actual band patterns obtained from cultured trophozoites of *P. olseni* and *P. honshuensis* after *Rsa* I and *Hinf* I digestions were almost identical to the deduced patterns and very similar to each other (Fig. 1). In contrast, deduced RFLP band patterns after *Dde* I digestion were quite different between the two species. A single combined band of 349–324 bp, and a single band of 666 pb were expected for *P. olseni* and *P. honshuensis*, respectively. Actual band patterns obtained from cultured trophozoites after *Dde* I digestion were almost identical to the deduced patterns (Fig. 1).

The fragments were detected at DNA concentrations $> 10^{-1}$ ng and $> 10^{-5}$ ng for *P. olseni* and *P. honshuensis*, respectively. When this method was applied to normal gill samples spiked with cultured trophozoites of the two species, the minimum detection levels were 100 cells per 10 mg sample for *P. olseni* and one cell for *P. honshuensis* (Fig. 2). Although the length of fragments obtained from the spiked samples slightly differed from those deduced from their sequences, it is because the fragments were loaded on gels without purification; high salt concentration used for enzyme digestions affected the migration distances in gels. In the spiked samples, the resultant bands were faint or absent in some lanes that must have contained detectable amount of trophozoite DNA (lane 4 in Fig. 2 A and lane 8 in Fig. 2B). It is probably because trophozoites did not evenly disperse in the suspensions, as cultured trophozoites formed massive clusters containing hundreds of cells.

Using this method, we found that all of three Manila clams from a clam farm in Hiroshima and two out of four clams from a tidal flat in Oita, were infected with both *P. olseni* and *P. honshuensis*. Other two clams from Oita were found to be infected with *P. olseni* only. *P. olseni* was detected in only one of three Manila clams from a

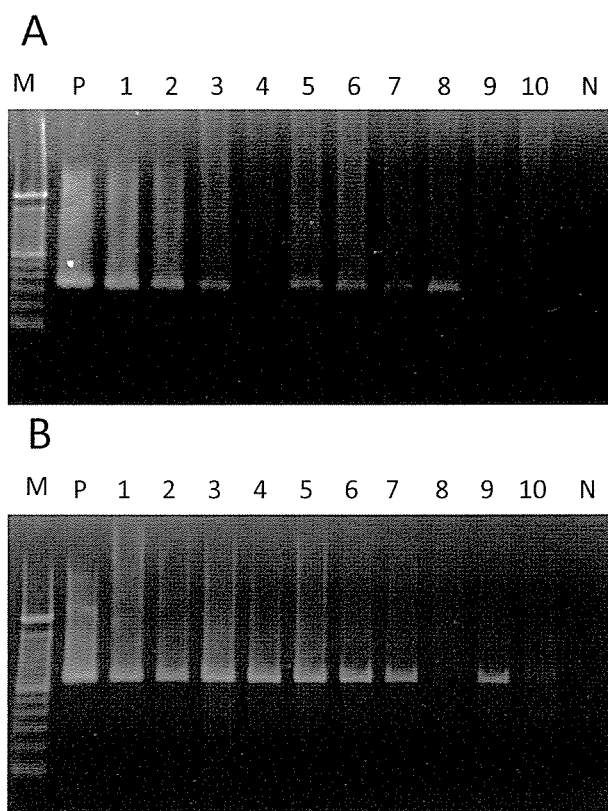


Fig. 2. PCR-RFLP electrophoretic profiles of PCR products of extracts from normal (= uninfected) gill samples spiked with *P. olseni* (A) and *P. honshuensis* (B) trophozoites. M, 100 bp marker; P, trophozoites only; N, negative control. The number of the trophozoites subjected to extraction in lane 1 was 10^9 (serially diluted 1:10).

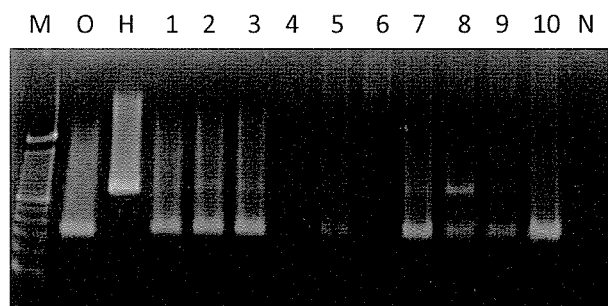


Fig. 3. PCR-RFLP analysis of Manila clams from three localities. M, 100 bp marker; O, positive control (*P. olseni*); H, positive control (*P. honshuensis*); 1–3, Hiroshima; 4–6, Kumamoto; 7–10, Oita.

tidal flat in Kumamoto, and *P. honshuensis* was not found in any clams from this site (Fig. 3).

Discussion

P. olseni can be distinguished from congeneric species by conducting PCR-RFLP with *Hinf* I and *Rsa* I⁶⁾. However, *P. olseni* and *P. honshuensis* were not distinguished using these enzymes. We developed a PCR-

RFLP method using the *Dde* I restriction enzyme that was used to distinguish these two species. We found that the intensity of *P. olseni* and *P. honshuensis* infection in Manila clams was approximated using the electrophoretic profile generated by PCR-RFLP. However, it was not achieved to accurately quantify the number of *Perkinsus* cells using this method, because the trophozoites formed massive clusters in medium. Quantitative surveillance of infection intensity are needed to estimate the impact of these parasites on clam populations. Given this, future research should focus on the development of methods for quantification. Very recently, species-specific mixed primers for *P. marinus* and *P. honshuensis* were developed by Moss⁸⁾ according to the OIE manual¹⁾. Species-specific PCR primer could be developed for *P. honshuensis* as well. By using species specific primers, semi-quantitative assays will become possible.

We used the new method to confirm the presence of parasites in Manila clams from several different localities around Japan. We found that Manila clams in the western Seto Inland Sea were infected with both *P. olseni* and *P. honshuensis*, though predominantly with *P. olseni*. Previously, *P. honshuensis* was reported only from Mie Prefecture. Detection of *P. honshuensis* had not been attempted in areas other than the original locality of the parasite until this study. Thus, this is the first report of *P. honshuensis* outside Mie. *P. olseni* may be widely distributed in other areas in Japan and in the Far East, as previously suggested⁵⁾. However, more detailed investigations are needed to accurately determine the distribution of the two species. The Manila clams from Yatsushiro Bay appeared to be infected with *P. olseni* only. However, considering that the low infection levels of *Perkinsus* in the clams from the site and the limited number of samples, the absence of *P. honshuensis* in Yatsushiro Bay is not definitive.

Acknowledgments

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分子進化に伴い変化する IHNV の病原性

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2006年に分離された IHNV 日本株の G タンパク質遺伝子を解析した結果、IHNV 日本株には静岡系および長野系が存在し、これらは日本のニジマス養殖環境下で今なお進化していることが示された。2006年分離株のニジマスに対する病原性を比較した結果、静岡系株での累積死亡率は76%以上であったが、長野系株では20-40%、また比較に用いた1976年の分離株では10%以下と、各株の病原性に差異が認められた。従って、ニジマス養殖環境下での IHNV の分子進化に伴い、IHNV のニジマスに対する病原性も変化していると考えられた。

魚病研究, 44 (4), 159-165 (2009)

アユ卵の水カビ病における塩化カリウムの抗真菌効果

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加地奈々・名倉 盾

KCl の水カビ病原菌への影響及びアユ卵の水カビ病発生防除効果について検討した。0.24% までの KCl は、水カビ病原菌の菌糸の伸長及び遊走子の発芽を抑制しない一方、遊走子の運動を0.03% で1分以内に顕著に抑制した。KCl 溶液中で、アユ卵を受精後から発眼期まで飼育した結果、0.06% 及び0.12% では水カビ病の発生が抑制された。また、この濃度で発眼率、孵化率及び奇形率に悪影響はみられなかった。KCl はアユ卵の水カビ病発生防除に有効であると考えられた。

魚病研究, 44 (4), 166-171 (2009)

生体染色と感染実験による *Enteromyxum leei* の海水中における生残能力の推定

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粘液胞子虫性やせ病原原因虫の1種 *Enteromyxum leei* の栄養体を蛍光色素で生体染色することにより、*in vitro* の *E. leei* の生死判定法を開発した。この染色法と未感染トラフグまたはクサフグへの感染実験を併用して、*E. leei* の海水中における生残能力を調べた。*In vitro* では20°C で12~24時間以内に失活したが、感染実験では24時間後で感染力を保持している場合もあった。*E. leei* の海水中での生残時間は、条件にもよるが約24時間以内であると推定された。

魚病研究, 44 (4), 172-177 (2009)

浸漬法によるヒラメ稚魚への *Tenacibaculum maritimum* 実験感染法の検討

西岡豊弘・渡辺研一・佐野元彦

ヒラメ稚魚に対する浸漬法による *T. maritimum* の実験感染条件について検討した。攻撃菌濃度が $10^{7.9}$ CFU/mL の時、いずれの試験水温 (15°C, 20°C, 25°C) でも80%以上の死亡率となった。 $10^{6.9}$, $10^{5.9}$ CFU/mL 攻撃ではどの水温でも死亡率はそれぞれ20-40%, 0%と低かった。死亡魚には口部の発赤や体表のびらんが認められ、患部より菌が再分離された。魚体通過させ、シードロット法で保存した菌株を使用したことが、強い感染力と再現性のある結果をもたらしたと考える。

魚病研究, 44 (4), 178-181 (2009)

わが国におけるサケ・マス類の病原ウイルス、特に伝染性サケ貧血症ウイルスの浸潤状況

笠井久会・岩脇周平・吉水 守

伝染性サケ貧血症ウイルス (ISAV) の浸潤状況を明らかにすることを目的に、2005~2007年にかけてサケ科魚類8種5,967尾のメス親魚から体腔液を採取し、ASK および ASE 細胞を用いてウイルスの分離培養を試みた。その結果、ISAV はいずれの検体からも分離されなかったが、3魚種116尾から伝染性造血器壊死症ウイルス (IHNV) が、また3魚種14尾から伝染性臓腑壊死症ウイルス (IPNV) が分離された。従って、ISAV は日本未侵入であると考えられた。

魚病研究, 44 (4), 182-184 (2009)

アサリに寄生する *Perkinsus olseni* と *P. honshuensis* の PCR-RFLP による識別法の開発

高橋美希・良永知義・脇 司

下川 潤・小川和夫

アサリに寄生するパーキンサス属原虫2種の PCR-RFLP による識別法を開発した。未感染のアサリの鰓と培養した栄養体を混合した試料では、*P. olseni* と *P. honshuensis* の検出限界は、アサリ鰓 10 mg あたりそれぞれ100細胞と1細胞であった。この方法によって、瀬戸内海西部のアサリにこの2種の混合感染が確認された。*P. honshuensis* は本虫が最初に確認された三重県以外にも広く分布することが示唆された。

魚病研究, 44 (4), 185-188 (2009)