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Short Communication

Update of information on perkinsosis in NW Mediterranean coast: Identification of *Perkinsus* spp. (Protista) in new locations and hosts

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ABSTRACT

This study addressed perkinsosis in commercially important mollusc species in the western Mediterranean area. *Perkinsus olseni* was found in Santa Gilla Lagoon (Sardinia) infecting *Ruditapes decussatus*, *Cerastoderma glaucum* and *Venerupis aurea*, in Balearic Islands infecting *Venus verrucosa* and in Delta de l'Ebre (NE Spain) parasitising *Ruditapes philippinarum* and *R. decussatus*. *Perkinsus mediterraneus* was detected infecting *Ostrea edulis* from the Gulf of Manfredonia (SE Italy) and Alacant (E Spain), *V. verrucosa* and *Arca noae* from Balearic Islands and *Chlamys varia* from Balearic Islands, Alacant and Delta de l'Ebre.

1. Introduction

Protozoan parasites of the genus *Perkinsus* have caused important mortalities in different mollusc species around the world resulting in severe economic losses (Andrews, 1988; Choi and Park, 2010; Villalba et al., 2011; Waki et al., 2012). The genus *Perkinsus* is included in the group Perkinsidae, which is assigned to the Protalveolata, in the higher rank group Alveolata, within the super group Sar (Adl et al., 2012). Seven species of *Perkinsus* are considered "valid" (Moss et al., 2008): *P. marinus*, *P. olseni*, *P. qugwadi*, *P. chesapeaki*, *P. mediterraneus*, *P. honshuensis* and *P. beihaiensis*. Three species of *Perkinsus* have been identified in Mediterranean waters: *P. mediterraneus* infecting the oyster *Ostrea edulis* (Casas et al., 2004) and the clam *Chamelea gallina* (Moss et al., 2008) from Balearic Islands (Spain); *P. olseni* infecting clams *Ruditapes philippinarum* from Venice lagoon (NW Italy) (Abollo et al., 2006), *Ruditapes decussatus* and *R. philippinarum* from Delta de L'Ebre (Catalonia, NE Spain) (Elandaloussi et al. 2009a, b) and both clam species from the Thau and Leucate lagoons of the French Mediterranean coast (Arzul et al., 2012); and *P. chesapeaki* infecting *R. decussatus* along the Mediterranean coast of France (Arzul et al., 2012) and cockles *Cerastoderma edule* in Catalonia (NE Spain) (Carrasco et al., 2014). Additionally, a number of molluscan species have been reported hosting unidentified *Perkinsus*-like parasites, including *Venerupis aurea* and *Pecten maximus* from the French coast (Goggin, 1992), and *V. aurea, C. edule*, *Callista chione*, *Mytilus galloprovincialis*, *Crassostrea gigas*, *Venus verrucosa*, *Chamelea gallina* and *Musculista senhousia* from the Italian coasts (da Ros and Canzonier, 1985; Berrilli et al., 1998; Canestri-Trotti et al., 1999, 2000a, 2000b). Identification of the *Perkinsus* species involved in each case is important for accurate risk assessment and disease management. Morphological characters do not allow conclusive species discrimination within this genus and its taxonomy is mostly based on the sequence of regions of the rDNA gene complex (Villalba et al., 2004; Moss et al., 2008); therefore species-specific diagnostic tools have been designed to target these sequences and include the polymerase chain reaction (PCR), restriction fragment length polymorphism analysis (RFLP), and *in situ* hybridisation (ISH) with DNA probes (Villalba et al., 2011).

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This article focuses on perkinsosis-affected mollusc species with commercial interest, occurring in areas of the West Mediterranean coast. *P. olseni* and *P. mediterraneus* have been detected in new hosts and new locations, which highlights the need for assessing the risk of these parasites for their respective host populations.

2. Material and methods

Mollusc samples were collected with two main objectives, a set of samples (A) to identify the species of *Perkinsus* occurring in different mollusc species from different locations, and another set of samples (B) to analyse perkinsosis affecting the scallop *Chlamys varia*, a species of increasing interest for aquaculture, taking also oysters *O. edulis* as a reference because of their known susceptibility to infection with *P. mediterraneus*. Table 1 summarises information on the samples. The set A samples were collected from natural beds in Maó (Menorca, Balearic Islands, Spain), Port d'Andratx (Mallorca, Balearic Islands), Delta de l'Ebre (Catalonia, NE Spain), Santa Gilla Lagoon (Sardinia, Italy) and Gulf of Manfredonia (Puglia, SE Italy), by different scientists, without a previous common plan; thus different sample types were obtained (live specimens, tissues from RFTM positive individuals preserved in 95% ethanol and tissues embedded in paraffin blocks) which had to be diagnosed with different procedures (PCR-RFLP, ISH or histology). The set B samples were collected from a farming area in Alacant (País Valencià, E Spain), a natural bed in Delta de l'Ebre and a natural bed in Maó.

Live specimens in sets A and B were processed with standard histological procedures to produce sections stained with Harris' haematoxylin and eosin (Howard et al., 2004). After taking the portion for histological analysis, a piece of gill tissue was collected and preserved in 96% ethanol for molecular analysis. DNA extractions were performed employing the Wizard Genomic DNA Purification Kit (Promega) for the samples of set A and the DNeasy Blood and Tissue Kit (Qiagen) for the samples of set B. Genomic DNA was also purified from the paraffin blocks using QIAamp DNA FFPE Tissue (Qiagen) according to the manufacturer's instructions.

To identify *Perkinsus* species, rRNA ITS region was amplified using the generic PCR assay for *Perkinsus* spp described by Casas et al. (2002). Subsequently, the RFLP assay described by Abollo et al. (2006) was performed on the PCR amplicons. For the set A samples, PCR products representing each different banding pattern by RFLP and those in which DNA concentrations were not high enough to carry out the RFLP assays, were ligated into the cloning vector pCR2.1 at 14 °C overnight and transformed into *E. coli* One Shot Top 10F′ Chemically Competent cells (Invitrogen Life Technologies™). Transformed cells were screened by PCR as described above and the positive clones were sequenced by the company Secugen (Madrid). For the set B samples, purified PCR amplicons (QIAquick PCR Purification Kit of QIAGEN) from 4 scallops *C. varia* collected from Alacant (3 of the first sample and 1 of the second sample) and 3 flat oysters (1 of each sample) were sequenced by the Sequencing Service of Valencia University (Spain). All sequences generated were searched for similarity to those previously deposited in GenBank using the BLAST tool (Altschul et al., 1997) available at the National Center for Biotechnology Information's website (USA).

For diagnosis of *P. mediterraneus* with an ISH assay, an antisense probe targeting the LSU rDNA and LSU rRNA was designed by alignment of sixty LSU rDNA sequences from the seven valid *Perkinsus* species. A sequence of 19 nucleotides that was appropriate for probe development was identified for *P. mediterraneus* and *P. honshuensis* that had at least three differences from the other species. The probe Pmed_PhonLSU410 5′-AGACAGAGGCGGGCAGCAA-3′ binds near position 410 of the aligned LSU rRNA gene sequences. A probe sequence unique to *P. mediterraneus* that would not bind to the species *P. honshuensis* could not be identified as the LSU rDNA sequences of these two species are very similar. Specificity of the *P. mediterraneus/P. honshuensis* probe was determined by testing against *Perkinsus* sp.-infected reference tissues, including *P. marinus* in *Crassostrea virginica*, *P. chesapeaki* in *Mya arenaria*, *P. olseni* in *R. philippinarum*, and *P. beihaiensis* in *Crassostrea hongkongensis*. In order to confirm the positive results for *P. mediterraneus*, obtained by PCR-RFLP or DNA sequencing, paraffin blocks corresponding to *O. edulis* from Gulf of Manfredonia, and *O. edulis*, *C. varia* and *A. noae* from Port d'Andratx were employed for ISH assay, using the *P. mediterraneus/P. honshuensis*-specific probe (PMed_PhonLSU410) labelled with digoxigenin, as described by Ramilo et al. (2014).

Table 1
Characteristics of the mollusc samples, number of specimens in which *Perkinsus mediterraneus*, *Perkinsus olseni* and both parasites were detected in the set A samples and the number of specimens with positive detection of *Perkinsus* sp. by histology in the set B samples.

Species	Location	Sampling date	Type of sample (N)	Diagnostic test	*P. mediterraneus*	*P. olseni*	Both spp.
Set of samples A							
Venus verrucosa	Maó	September 2007	Live specimens (31)	Histology, PCR–RFLP	3	2	14
Ostrea edulis	Maó	September 2007	Live specimens (32)	PCR–RFLP	25	0	0
Ruditapes philippinarum	Delta de L'Ebre	September 2007	Live specimens (8)	PCR–RFLP	0	4	0
Ruditapes decussatus	Delta de L'Ebre	September 2007	Tissues in ethanol (3)	PCR–RFLP	0	3	0
Ruditapes decussatus	Santa Gilla Lagoon	October 2007	Tissues in ethanol (5)	PCR–RFLP	0	5	0
Venerupis aurea	Santa Gilla Lagoon	October 2007	Tissues in ethanol (2)	PCR–RFLP	0	2	0
Cerastoderma glaucum	Santa Gilla Lagoon	October 2007	Tissues in ethanol (3)	PCR–RFLP	0	3	0
Ostrea edulis	Gulf of Manfredonia	November 2008	Paraffin block (1)	Histology, ISH	1	0	0
Arca noae	Port d'Andratx	September 2011	Paraffin block (1)	Histology, ISH	1	0	0
Chlamys varia	Port d'Andratx	November 2011	Paraffin block (1)	Histology, ISH	1	0	0

Species	Location	Sampling date	Type of sample (N)	Diagnostic test	*Perkinsus* sp. (histology)
Set of samples B					
Chlamys varia (2010 recruits)	Alacant	December 2011	Live specimens (30)	Histology, PCR–RFLP[a]	9
Chlamys varia (2011 recruits)	Alacant	December 2011	Live specimens (30)	Histology, PCR–RFLP[a]	1
Chlamys varia	Delta de L'Ebre	March 2012	Live specimens (29)	Histology	2
Chlamys varia	Delta de L'Ebre	May 2012	Live specimens (23)	Histology	2
Chlamys varia	Maó	July 2012	Live specimens (47)	Histology	7
Ostrea edulis (2008 recruits)	Alacant	November 2011	Live specimens (30)	Histology, PCR	2
Ostrea edulis (2009 recruits)	Alacant	November 2011	Live specimens (30)	Histology, PCR	3
Ostrea edulis (2010 recruits)	Alacant	November 2011	Live specimens (30)	Histology, PCR	3

[a] Restriction fragment length polymorphism (RFLP) assays were performed only with some but not all specimens of the sample.