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Species-specific diagnostic assays for *Bonamia ostreae* and *B. exitiosa* in European flat oyster *Ostrea edulis*: conventional, real-time and multiplex PCR

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ABSTRACT: *Bonamia ostreae* and *B. exitiosa* have caused mass mortalities of various oyster species around the world and co-occur in some European areas. The World Organisation for Animal Health (OIE) has included infections with both species in the list of notifiable diseases. However, official methods for species-specific diagnosis of either parasite have certain limitations. In this study, new species-specific conventional PCR (cPCR) and real-time PCR techniques were developed to diagnose each parasite species. Moreover, a multiplex PCR method was designed to detect both parasites in a single assay. The analytical sensitivity and specificity of each new method were evaluated. These new procedures were compared with 2 OIE-recommended methods, viz. standard histology and PCR-RFLP. The new procedures showed higher sensitivity than the OIE recommended ones for the diagnosis of both species. The sensitivity of tests with the new primers was higher using oyster gills and gonad tissue, rather than gills alone. The lack of a 'gold standard' prevented accurate estimation of sensitivity and specificity of the new methods. The implementation of statistical tools (maximum likelihood method) for the comparison of the diagnostic tests showed the possibility of false positives with the new procedures, although the absence of a gold standard precluded certainty. Nevertheless, all procedures showed negative results when used for the analysis of oysters from a *Bonamia*-free area.

KEY WORDS: Bonamiosis · *Bonamia exitiosa* · *Ostrea edulis* · Conventional PCR · Real-time PCR · Multiplex PCR · Bivalve

INTRODUCTION

Bonamiosis is a serious disease that causes mass mortalities of different species of oysters around the world. It is caused by protozoan parasites of the genus *Bonamia*, which is included in the phylum Haplosporidia (Perkins 1990, Burreson & Ford 2004). Two species of *Bonamia*, *B. ostreae* (Pichot et al. 1980) and *B. exitiosa* (Hine et al. 2001), are highly pathogenic and are widespread, the former in the northern hemisphere and the latter in both northern and southern hemispheres. *B. ostreae* has been reported infecting flat oyster *Ostrea edulis* of the Pacific (Katkansky et al. 1969, Elston et al. 1986, Friedman et al. 1989) and Atlantic coasts (Friedman & Perkins 1994, Zabaleta & Barber 1996) of the USA, as well as in Canada (Marty et al. 2006), France (Pichot et al. 1980), Spain (Polanco et al. 1984), the UK (Hudson & Hill 1991), The Netherlands (van Banning 1991), Ireland (Culloty & Mulcahy 2001), Italy (Narcisi et al. 2010) and Morocco (Belhsen et al. 2008). *B. exitiosa* was first reported infecting *O. chilensis* in New Zealand (Hine 1991, Doonan et al. 1994, Cranfield et al. 2005). *B. exitiosa* or *Bonamia* sp. resembling *B. exitiosa* were also found in *O. chilensis* from Chile (Kern 1993, Campalans et al.

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2000), *O. angasi* from Australia (Corbeil et al. 2006a), *O. puelchana* from Argentina (Kroeck & Montes 2005), *Crassostrea ariakensis* from the USA (Burreson et al. 2004), *O. edulis* from Spain (Abollo et al. 2008), Italy (Narcisi et al. 2010) and France (Arzul et al. 2011), and *O. stentina* from Tunisia (Hill et al. 2010). The parasite originally described as *Mikrocytos roughleyi* (Farley et al. 1988), which infects oysters *Saccostrea glomerata* in Australia, is considered to be closely related to *B. exitiosa* (Cochennec-Laureau et al. 2003, Carnegie & Cochennec-Laureau 2004, Hill et al. 2010). *B. perspora* is a further member of the genus. It has only been reported infecting the type host, *Ostreola equestris*, in North Carolina, USA (Carnegie et al. 2006).

The World Organisation for Animal Health (OIE) has included infections with *Bonamia ostreae* and *B. exitiosa* on the list of notifiable diseases (www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2013/). The European animal health regulation includes both species among those to be kept under surveillance, reporting *B. exitiosa* on the list of exotic diseases and *B. ostreae* on the list of non-exotic ones (Council of the European Union 2006). Both parasites co-occur in some areas of Europe and may cause mixed infections in the same oyster (Abollo et al. 2008). Therefore, the development of rapid, sensitive and specific diagnostic methods for bonamiosis is indispensable in order to carry out early detection of *Bonamia* spp. with the aim of preventing infection of healthy animals and dispersion of the agent to non-affected areas.

Different diagnostic methods for detection and identification of *Bonamia ostreae* and *B. exitiosa* have been developed, but all of them have certain limitations. Light microscopy-based methods such as examination of gills or heart tissue imprints or histological sections have been described, but these methods are slow (especially the latter technique) and require a trained observer to screen the samples, because of the similar morphological characteristics between the species (Hine et al. 2001, Abollo et al. 2008). Numerous researchers have noticed low sensitivity and specificity of these light microscopy techniques (O'Neill et al. 1998, Culloty et al. 2003, Diggles et al. 2003, da Silva & Villalba 2004, Balseiro et al. 2006, Marty et al. 2006). Molecular techniques used for identification of *Bonamia* spp., such as PCR or *in situ* hybridization, have been proven to be more sensitive than microscopical techniques, but most of them lack specificity for species identification. Thus, several PCR assays for detection of *Bonamia* spp. have been developed, like those recommended by the OIE (Carnegie et al. 2000, Cochennec et al. 2000), those described by Abollo et al. (2008) and Carnegie et al. (2006) or TaqMan® assays developed by Marty et al. (2006) and Corbeil et al. (2006b). However, none of these methods is specific for either *B. ostreae* or *B. exitiosa*. For this reason, differentiation between the 2 species has been achieved using PCR-RFLP assays (Hine et al. 2001, Cochennec-Laureau et al. 2003, Abollo et al. 2008), sequencing the products obtained by PCR assays or by means of light microscopic techniques, thus causing the diagnostic process to be more expensive and slower. Recently, species-specific PCR assays for *B. ostreae* have been described, including conventional PCR (cPCR) developed by Engelsma et al. (2010) and quantitative real-time PCR designed by Robert et al. (2009).

The aim of our study was to develop molecular tools for a specific, rapid and sensitive diagnostic of bonamiosis in the European flat oyster *Ostrea edulis*. Here we describe (1) a species-specific cPCR for *Bonamia exitiosa* and another for *B. ostreae*; (2) a species-specific real-time PCR for each of these species; and (3) a multiplex PCR for the diagnosis of both species simultaneously. Moreover, we compared the sensitivity and specificity of these new diagnostic assays with 2 OIE listed procedures (www.oie.int/en/international-standard-setting/aquatic-manual/access-online/), viz. the standard histological procedure and the PCR-RFLP assay, assuming that none of the compared procedures could be considered as a 'gold standard'.

MATERIALS AND METHODS

Oysters

Flat oyster *Ostrea edulis* spat that had been produced in the hatchery facilities of the Centro de Investigacións Mariñas and in those of a private company, and was derived from 5 different geographic origins, were deployed throughout the summer of 2005. Spat were hung from a raft located in Cambados, Ría de Arousa (Galicia, NW Spain), an area where *Bonamia ostreae* has occurred since the 1980s (da Silva et al. 2005) and where *B. exitiosa* was more recently detected with high prevalence (Abollo et al. 2008). A total of 137 oysters corresponding to the 5 origins were collected between March and September 2007 (first collection) for testing and comparison of diagnostic assays. In this case, only gills were used in the molecular assays, because gills had been frequently used in previous reports for molecular diag-

nosis of *Bonamia* spp. (Carnegie et al. 2000, Robert et al. 2009). Subsequently, 30 flat oysters were collected in October 2009 (second collection) from each of 3 areas affected by both *Bonamia* species, Cambados and O Grove in Ría de Arousa and Redondela in Ría de Vigo (90 oysters in total). In this case, gills plus gonad tissue were included for molecular diagnosis to enhance the study, after we had realised that *B. exitiosa* was more frequently found in the gonad area than in the gills. Finally, gonad and gill pieces from 30 oysters collected in May 2011 (third collection) from Limfjord (Denmark), where *B. ostreae* and *B. exitiosa* have not been detected, were added to the study to assess whether false positive diagnosis is produced by any diagnostic procedure.

Genomic DNA extraction

Oysters were shucked and a piece of meat was removed for histological processing (see below). A small piece of gill from each oyster of the first collection and a sample of gill plus gonad from each oyster of the second and third collections were preserved in 96% ethanol for molecular analysis. The weight of the preserved pieces was ca. 25 to 50 mg. The gonad piece included gonad follicles plus interfollicular connective tissue. DNA extractions were performed employing the commercial kit Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol, to isolate genomic DNA from animal tissue. DNA quality and quantity were checked in a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies).

Primer design

In this study, 4 specific primers were designed based on the 18S rDNA gene and on the first internal transcribed spacer (ITS1) sequence of *Bonamia ostreae* and *B. exitiosa*: BOSTRE-F, BOSTRE-R, BEXIT-F and BEXIT-R (Table 1). For this, 21 DNA sequences belonging to 17 different species deposited in GenBank were aligned using the programme Clustal W: *Ostrea edulis* (OEU88709), *Urosporidium crescens* (UA7852), haplosporidian-like parasites infecting *Ruditapes decussatus* (AY435093) and *Haliotis iris* (AF492442), *Haplosporidium louisiana* (U47851), *H. nelsoni* (X74131), *H. costale* (AF387122), *H. edule* (DQ458793), *H. pickfordi* (AY452724), *H. lusitanicum* (AY449713), *Minchinia tapetis* (AY449710), *M. chitonis* (AY449711), *M. teredinis* (U20319), *B. ostreae* (AF262995), *B. perspora* (DQ356000), *B. roughleyi* (AF508801), *B. exitiosa* from Australia (DQ312295), New Zealand (AF337563) and Spain (EU016528), *Bonamia* sp. from North Carolina (AY542903), and *Bonamia* sp. from Chile (AY860060). Subsequently, the primers were designed using the programme Primer 3 (Rozen & Skaletsky 2000). The forward primers BOSTRE-F and BEXIT-F target the 18S rDNA gene of both *B. ostreae* and *B. exitiosa*, whereas the reverse primers BOSTRE-R and BEXIT-R specifically target the ITS1 region of each species, respectively.

Conventional PCR assays for *Bonamia ostreae* and *B. exitiosa*

The optimal annealing temperature was determined for each assay with a temperature gradient and various incubation times. PCR assays were performed in a total volume of 25 µl containing 1 µl of genomic DNA (200 ng), PCR buffer at 1× concentration, 1.5 mM $MgCl_2$, 0.2 mM nucleotides (Roche Applied Science), 0.3 µM each specific primer for either *B. ostreae* (BOSTRE-F/R) or *B. exitiosa* (BEXIT-F/R) and 0.025 U μl^{-1} *Taq* DNA polymerase (Roche Applied Science). A positive control for *B. ostreae* or *B. exitiosa* and a negative control (no DNA) were used in each PCR assay. The PCR assays were carried out in a Tgradient thermocycler (Biometra), under the following reaction parameters: 94°C for 2 min, 35 cycles at a melting temperature of 94°C for 30 s, an annealing temperature of 55°C for *B. ostreae* and 58°C for *B. exitiosa* for 45 s, an extension temperature of 72°C for 1 min, followed by a final extension period of 72°C for 1 min. After PCR, 10 µl of amplified DNA were analysed by electrophoresis on 2% agarose gels, in 1% Tris acetate EDTA buffer, stained with ethidium bromide and scanned in a GelDoc XR documentation system (BioRad).

Table 1. Sequences of the primers designed in this study together with their position within the corresponding GenBank sequence. F: forward; R: reverse

Primer	Sequence (5′ to 3′)	Position (nt)
BOSTRE-F	TTACGTCCCTGCCCTTTGTA	1622–1641 (AF262995)
BOSTRE-R	TCGCGGTTGAATTTTATCGT	1810–1829 (AF262995)
BEXIT-F	GCGCGTTCTTAGAAGCTTTG	1707–1726 (DQ312295)
BEXIT-R	AAGATTGATGTCGGCATGTCT	1931–1951 (DQ312295)

Multiplex PCR

A combination of 3 primers, BOSTRE-F/BOSTRE-R/BEXIT-R, was used to simultaneously detect *Bonamia ostreae* and *B. exitiosa* in a multiplex PCR assay. As mentioned above, the forward primer BOSTRE-F targets DNA of both species, and its concentration was double that of each species-specific reverse primer. PCR assays were performed in a total volume of 25 µl containing 1 µl of genomic DNA (200 ng), PCR buffer at 1× concentration, 1.5 mM $MgCl_2$, 0.2 mM nucleotides (Roche Applied Science), 0.6 µM BOSTRE-F primer, 0.3 µM BOSTRE-R primer, 0.3 µM BEXIT-R primer and 0.05 U μl^{-1} *Taq* DNA polymerase (Roche Applied Science). In this case, a positive control for both species, *B. ostreae* and *B. exitiosa*, and a negative control (no DNA) were included in all PCR assays. The cycling protocol was: 94°C for 2 min, 35 cycles at 94°C for 30 s, 57.5°C for 45 s and 72°C for 1 min, followed by 72°C for 7 min. PCR products were separated as described above.

Real-time PCR for *Bonamia ostreae* and *B. exitiosa*

Species-specific real-time PCR assays were carried out on an iQ™5 Multicolour Real-Time PCR Detection System (Bio-Rad Laboratories) using the DNA-binding dye technique (SYBR Green). Reactions were performed in a total volume of 25 µl comprising 1 µl of genomic DNA (200 ng), 12.5 µl of FastStart Universal SYBR Green Master (Rox) from Roche Applied Science, 0.3 µM each specific primers for either *B. ostreae* (BOSTRE-F/R) or *B. exitiosa* (BEXIT-F/R) and 10 µl of distilled water. Negative control (no DNA) and positive controls (samples positive for *B. ostreae* or *B. exitiosa*) were included in each PCR assay. The amplification programme used for *B. ostreae* was: 95°C for 10 min, 35 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 1 min. The fluorescent signal was measured at the end of the annealing step of each cycle. In order to evaluate the analytical specificity of the amplified products, a melting curve was carried out with temperature increments of 0.5°C s^{-1} starting at 55°C and ending at 95°C and recording fluorescence at each change of temperature. Amplification was also confirmed by electrophoresis on 2% agarose gels, in 1% Tris acetate EDTA buffer, stained with ethidium bromide and scanned in a GelDoc XR documentation system (BioRad). The amplification programme for *B. exitiosa* was as described above but with some modifications: 95°C for 10 min, 35 cycles of 95°C for 30 s, 58°C for 45 s and 72°C for 1 min. The melting curve was generated in this case with temperature increments of 0.5°C s^{-1} starting at 58°C and ending at 95°C. In both real-time PCR assays, the cut-off was set at a C_t value that was the last cycle completely devoid of background noise. The resulting data were analysed using the software provided by the iQ5 system (Bio-Rad iQ™5 2.0 Standard Edition Optical System Software).

DNA sequencing

Products from the different PCR assays described in this work were cleaned for sequencing using the commercial Rapid PCR Purification system (Marligen Biosciences) according to the manufacturer's instructions. Sequencing was performed by Secugen (Madrid, Spain), and the chromatograms were analysed using the programme ChromasPro version 1.41 (Technelysium). All sequences generated were searched for similarity using BLAST through web servers of the US National Center for Biotechnology Information.

Analytical specificity of primer pairs

The analytical specificity of the primers was evaluated in the specific cPCR and multiplex PCR assays using genomic DNA extracted from flat oysters infected with *Bonamia exitiosa*, with *B. ostreae,* and co-infected with both species; the diagnosis of these oysters had been performed with histology and PCR-RFLP (Cochennec-Laureau et al. 2003) plus further sequencing. We also used *Haplosporidium nelsoni* and *Mikrocytos mackini* infecting *Crassostrea gigas*; *H. armoricanum* and *Perkinsus mediterraneus* parasitising *Ostrea edulis*; *Marteilia refringens* infecting *Mytilus galloprovincialis* and *P. olseni* infecting *Ruditapes decussatus* were also used. To test the specificity of the real-time PCR assays, DNA of oysters *O. edulis* with different levels of infection by *B. ostreae* and *B. exitiosa* and DNA of non-infected oysters *O. edulis* were used. Again, the diagnosis of these oysters had been performed with histology and PCR-RFLP (Cochennec-Laureau et al. 2003) plus further sequencing. PCR amplicons obtained were monitored by measuring the fluorescence and obtaining a C_t value for each sample, and subsequently a melting curve was generated to evaluate the analytical specificity of the amplified products. Specific melting temperature (T_m) values (mean ± SD) for *B. ostreae* and for *B. exitiosa* were estimated in both real-time PCR assays.

Analytical sensitivity of the PCR assays

Construction of plasmids

To evaluate the analytical sensitivity of the PCR assays and to prevent false negative results that might be caused by PCR inhibitors, 3 plasmids were constructed. Thus, cPCR was performed to construct plasmid-1 (P-1) for *Bonamia ostreae* using the primers BOSTRE-F/R and to construct plasmid-2 (P-2) and plasmid-3 (P-3) for *B. exitiosa* using the primers BEXIT-F/R and BOSTRE-F/BEXIT-R, respectively. PCR products were ligated into cloning vector pGEM®-T vector systems (Promega) at 4°C overnight and transformed into *E. coli* One Shot Top 10F′ Chemically Competent cells (Invitrogen Life Technologies™). Transformed cells were screened by PCR using the primers described above. Plasmid DNA of positive clones was purified using the High Pure Plasmid Isolation Kit (Roche). DNA quality and quantity was checked in a Nanodrop® ND-1000 spectrophotometer.

Conventional and multiplex PCR

Ten-fold serial dilutions of plasmids (from 10 ng to 1 ag) were used to evaluate the analytical sensitivity of the cPCR (P-1 and P-2) and multiplex PCR (P-1 and P-3). To evaluate whether host DNA interferes in PCR reactions, the same dilutions mixed with 200 ng of DNA from the gills of a non-infected oyster *Ostrea edulis* were made.

Real-time PCR

To determine the detection threshold of real-time PCR for *Bonamia ostreae* and for *B. exitiosa*, standard curves (DNA concentration versus C_t) were generated in duplicate, in both cases. Ten-fold serial dilutions of P-1 and P-2 ranging from 1 pg to 1 ag were used as well as 10-fold serial dilution of the same plasmids with 200 ng of DNA from the gills of a non-infected oyster *Ostrea edulis*. The amplification efficiency (E) was calculated from the slope of the standard curve, using the following formula (Pfaffl 2001): $E = 10^{-1/slope} - 1$.

Comparison of PCR assays with other diagnostic techniques

In order to assess the sensitivity and efficiency of the new molecular diagnostic procedures described in this work, the 137 oysters from the first collection were diagnosed for *Bonamia ostreae* and *B. exitiosa* by cPCR, multiplex PCR and real-time PCR, and the results were compared with diagnosis by 2 OIE-recommended techniques: histological analysis and the PCR-RFLPs assay described by Cochennec-Laureau et al. (2003). Molecular analyses of this comparison were based on DNA extracted from oyster gills. Subsequently, the 90 oysters from the second collection were diagnosed with the same procedures as above, except with real-time PCR, to assess whether the involvement of gonad tissue in the molecular diagnosis improves their sensitivity. Finally, the 30 oysters from the third collection were diagnosed with every procedure to assess whether false positives are produced with oysters from a non-affected area.

Histological assay was performed using a transverse section (ca. 5 mm thick) of each oyster containing gills, visceral mass and mantle lobes fixed in Davidson's solution, dehydrated in an ethanol series and embedded in paraffin. Histological sections (5 µm thick) were stained with Harris's haematoxylin and eosin (Howard & Smith 1983) and observed under a light microscope (1000× magnification) for the specific diagnosis of *Bonamia exitiosa* and *B. ostreae*.

To carry out the PCR-RFLP, the DNA samples were amplified in a PCR assay according to Cochennec et al. (2000). PCR was performed in a total volume of 25 µl containing 1 µl of genomic DNA (200 ng), PCR buffer at 1× concentration, 1.5 mM $MgCl_2$, 0.2 mM nucleotides (Roche Applied Science), 0.3 µM Bo/Boas primers and 0.025 U μl^{-1} *Taq* DNA polymerase (Roche Applied Science). The PCR was carried out in a TGradient thermocycler (Biometra) using the cycling protocol: 94°C for 2 min, 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by 72°C for 7 min. To identify *Bonamia* species, an RFLP assay was performed with positive PCR products. All restriction reactions were carried out in a final volume of 15 µl containing 2400 ng of DNA, 1.5 µl of enzyme buffer and 0.5 µl (5 U) of *Bgl*I restriction enzyme (Takara Bio). The digestions were performed for 3 h at 37°C, which was followed by 20 min at 65°C to inactivate the enzyme. To visualize the restriction patterns, digested samples (15 µl) were mixed with 1 µl loading buffer and subjected to electrophoresis through 2% agarose gels, stained with ethidium bromide and run at 60 V for 2 h. A 50 bp ladder (Roche Applied Science) was included as a molecular weight marker. In some cases, the PCR amplification was poor (below 2400 ng of DNA), resulting in insufficient amplicon to subsequently perform RFLP. Those cases were considered negative for the diagnosis of

B. ostreae or *B. exitiosa* even though detection of DNA of a *Bonamia* parasite occurred.

Diagnostic sensitivity and specificity

The sensitivity and specificity of the diagnostic procedures for *Bonamia ostreae* and *B. exitiosa*, assuming that none of them could be considered as a gold standard, was estimated through the maximum likelihood method using the TAGS programme (Pouillot et al. 2002). The PCR-RFLP diagnostic test was excluded because of the cases in which the PCR amplicon was insufficient to perform RFLP. The diagnostics corresponding to each collection period (first and second) were treated separately because of the differences in the organs involved for molecular diagnostic procedures. The maximum likelihood method was implemented through the website www.epi.ucdavis.edu/diagnostictests/query.htm, created by the Graduate Group in Epidemiology (School of Veterinary Medicine, University of California in Davis, CA, USA). All diagnostic procedures were compared together with this maximum likelihood method; it involved 4 diagnostic tests (species-specific cPCR, multiplex PCR, species-specific real-time PCR and histology) and 5 populations (the 5 geographic origins in the same raft) for the comparison of the first collection (2007), and 3 tests (species-specific cPCR, multiplex PCR and histology) and 3 populations (the 3 locations) for the second collection (2009).

RESULTS

Analytical specificity of primer pairs

To evaluate the analytical specificity of the pair of primers BOSTRE-F/R and BEXIT-F/R, PCR assays for *Bonamia ostreae* and *B. exitiosa*, respectively, were performed using DNA templates of *Ostrea edulis* infected with *B. ostreae*, *B. exitiosa*, co-infections by both species and with DNA of other more or less closely related parasites. When BOSTRE-F/R were tested (Fig. 1A), a 208 bp PCR product was observed only in samples with *B. ostreae* DNA (lanes 1 and 3), while no amplification was observed in samples of *O. edulis* infected with *B. exitiosa* or in other tested parasites (lanes 2 and 4 to 9). In the same way, when the BEXIT-F/R pair of primers were used (Fig. 1B), positive results were observed in samples where DNA of *B. exitiosa* was present (lanes 2 and 3), obtaining a 246 bp PCR product, which was absent in the other

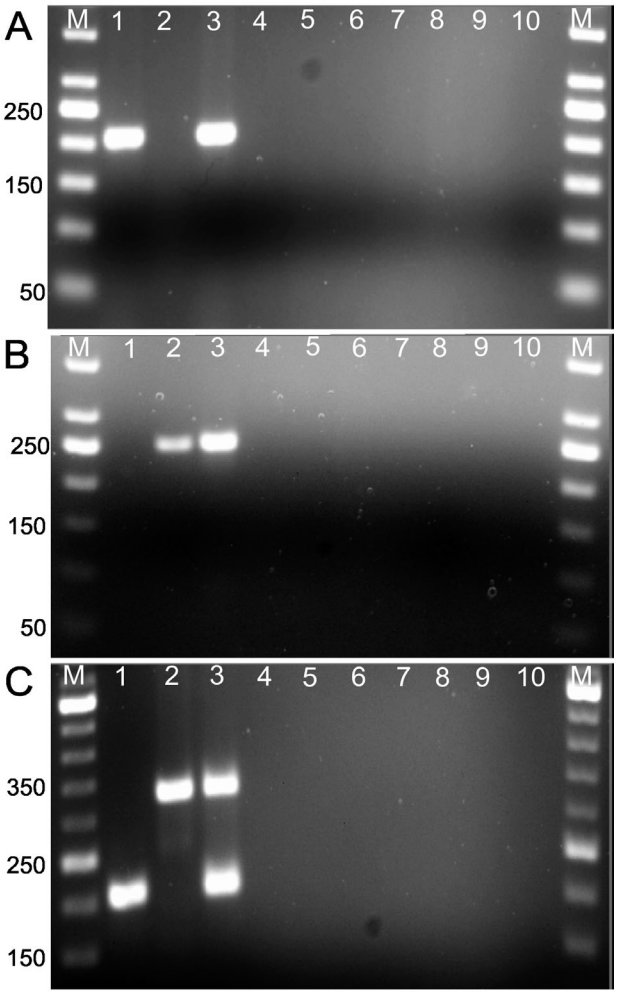

Fig. 1. Agarose gel electrophoresis after PCR assays performed to assess analytical specificity. (A) PCR using the primer pair BOSTRE-F/BOSTRE-R. (B) PCR with the primer pair BEXIT-F/BEXIT-R. (C) Multiplex PCR performed with the primers BOSTRE-F/BOSTRE-R/BEXIT-R. Lanes 1 to 5: *Ostrea edulis* infected with *Bonamia ostreae*, *B. exitiosa*, both *Bonamia* spp., *Haplosporidium armoricanum* and *Perkinsus mediterraneus*, respectively; lanes 6 and 7: *Crassostrea gigas* infected with *Haplosporidium nelsoni* and *Mikrocytos mackini*, respectively; lane 8: *Ruditapes decussatus* infected with *Perkinsus olseni*; lane 9: *Mytillus galloprovincialis* infected with *Marteilia refringens*; lane 10: negative control; M: 50 bp molecular marker

cases, including *O. edulis* parasitised by *B. ostreae* (lane 1 and lanes 4 to 9). The sequence of the PCR-amplification products showed 100% homology with *B. ostreae* and *B. exitiosa*, respectively

Analytical specificity of multiplex PCR

The analytical specificity of multiplex PCR was demonstrated using DNA of *Ostrea edulis* infected

with *Bonamia ostreae* or infected with *B. exitiosa*, obtaining only a 208 bp or a 330 bp product, respectively (Fig. 1C, lanes 1 and 2). When DNA templates of *O. edulis* co-infected with *B. ostreae* and *B. exitiosa* were analysed, both amplification products were observed (Fig. 1C, lane 3). This PCR multiplex assay is species-specific for *B. ostreae* and *B. exitiosa* since it did not amplify the DNA of other more or less related parasites (Fig. 1C, lanes 4 to 9). The sequence of the PCR-amplification products showed the correct correspondence with *B. ostreae* and *B. exitiosa* in each case.

Real-time PCR analytical specificity

The ability of the specific BOSTRE-F/R and BEXIT-F/R primers to detect *Bonamia ostreae* and *B. exitiosa* in real-time PCR was evaluated, using DNA of oysters with different levels of infection with those parasites and DNA of non-infected oysters. Thus, positive samples for *B. ostreae* amplified with BOSTRE-F/R produced a specific melting peak at 78.25 ± 0.25°C (mean ± SD; Fig. 2A), whereas DNA of *Ostrea edulis* infected with *B. exitiosa* generated a melting peak at 74.75 ± 0.25°C when BEXIT-F/R primers were used (Fig. 2B). In order to confirm the specific sizes of the amplification products, 2% agarose gel electrophoresis was carried out, and the bands observed were of the expected size for *B. ostreae*, 208 bp, and for *B. exitiosa*, 246 bp. Amplification products were also sequenced, and their identity was confirmed. Positive DNA for *B. ostreae* or for *B. exitiosa* yield a positive C_t value in each specific real-time assay, whereas DNA from non-infected oysters or negative control (without DNA) did not produce any C_t value (data not shown).

Analytical sensitivity of conventional PCR and multiplex assays

The analytical sensitivity of the primers for *Bonamia ostreae* and *B. exitiosa* was tested using the plasmids. Furthermore, a similar assay was performed by adding DNA of a non-infected oyster *Ostrea edulis*. The species-specific cPCR assays for *B. ostreae* and *B. exitiosa* were able to detect as little as 1 fg of P-1 and P-2, respectively (Fig. 3A,B) whereas multiplex PCR achieved amplification up to 100 fg of P-1 and P-3. Similar results were obtained when 200 ng DNA of non-infected oyster were added (Fig. 3C).

Analytical sensitivity of real-time PCR

Standard curves for $\log_{10}$[DNA] of 10-fold dilution series versus mean C_t were constructed for both *Bonamia* species. The *B. ostreae* standard curve displayed an amplification efficiency of 104% for the P-1 assay and a linearity (R^2) of 0.98. When P-1 with oyster DNA was tested, similar results were obtained (E = 103%, R^2 = 0.97). The detection limit of the real-time PCR was as low as 1 fg in either case (Fig. 4A). Lower DNA concentration values gave non-linear C_t values, thus affecting reproducibility. The highest C_t value within linearity range was 31. Regarding the

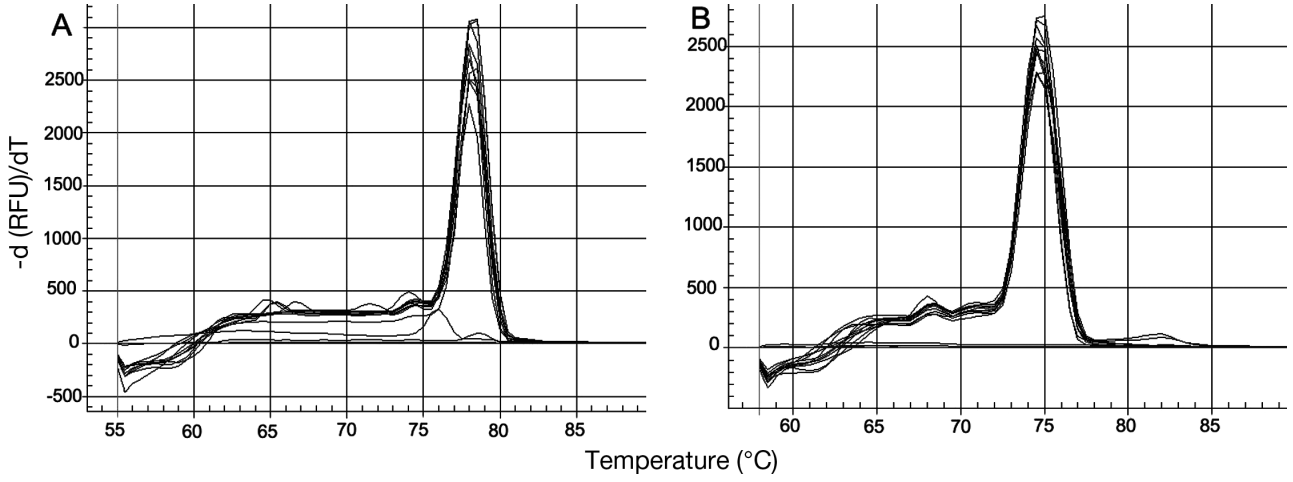

Fig. 2. *Bonamia* spp. Melting curves of real-time SYBR Green PCR products for (A) *B. ostreae* and (B) *B. exitiosa*. RFU: relative fluorescence units

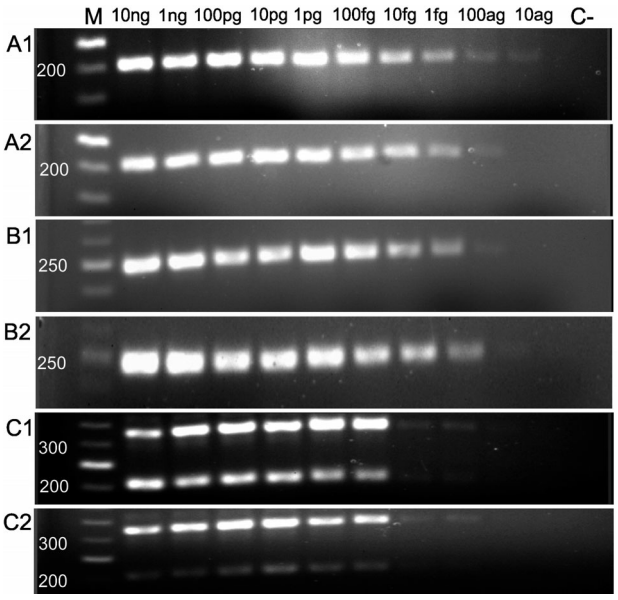

Fig. 3. *Bonamia* spp. Agarose gel electrophoresis after PCR assays performed to evaluate the analytical sensitivity of the cPCR assays using 10-fold serial dilution of plasmids (from 10 ng to 1 ag; panels A1, B1 and C1) and the same dilutions in the presence of 200 ng of DNA of non-infected oyster *Ostrea edulis* (panels A2, B2 and C2). (A1,A2) cPCR assay for *B. ostreae* with plasmid-1. (B1,B2) cPCR assay for *B. exitiosa* with plasmid-2. (C1,C2) Multiplex PCR assay with plasmid-1 and plasmid-3. M: 50 bp molecular marker. C-: negative control

B. exitiosa standard curve, the amplification efficiency was 104% for the assay carried out with P-2 and 108% for P-2 plus DNA of non-infected oyster, whereas the linearity of the assays was 0.96 and 0.98, respectively. The limit of detection for real-time PCR for *B. exitiosa* was lower than for *B. ostreae*, amplifying up to 10^{-1} fg (Fig. 4B). Again, lower DNA concentration values gave non-linear C_t values, thus affecting reproducibility. The highest C_t value within linearity range was 30.78.

Comparison of techniques

First oyster collection

Gills were used for molecular diagnostics. *Bonamia ostreae* was the only *Bonamia* species detected in oysters deriving from Ría de Arousa; *B. exitiosa* was the only species in oysters deriving from Ría de Ortigueira, and both parasites were found in oysters of the remaining 3 origins. Table 2 shows the number of positive cases of infection with *B. ostreae* and *B. exitiosa* corresponding to each diagnostic test. More positive cases of *B. ostreae* were detected with the 3 new PCR methods than with the 2 OIE-recommended ones (histology and PCR-RFLP). Regarding *B. exitiosa*, species-specific cPCR and real-time PCR also showed higher numbers of positives than the OIE-recommended tests, but PCR-RFLP provided 2 more positive cases than multiplex PCR. The highest number of positive cases corresponded to real-time PCR for either parasite. Nevertheless, the C_t values of 23 positive cases for *B. ostreae* by real-time PCR were higher than the highest limit of proven reproducibility (31.00); 7 of those cases were also found positive by at least one other test. Regarding *B. exi-*

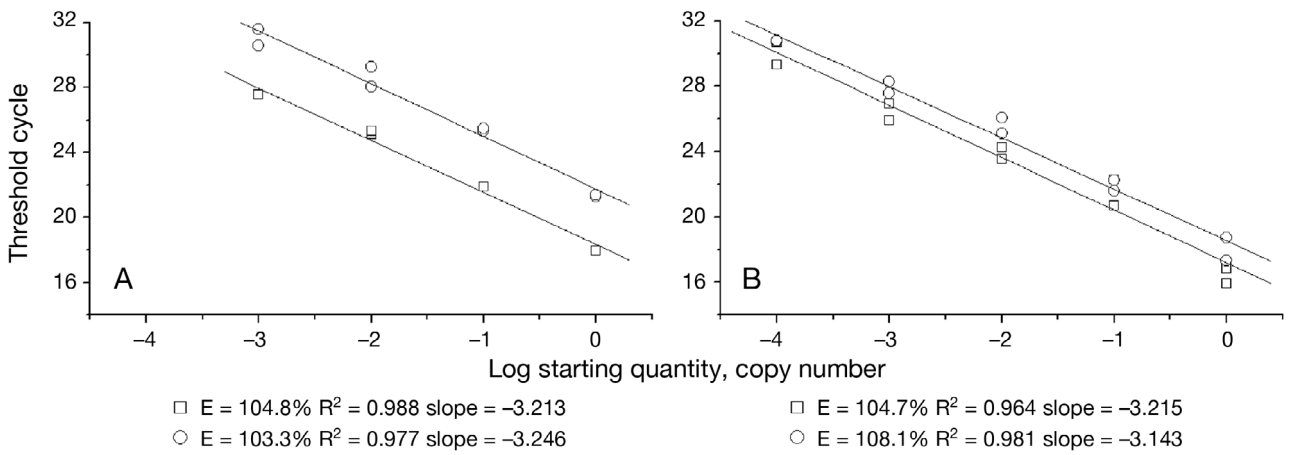

Fig. 4. *Bonamia* spp. Standard curves obtained with real-time PCR for quantification of (A) *B. ostreae* with plasmid-1 and (B) *B. exitiosa* with plasmid-2. The *x*-axis is based on a log scale for copy number derived from 10-fold serial dilutions of each plasmid ranging from 1 pg to 1 ag (0 = 1 pg, –1 = 100 fg, –2 = 10 fg, –3 = 1 fg, –4 = 100 ag, –5 = 10 ag, –6 = 1 ag). The *y*-axis represents the critical threshold, where fluorescence was measured and used to generate a standard curve. Circles: plasmid; squares: plasmid plus DNA of non-infected oyster *Ostrea edulis*

Table 2. *Ostrea edulis*. Number of oysters with positive diagnosis of *Bonamia ostreae*, *B. exitiosa* and *Bonamia* (at least 1 of the *Bonamia* spp.) corresponding to each diagnostic procedure. The column at the right corresponds to the number of positive cases detected by at least one diagnostic procedure. The percentages of oysters with positive diagnosis are shown in parentheses. Values for species-specific cPCR and real-time PCR correspond to assays using the primer pair BOSTRE-F/R for diagnosis of *B. ostreae*, BEXIT-F/R for diagnosis of *B. exitiosa* and grouping both assays for diagnosis of bonamiosis. Molecular diagnostic procedures for the collection period September 2006–June 2007 were based on DNA extracted from gills; those for October 2009 were based on DNA extracted from gills plus gonads

Collection period Targeted diagnosis	Histology	PCR-RFLP	PCR Bo/Boas	Multiplex PCR	Species-specific cPCR	Species-specific real-time PCR	At least 1 test
Sept 2006–June 2007 (N = 137)							
B. ostreae	49 (36)	49 (36)		66 (48)	81 (59)	96 (70)	98 (72)
B. exitiosa	29 (21)	34 (25)		32 (23)	55 (40)	66 (48)	78 (57)
Bonamia	72 (53)		88 (64)	85 (62)	97 (71)	110 (80)	116 (85)
Oct 2009 (N = 90)							
B. ostreae	11 (12)	13 (14)		42 (47)	44 (49)		55 (61)
B. exitiosa	23 (26)	21 (23)		40 (44)	62 (69)		66 (73)
Bonamia	33 (37)		50 (56)	64 (71)	77 (86)		80 (89)

tiosa, the C_t values of 24 positive cases by real-time PCR were higher than the highest limit of proven reproducibility (30.78); 4 of those cases were also found positive by at least one other test. The max C_t value of detection was 33.13 for *B. ostreae* and 33.48 for *B. exitiosa*. A total of 97 oysters were found infected with *B. ostreae* and 78 oysters with *B. exitiosa* by at least one diagnostic test, but no single test gave positive detection for all of those positive cases. One of the 49 oysters found infected with *B. ostreae* by histology was not found infected with this parasite by species-specific cPCR, real-time PCR or multiplex PCR, but these 3 molecular tests gave a positive diagnosis for *B. exitiosa* in that particular oyster, thus the histological diagnosis could misidentify the *Bonamia* species. Three more oysters found positive for *B. ostreae* by histology were found negative for this parasite by multiplex PCR; 2 of them were found co-infected with both parasites by histology, species-specific cPCR and real-time PCR, but multiplex PCR only detected *B. exitiosa* in them. In the 29 oysters found infected with *B. exitiosa* by histology, in 2, 4 and 8 of them this parasite was not detected by real-time PCR, species-specific cPCR and multiplex PCR, respectively; one of those oysters was found infected with *B. ostreae* by the molecular tests, thus the histological diagnosis could misidentify the *Bonamia* species; 4 of those oysters were found co-infected with both parasites by histology, species-specific cPCR and real-time PCR, but multiplex PCR only detected *B. ostreae* in them. Forty-one cases of co-infection with both parasites were detected by at least one diagnostic test, of which multiplex PCR detected co-infection in 18 oysters, infection with just *B. ostreae* in 14 oysters, with just *B. exitiosa* in 6 oysters and no infection in 3 oysters. PCR-RFLP yielded a much lower number of infection cases with either parasite than species-specific cPCR and real-time PCR; however, the PCR assay performed with Bo/Boas primers previous to RFLP analysis produced an amplicon (i.e. it did detect *Bonamia* sp.) that was insufficient to allow subsequent RFLP analysis in 17 cases, thus those cases were classified as negative for *B. ostreae* and *B. exitiosa* because allocation to a species was not possible with the PCR-RFLP procedure.

The values of sensitivity and specificity of 4 diagnostic tests estimated by the maximum likelihood method are shown in Table 3. Sensitivity values from histology and multiplex PCR <0.90 occurred for both

Table 3. *Bonamia* spp. Values of sensitivity (Se) and specificity (Sp) of 4 diagnostic tests for *B. ostreae* and *B. exitiosa*, corresponding to the first and the second oyster collection, estimated by the maximum likelihood method. Values for species-specific cPCR and real-time PCR correspond to assays using the primer pair BOSTRE-F/R for diagnosis of *B. ostreae* and BEXIT-F/R for diagnosis of *B. exitiosa*. Values >0.90 are shown in **bold**

	First collection		Second collection	
	B. ostreae	*B. exitiosa*	*B. ostreae*	*B. exitiosa*
Histology				
Se	0.64	0.54	0.41	0.62
Sp	**0.98**	**0.96**	**1.00**	**1.00**
Multiplex PCR				
Se	0.82	0.71	**1.00**	0.86
Sp	**1.00**	**1.00**	0.76	0.85
Species-specific cPCR				
Se	**1.00**	**0.93**	**1.00**	**1.00**
Sp	**0.91**	0.86	0.73	0.53
Species-specific real-time PCR				
Se	**0.99**	**1.00**	–	–
Sp	0.72	0.77	–	–

parasites, which would involve some false negative diagnostics, while values from species-specific cPCR and real-time PCR were >0.90 for both parasites. The estimated specificity values from multiplex PCR and histology were 1.00 and close to 1.00, respectively, for both parasites, while specificity values from species-specific cPCR and real-time PCR for *Bonamia exitiosa* were lower, which suggests some false positives detected by the latter 2 techniques.

Second and third oyster collections

Gill and gonad tissues were used for molecular diagnosis. Both *Bonamia ostreae* and *B. exitiosa* were found in oysters from every investigated culture area. The differences between the number (and percentage) of positive cases of either parasite detected with the new PCR methods and that with the 2 OIE-recommended ones (histology and PCR-RFLP) were higher than in the first oyster collection (Table 2). The highest number of positive cases corresponded to species-specific cPCR for either parasite. Again no diagnostic test gave positive results in all cases of infection with either parasite that were detected by at least one test. Every case of infection with *B. ostreae* detected by histology was also detected by multiplex PCR and species-specific cPCR; every case of infection with *B. exitiosa* detected with histology was detected by species-specific cPCR, but 3 cases were not detected by multiplex PCR, one of which corresponded to a case of co-infection with both parasites for which multiplex PCR detected only *B. ostreae*. Regarding the 37 cases of co-infection with both parasites detected by at least one diagnostic test, PCR multiplex detected co-infection in 15 oysters, infection with just *B. ostreae* in 12 oysters, with just *B. exitiosa* in 7 oysters and no infection in 3 oysters. As in the first collection, the PCR assay performed with Bo/Boas primers previous to RFLP analysis produced an amplicon (i.e. it did detect *Bonamia*) that was insufficient to allow subsequent RFLP analysis in 17 cases, which were considered negative for *B. ostreae* and *B. exitiosa*.

The values of sensitivity and specificity of 3 diagnostic tests estimated by the maximum likelihood method are shown in Table 3. The sensitivity values of histology were well below 0.90 for both parasites, which indicates some false negative diagnostics; however, the sensitivity value of multiplex PCR for both parasites increased substantially, in the absence of real-time PCR, with regard to the first collection. Sensitivity of species-specific cPCR was maximal for both parasites. The specificity values of histology were 1.00 for *Bonamia ostreae* and *B. exitiosa*; however, multiplex PCR and species-specific cPCR showed specificity values below 0.90 for both parasites, lower than in the first collection, which would involve false positives by these 2 tests for both parasites.

The 5 diagnostic tests provided negative results for *Bonamia ostreae* and *B. exitiosa* for the oysters from the third collection, which had been harvested in a non-affected area.

DISCUSSION

Here we have described a new specific cPCR procedure for detection of *Bonamia ostreae* and the first specific cPCR method for *B. exitiosa*. Both cPCR procedures have been tested with other parasites belonging to the phylum Haplosporidia and other parasites of molluscs of the genera *Perkinsus, Mikrocytos* and *Marteilia*, showing their analytical specificity. The primers described in this study, BOSTRE-F/BOSTRE-R and BEXIT-F/BEXIT-R, were designed to target the 18S-ITS 1 rDNA sequence. The species-specific primers for *B. ostreae* and those for *B. exitiosa* were successfully applied in real-time SYBR Green PCR assays, giving a different melting peak for each species. The primer and probes used for TaqMan® Real Time PCR assays described by Marty et al. (2006) and Corbeil et al. (2006b) were designed to target the 18S rRNA gene and are not able to differentiate between *Bonamia* species. However, quantitative real-time PCR described by Robert et al. (2009) based on the actin 1 gene showed a specific melting peak at 81.7 ± 0.21°C for *B. ostreae* but did not detect *B. exitiosa*. Moreover, a multiplex PCR method was designed for the first time to detect *B. ostreae* and *B. exitiosa* in a single assay, using a cocktail of 3 primers: BOSTRE-F which can target to the 18S rDNA sequence of either species; BOSTRE-R, a specific primer for the ITS1 sequence of *B. ostreae*; and BEXIT-R, which is specific for the ITS1 region of *B. exitiosa*.

The 2 species-specific cPCRs are tools capable of detecting very low quantities of DNA from *Bonamia ostreae* and *B. exitiosa*, even in the presence of DNA of non-infected oysters. Multiplex PCR is also a sensitive technique that can detect very low quantities of DNA from either *Bonamia* sp. Nevertheless, in cases of co-infection by both *Bonamia* spp., competition between DNA of *B. exitiosa* and *B. ostreae* for the primers and other reaction components of multiplex assay could explain a lower analytical sensitivity than cPCR, even though the multiplex PCR reaction was adjusted with a double concentration of *Taq* poly-

merase and the common primer BOSTRE-F, as well as an optimum T_m for all primers. The values of the detection limit obtained for real-time PCR assays were similar to those obtained with the specific cPCR assays. Standard curves (DNA concentration versus C_t) generated for *B. ostreae* and *B. exitiosa* were accurate and reproducible since they showed high amplification efficiency and acceptable linearity. Our results agreed with those of Robert et al. (2009), who did not find significant differences of sensitivity when a standard curve of *B. ostreae* was obtained with plasmids alone or with plasmids in the presence of DNA from non-infected oysters. The real-time PCR assay could be used as a quantitative method if it were calibrated with known numbers of parasites. To do this, the parasites would have to be isolated from heavily infected oysters (Mialhe et al. 1988); as long as a procedure for *in vitro* culture of the parasite is not available. Using parasites instead of plasmids would improve the detection limit estimations (Sivaganesan et al. 2008). The detection limits of cPCR assays using Bo/Boas (Cochennec et al. 2000), CF/CR (Carnegie et al. 2000), BOG-F/BOG-R (Abollo et al. 2008), Bo-1/Bo-4 (Marty et al. 2006) or BoosF03/BoosR03 primers (Engelsma et al. 2010) for DNA of *Bonamia* spp. have not been established, nor have those of the primers used in real-time TaqMan® PCR assays described by Marty et al. (2006) and Corbeil et al. (2006b).

The comparisons between diagnostic tests revealed that the 3 new molecular diagnostic tests described in this study provided more positive results (showed higher sensitivity) than the 2 OIE-recommended tests. How many (if any) of the positive cases detected by the new tests corresponding to negative cases by the OIE-recommended tests were false positives could not be determined in the absence of a gold standard test. Because of the large diagnostic differences between some tests, the application of statistics to estimate sensitivity and specificity led us to infer that there were some false positives (low specificity) provided by the new molecular methods and false negatives (low sensitivity) by the OIE-recommended ones, which is a matter of likelihood but not of certainty. The absence of a gold standard diagnostic test precludes certainty.

The differences in sensitivity between the tests with the new primers and the OIE recommended ones increased with the addition of gonads compared to using just gills for diagnosis. Therefore, the choice of organs for diagnosis of infection with either *Bonamia exitiosa* or *B. ostreae* is crucial, keeping in mind that *B. exitiosa* is more frequently located in the gonads than *B. ostreae*, while the latter is more frequently located in the gills than *B. exitiosa* (our unpublished results); an obvious recommendation is to include as many organs as possible in the diagnostic assays. Molecular tests with high analytical sensitivity could carry the risk of false positives through various ways (Burreson 2008). Considering the analytical specificity of the new primers, the lack of positive cases in the sample from the Danish oyster bed would strengthen the unlikelihood of false positives from a *Bonamia*-free area. Therefore, another recommendation would be to minimise the chance of contamination of non-infected individuals with parasite DNA from infected ones during sampling, dissection and analytical processes. Particular attention should be given to establishing a superior C_t value limit above which positive diagnostics are not reliable when performing real-time PCR assay. That limit would depend on the equipment, reagents and operators. Some of the positive diagnostics by real-time PCR corresponded to C_t values higher than the highest limits of proven reproducibility. A similar problem was described by Robert et al. (2009) when quantifying *B. ostreae* in *Ostrea edulis* with real-time PCR. A way to deal with cases of real-time PCR positive diagnosis with C_t values around the highest limit of proven reproducibility would be repeating the assay or confirming the result with another diagnostic test.

The lower sensitivity of multiplex PCR with regard to species-specific cPCR and real-time PCR was mostly due to oysters found co-infected with *Bonamia ostreae* and *B. exitiosa* by other tests. In a significant number of those cases, multiplex PCR detected just one of the parasites, typically *B. ostreae*, but not both, to the detriment of sensitivity. Likely, when DNA of both parasites occurred, there was competition for the forward primer between them, frequently leading to detectable amplification of DNA of just one of the parasites. These results suggest that the multiplex PCR test should be improved. Alhassan et al. (2005) designed a multiple PCR test to diagnose the protozoan parasites *Babesia caballi* and *B. equi* that, like in our multiplex PCR test, had a unique forward primer. Those authors also observed a decrease in the sensitivity of the multiplex PCR test in co-infections with *B. caballi* and *B. equi*.

The PCR-RFLP test showed a restricted applicability for species discrimination because of the insufficient amplification to perform subsequent RFLP in a significant number of cases. The number of cases of amplification with Bo/Boas primers (detection of DNA of at least one of the parasites) was lower than that with the new primers. Histology was the diagnostic test with the lowest values of sensitivity but its

high values of specificity support that it is a reliable technique to distinguish *Bonamia ostreae* and *B. exitiosa* with appropriate training. The sensitivity of the 2 OIE-recommended tests involved in this study have been evaluated in previous reports through comparison with other diagnostic techniques (Carnegie et al. 2000, Diggles et al. 2003, Balseiro et al. 2006, Corbeil et al. 2006b, Marty et al. 2006, Lynch et al. 2008, Robert et al. 2009).

The new species-specific cPCR and real-time PCR allow diagnosis of *Bonamia exitiosa* and *B. ostreae* with higher sensitivity and are quicker than the OIE-recommended tests. Thus, the new molecular diagnostic methods could replace the OIE-recommended tests in epidemiological studies. Availability of equipment would influence the choice between the new tests. Real-time PCR provides the possibility of quantitative estimations of infection intensity. In areas where both *B. ostreae* and *B. exitiosa* occur, multiplex PCR is a cheaper option, saving time and effort, to diagnose both parasites because it allows diagnosis of both species in a single assay, although its sensitivity decreases for mixed infections. The OIE recommended cPCR with Bo/Boas primers would be useful to cover the possibility of the occurrence of a *Bonamia* species different from *B. ostreae* and *B. exitiosa*. Reporting the occurrence of either parasite in an area for the first time should not be based exclusively on a cPCR or real-time PCR positive result but should be further confirmed with the observation of the parasite in host tissues through histology, tissue imprints or *in situ* hybridization (Burreson 2008). Sequences of *Bonamia* spp. from different geographic origins were considered in designing the new primers; nevertheless, intercalibration or multilaboratory comparisons of the new procedures are advisable with the involvement of different host species and multiple geographic origins to test the reliability of the new procedures.

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