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Perkinsus olseni and *P. chesapeaki* detected in a survey of perkinsosis of various clam species in Galicia (NW Spain) using PCR–DGGE as a screening tool

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ABSTRACT

A survey on perkinsosis was performed involving 15 locations scattered along the Galician coast (NW Spain) and four clam species with high market value (*Ruditapes decussatus*, *Ruditapes philippinarum*, *Venerupis corrugata* and *Polititapes rhomboides*). The prevalence of *Perkinsus* parasites was estimated by PCR using genus-specific primers. The highest percentage of PCR-positive cases for perkinsosis corresponded to clams *R. decussatus* and *V. corrugata*, while lower values were detected in *R. philippinarum* and no case was found in *P. rhomboides*. The discrimination of *Perkinsus* species was performed by PCR–RFLP and by a new PCR–DGGE method developed in this study. *Perkinsus olseni* was identified in every clam species, except in *P. rhomboides*, using both PCR–DGGE and PCR–RFLP. Additionally, *Perkinsus chesapeaki* was only detected by PCR–DGGE infecting two Manila clams *R. philippinarum* from the same location, reporting the first case in Galicia. *P. chesapeaki* identification was further confirmed by *in situ* hybridisation assay and phylogenetic analysis of ITS region and LSU rDNA.

1. Introduction

Perkinsosis is a disease affecting numerous mollusc species worldwide, causing important mortalities and economic losses. The use of molecular tools has contributed to discriminate *Perkinsus* species and to record them in new hosts and locations. Nowadays, seven *Perkinsus* species are considered valid: *P. marinus* (Mackin et al., 1950), *P. olseni* (Lester and Davis, 1981), *P. qugwadi* (Blackbourn et al., 1998), *P. chesapeaki* (McLaughlin et al., 2000), *P. mediterraneus* (Casas et al., 2004), *P. honshuensis* (Dungan and Reece, 2006) and *P. beihaiensis* (Moss et al., 2008). Two of them, *P. marinus* and *P. olseni*, have a severe impact on bivalve mollusc populations and are included in the list of reportable diseases of aquatic animals by the World Organisation for Animal Health. Three species of the genus have been reported from Europe: *P. olseni*, a cosmopolitan species infecting various mollusc species along the coasts of Europe (Azevedo, 1989; Abollo et al., 2006; Elandaloussi et al., 2009; Arzul et al., 2012; Ramilo et al., 2015); *P. mediterraneus*, originally described infecting oysters *Ostrea edulis* (Casas et al., 2004) from Balearic Islands (Spain) and then found in other hosts and Mediterranean locations of Spain and Italy (Valencia et al., 2014; Ramilo et al., 2015); and *P. chesapeaki*, a species that had been reported from various hosts in the Atlantic coast of USA (McLaughlin et al., 2000; Coss et al., 2001; Dungan et al., 2002; Pecher et al., 2008; Reece et al., 2008; Marquis et al., 2015) and recently found in clams *Ruditapes decussatus* and *Ruditapes philippinarum* from France (Arzul et al., 2012), cockles *Cerastoderma edule* from Spain (Carrasco et al., 2014) and mud ark cockles *Anadara trapezia* from Australia (Dang et al., 2015). Perkinsosis is an endemic disease in Galicia (NW Spain) affecting various clam species of clams and *P. olseni* is the only species of the genus reported thus far (Robledo et al., 2000; Casas et al., 2002; Balseiro et al., 2010).

Perkinsus infections can be diagnosed by incubating host tissues in Ray's fluid thioglycollate medium (RFTM) (Ray, 1966); the RFTM assay is more sensitive than standard histology, but it does not allow discrimination between *Perkinsus* spp. Additionally, PCR assays have been developed to be genus-specific (Casas et al., 2002; Robledo et al., 2002; Gauthier et al., 2006) and species-specific, such as those developed for *P. marinus* (Robledo et al., 1998; Audemard et al., 2004; De Faveri et al., 2009), *P. olseni* (De la Herrán et al., 2000; Robledo et al., 2000; Park et al., 2002), *P. chesapeaki* (Coss et al., 2001) and *P. beihaiensis* (Moss et al., 2008). Moreover, restriction fragment length polymorphism (RFLP) assays have been also designed for differential diagnosis of

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Perkinsus species (Kotob et al., 1999; Abollo et al., 2006) and *in situ* hybridisation (ISH) assays for the detection of *Perkinsus* species on histological sections (Moss et al., 2006, 2008; Navas, 2008; Ramilo et al., 2015). In this study, another molecular procedure, based on denaturing gradient gel electrophoresis (DGGE), has been applied for the first time to simultaneously detect various *Perkinsus* species. DGGE is a molecular technique used to separate DNA fragments of the same length but with different sequences. This method has a high power of discrimination, detecting until single base variations in DNA (Fisher and Lerman, 1983; Lerman and Beldjord, 1999). DGGE analysis have been widely used in microbial ecology studies in different environments (Muyzer and Samala, 1998) including aquaculture systems (Pintado et al., 2010; Prol-García et al., 2010, 2014); its application is not widespread in parasitological studies, it has been used to identify Quahog Parasite Unknown (QPX) infecting clams *Mercenaria mercenaria* (Gast et al., 2006, 2008), for the discrimination of three species and one genotype of the protistan genus *Cryptosporidium* (Satoh and Nakai, 2007), for the discrimination of *Eimeria* species (Martynova-Vankley et al., 2008) and to display sequence variation of nematode parasite species (Gasser et al., 1996, 1998).

The above-referred recent reports of *Perkinsus* spp. in new areas and new hosts encouraged us to perform a survey of perkinsosis along Galician coast, involving four clam species with high market value, using sensitive and specific diagnostic tools. A new PCR–DGGE assay was developed to discriminate *Perkinsus* species, which was compared with the well-known PCR–RFLP assay described by Abollo et al. (2006). DGGE–PCR results revealed for the first time the presence of *P. chesapeaki* in Galicia (NW Spain), infecting the Manila clam *R. philippinarum*.

2. Material and methods

2.1. Mollusc samples

Samples involving four clam species were collected between April and June 2010 from 15 beds scattered along Galician coast: grooved carpet-shell clams *R. decussatus* were obtained from O Barqueiro, Foz, Ortigueira, Viveiro, A Toxa and Lourizán; Manila clams *R. philippinarum* were collected from Carril, Pontedeume, Camariñas and Arcade; pullet carpet-shell clams *Venerupis corrugata* were obtained from A Coruña, Illa de Arousa, Ferrol, Vilanova de Arousa and Cangas; and banded clams *Polititapes rhomboides* were collected from A Illa de Arousa (Fig. 1). Samples consisting of 30 clams from each site were collected and, once in the laboratory, the clams were placed in tanks with filtered seawater and aeration, overnight, to favour sediment expelling before being processed for histological and molecular procedures.

2.2. Histology

A section (ca. 5 mm thick) of the meat of each specimen containing gills, visceral mass, mantle lobes and foot was fixed in Davidson's solution, dehydrated in an ethanol series and embedded in paraffin. Paraffin blocks were stored in case histological examination or ISH assay were needed.

2.3. Genomic DNA extraction

Small pieces of gills from the clams were preserved in 96% ethanol for molecular analysis. Moreover, samples from *in vitro* non-clonal cultures of *P. olseni*, *P. mediterraneus*, *P. chesapeaki* and *P. marinus* were also employed for the development and validation of the DGGE technique. DNA purifications were performed employing the commercial kit Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol. DNA quality and quantity was checked with a spectrophotometer Nanodrop® ND-1000 (Nanodrop technologies, Inc).

2.4. Molecular procedures

PCR assays using the generic primers PerkITS750/PerkITS85 (Casas et al., 2002) (Table 1) were carried out first to detect *Perkinsus* spp. in all sampled clams. Subsequently, all positive cases were analysed to identify *Perkinsus* species by the well-known RFLP analysis, as described by Abollo et al. (2006), and by a new PCR–DGGE assay described next. Eight random PCR products were also sequenced to confirm the identity of the restriction profile obtained.

2.4.1. PCR–DGGE assay

2.4.1.1. Primer design. To develop the DGGE method for *Perkinsus* spp. diagnosis, new genus-specific primers, named PerkLSU-F and PerkLSU-R (Table 1), were designed based on the LSU rDNA sequence of *Perkinsus* genus. This region shows lower intraspecific variability than ITS region, which is used for diagnosis of *Perkinsus* spp. For this, the *Perkinsus* spp. DNA sequences shown in Table 2 were aligned using the programme Clustal W (Kumar et al., 2004) and, subsequently, the primers were designed using the programme Primer 3 (Rozen and Skaletsky, 2000).

2.4.1.2. PCR assay. In order to obtain a pattern to be used as a reference for each *Perkinsus* species, PCRs were performed with *in vitro* cultured cells of *P. olseni*, *P. chesapeaki*, *P. marinus* and *P. mediterraneus*. Subsequently, DNA from every *Perkinsus* PCR-positive clam was selected to perform PCR–DGGE assay. Those DNA samples were amplified using the primers PerkLSU-F/PerkLSU-R. The forward primer was used with a GC clamp attached to the 5′ end (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAC GGGGGG-3′). The PCR mix solution was prepared as described above. All PCRs were carried out under the following reaction parameters: 94 °C for 2 min, 40 cycles at a denaturation temperature of 94 °C for 30 s, an annealing temperature of 55 °C for 45 s and an extension temperature of 72 °C for 1 min, followed by a final extension of 72 °C for 7 min. Positive control for *Perkinsus* sp. and a negative control (no DNA) were also used and 10 μl of each amplified DNA were analysed by electrophoresis on 2% agarose gels.

2.4.1.3. DGGE. PCR products were analysed by DGGE using a Bio Rad DCode for electrophoresis. One hundred nanograms of each PCR amplified product were loaded on 8% (w/v) polyacrylamide gels in TAE 1X with two concentrations of gradients: 20–80% and 30–60% gradient urea-formamide (100% corresponded to 7 M urea and 40% (v/v) formamide), increasing in the direction of electrophoresis. Additionally, 6% polyacrylamide gels with a denaturing gradient of 20–80% and 30–70% were also tested in order to establish the best concentration of polyacrylamide and denaturing gradient for *Perkinsus* species. Gels were run at 60 °C for 10 min to 20 V and 17 h at 100 V, stained with ethidium bromide for 15 min and rinsed for 20 min in distilled water. PCR products derived from *in vitro* cultured cells of *P. olseni*, *P. chesapeaki*, *P. marinus* and *P. mediterraneus* were included in all gels as reference of each species. DGGE gels were scanned in a GelDoc XR documentation system (BioRad). To confirm the specific identity, different patterns of bands were cut out with a sterile scalpel. All bands were washed with 200 μl of sterile water and DNA was eluted in 50 μl of the same solution at 4 °C for 5 days. After this, 5 μl of each band were re-amplified using the primer PerkLSU-R/PerkLSU-F without the GC clamp, using the same conditions described above, and then processed for sequencing.