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
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 gastropods*. Infection of the Manila clam Ruditapes philippinarum with P. olseni has been reported in the Far East, including Japan, Korea, China, and southwestern Europe. The majority of Manila clam populations in Japan are infected with P. olseni*. Furthermore, P. olseni is thought to have caused the decline in Manila clam production in Japan and Korea in the 1980s. Recently, a new species of parasite, P. honshuensis, was described in Manila clams in Mie Prefecture*. 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*. 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 6) 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 > 10^5 cells/g gill weight), while those from Yatsushiro Bay were very lightly infected (prevalence 44%, geometric mean intensity of infection ~10^4 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 PCR*.

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 x g, 5 min) 10-day-old cultures of trophozoites of the two Perkinsus strains and extracted DNA from each pellet (~10^5 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 µL 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 × 10⁶ 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 PerktTS-750. We compared the nucleotide sequences of the ITS region of congeneric species registered in GenBank (P. olseni, DQ570464–DQ570472 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 µL containing 16 µL of the DNA extract, dNTP Mixture (0.2 mM per nucleotide, TaKaRa, Japan), 0.75 µM of each primer, and 0.025 units/µL TaKaRa Ex Taq™ (Hot start version; TaKaRa). The extract of cultured cells was diluted, and the amounts of DNA were adjusted to 10⁻⁶–10 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 µL 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 µL for 4 h at 37°C. Following digestion the fragments were loaded onto a 1.5% agarose gel stained with TAE™ (Hot start version; TaKaRa). The extract of cultured cells was digested, and the amounts of DNA were adjusted to 10⁻⁶–10 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 µL 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 µL 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 were 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 > 10⁻⁷ 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...
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A

![PCR-RFLP electrophoretic profiles of PCR products of extracts from normal (uninfected) gill samples spiked with *P. olseni* (A) and *P. honshuensis* (B) trophozoites.](image)

B

![PCR-RFLP analysis of Manila clams from three localities.](image)

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 I*. 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 Moss according to the OIE manual. 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 suggested. 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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References

分子進化に伴い変化する IHNV の病原性
望月万美子・金 亨樫・笠井久会
西澤重良・吉水 守
2006年に分離された IHNV 日本株の Δ タンパク質遺伝子を解析した結果、IHNV 日本株には静岡県および長野県が存在し、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、静岡県系での累積死亡率は76%以上であったが、長野県系では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)

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
横山 博・筒井栄・柳田哲夫・小川和夫
粘液脳子虫性やせ病の原因虫の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 での
E. leei の生死判定法を開発した。この染色法は未感染
トラフガまたはヌサフガへの感染実験を併せて、E. leei
の海水中における生残能力を調べた。in vitro では20℃
で12-24時間以内に失活したが、感染実験では24時間後
で感染を保持している陽性株もあった。E. leei の海水中
での生残時間は、条件によっては約24時間以内であると
推定された。
魚病研究. 44 (4), 172-177 (2009)

アユ卵の水カリ病における塩化カリウムの抗真菌効果
三浦正之・細井喜司雄・大野平祐
加地奈々・名取 盾
KCIの水カリ病原因菌への影響及びアユ卵の水カリ病
発生防止効果について検討した。0.24%までのKCIは、
水カリ病原因菌の伸長および遊泳の発育を抑制しない一方、遊泳の運動を0.03%で1分以内に顕著に抑
制した。KCI浸漬で、アユ卵を受精卵から発育期まで
飼育した結果、0.06%及び0.12%では水カリ病の発生が
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。KCIはアユ卵の水カリ病
発生防止に有効であると考えられた。
魚病研究. 44 (4), 166-171 (2009)

メンシェイクに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10⁷ CFU/mL
の時、いずれの試験水温（15℃、20℃、25℃）でも60%
以上の死亡率となった。10⁸、10⁹ CFU/mL 前後では、こ
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。魚体通過率が0.03%で保存した
菌株を使用することが可能で、強い感染力と再現性
のある結果を得たと考察される。
魚病研究. 44 (4), 178-181 (2009)

ワグナロにおけるサケ・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調試験
笠井久会・岩原周平・吉水 守
伝染性サケ貧血症ウイルス（ISAV）の浸調状況を明らか
にするために、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検査を試みた。
その結果、ISAV はいずれの検体からも分離されなかった
が、魚種116尾から伝染性造血組織死症ウイルス
（IHNV）が、また魚種14尾から伝染性脳腫死症ウイ
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・前 司
下川 源・小川和夫
アサリ寄生する P. olseni と P. honshuensis の PCR-
RFLP による識別法を再開発した。未感染のアサリの餌と
培養した栄養体を混和した試料では、P. olseni と P.
honshuensis の菌株は、アサリ餌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、関
内外海西部のアサリにここの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)