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Phylogenetic relationship of *Perkinsus olseni* from the Ebro Delta, Spain, to other *Perkinsus* species, based on ribosomal DNA sequences

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ABSTRACT: The phylogenetic relationship of *Perkinsus olseni* originating from the Ebro Delta, Spain, to other *Perkinsus* spp. was investigated using the nontranscribed spacer (NTS) region and the internal transcribed spacer (ITS) region (including ITS1, 5.8S and ITS2) of the ribosomal DNA sequences. These 2 molecular markers (NTS and ITS) were sequenced from prezoosporangia of *Perkinsus* sp. originating from Manila clam *Ruditapes philippinarum* from the Ebro Delta. The sequence of the 5.8S ITS region of the ribosomal RNA gene was 100% similar to that of *P. olseni*. Higher genetic variability was found for the NTS sequence, with 80.7 to 81.8% similarity to *P. olseni*. The NTS sequence of a *P. olseni* isolate previously detected in *R. decussatus* from the same area was also obtained and showed 81% identity with our isolate. Evidence obtained from phylogenetic analysis of the 5.8S ITS and NTS aligned sequences appears to indicate that *P. olseni* strains group together according to their host rather than their geographic origins within a well-resolved *P. olseni* clade.

KEY WORDS: *Perkinsus olseni* · *Ruditapes philippinarum* · Manila clam · Ebro Delta · Spain · Nontranscribed spacer · NTS · ITS rRNA

INTRODUCTION

Perkinsus olseni has a broad geographic range and infects a wide range of molluscs, including Manila clam *Ruditapes philippinarum*. Interestingly, co-infection of the Manila clam with *P. olseni* and *P. honshuensis* has been detected in Japan (Dungan & Reece 2006). It has been hypothesised that *P. olseni* might have been introduced into European waters through the transfer of *R. philippinarum* from Asia (Hine 2001). The recent description of *P. honshuensis* in Japanese Manila clam (Dungan & Reece 2006) raises the concern about a possible transfer in Europe. Manila clam cultures in Spanish Mediterranean waters are exclusively located in the 2 bays of the Ebro Delta, Fangar and Alfacs, the northern and southern arms of the delta, respectively. A recent survey of clams from farms and natural harvest grounds showed the presence of the protozoan parasite *Perkinsus* sp. in *R. philippinarum* from the Ebro Delta (Elandaloussi et al. 2008), and the parasite infecting the carpet-shell clam *R. decussatus* was identified as *P. olseni* (Elandaloussi et al. 2009). Current practices in the Ebro Delta include the importation of seeds and juveniles for fattening, and with the planned intensification of clam cultivation in this area, this activity might be expected to increase the risk of introducing exotic species of *Perkinsus*. However, to date, only *P. olseni* and *P. mediterraneus* have been described in Mediterranean waters (Casas et al. 2004, Abollo et al. 2006, Elandaloussi et al. 2009).

The reference methods for the detection of *Perkinsus* spp. are histological examination of sample tissue and diagnosis by culture in fluid thioglycollate medium (FTM) (Ray 1963). Ultrastructural observation of the zoospores using transmission electronic microscopy (TEM) does not have taxonomic value since other protists such as *Colpodella* spp. are difficult to distinguish from *Perkinsus* spp. based on morphological features

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(Siddall et al. 2001). The development of molecular methods has facilitated the taxonomic identification of *Perkinsus* spp., and analysis of such molecular sequences has permitted the study of the phylogenetic relationship of *Perkinsus* spp. with other protists. The most common molecular markers for *Perkinsus* spp. discrimination are the 5.8S sequence of the internal transcribed spacer (ITS) region and the nontranscribed spacer (NTS) region of ribosomal DNA (rDNA); the comparison of ribosomal sequences with reference sequences constitutes the only confirmatory method for the identification of *Perkinsus* spp. (Reece et al. 1997, Kotob et al. 1999, Robledo et al. 1999, Coss et al. 2001, Casas et al. 2002, Murrell et al. 2002, Burreson et al. 2005, Park et al. 2005).

In the present study, we report the species identity of *Perkinsus* sp. found in *Ruditapes philippinarum* from Spanish Mediterranean waters based on an analysis of NTS and ITS (including ITS1, 5.8S and ITS2) sequences, and the phylogenetic relationship of *P. olseni* strains from this area to other *Perkinsus* spp.

MATERIALS AND METHODS

Isolation of prezoosporangia. Clams were collected from the shallow areas of the Fangar Bay located on the Ebro Delta in June 2006. For induction of prezoosporangia, 24 whole clams were individually incubated in 20 ml FTM supplemented with streptomycin (500 µg ml^{-1}) and penicillin G (500 U ml^{-1}) (Ray 1963) for 7 d at room temperature in the dark. Hypnospores formed in the FTM were harvested by centrifuging at 1500 × *g* for 10 min and washing 3 times using sterilised seawater.

DNA extraction, PCR amplification and sequencing. Genomic DNA was extracted from prezoosporangia using the DNeasy tissue kit (Qiagen). Extracted DNA was further purified using the Geneclean Turbo kit (Qbiogene). The NTS and ITS regions of the ribosomal RNA (rRNA) gene were amplified from isolated DNA by PCR amplification using the *Perkinsus* genus-specific NTS primer set PKnts (Park et al. 2005) and the *Perkinsus*-specific ITS-region primers PerkITS85 and PerkITS750 (Casas et al. 2002), respectively. PCR reactions were carried out in 50 µl final volume with the GoTaq polymerase (Promega) following the instructions of the manufacturer. Amplification of the NTS region was carried out as follows: an initial denaturation for 2 min at 94°C followed by 30 cycles (1 min at 94°C, 1 min at 57°C and 1 min at 70°C) and a final extension of 5 min at 70°C. Amplification parameters for the ITS region were as follows: an initial denaturation for 4 min at 94°C followed by 35 cycles (1 min at 94°C, 1 min at 53°C and 3 min at 68°C) and a final extension of 5 min at 68°C. DNA from *P. olseni* prezoosporangia originating from infected carpet-shell clams obtained in a previous study (Elandaloussi et al. 2009) was used for PCR amplification of the NTS region and as a positive control in PCR reactions for the amplification of the ITS region. All PCR products were electrophoresed and visualised under UV light on 1% agarose gel stained with ethidium bromide. The resulting PCR products were purified from primers, nucleotides, polymerases and salts using the QIAquick PCR purification kit (Qiagen) following the instructions of the manufacturer, and sequenced by Macrogen.

Sequence analyses. To determine the taxonomic affiliation of *Perkinsus* sp. isolated from the clams, the resulting NTS and ITS consensus sequences were analysed using the basic local alignment search tool (BLAST) of the National Center for Biotechnology Information (NCBI) and were aligned to available sequences for *Perkinsus* spp. in the GenBank database using the CLUSTAL-W algorithm (Thompson et al. 1994) in the BioEdit 7.0.5.2 software package (Hall 1999). Neighbour-joining (NJ) and maximum parsimony (MP) analyses were conducted using MEGA version 3.1 software (Kumar et al. 2004) with 1000 replicates for bootstrap values. Bayesian inference was also performed using MrBayes version 3.2 (Huelsenbeck et al. 2001) with the best-fit model and parameters according to jModelTest version 0.1.1 (Posada 2008).

RESULTS

Perkinsus olseni species-specific PCR assays were negative for all samples tested when using the DNA extracted with the DNeasy tissue kit, but gave positive results when using the same DNA that had been further purified by the Geneclean Turbo kit. The sequences of the NTS and the ITS (including ITS1, 5.8S and ITS2) rRNA genes of *Perkinsus* sp. prezoosporangia isolated from *Ruditapes philippinarum* collected in Fangar Bay were 463 and 578 bp in length, respectively (GenBank accession nos. FJ626860 and FJ481986, respectively). The NTS sequence of the isolate of *P. olseni* prezoosporangia isolated from *R. decussatus* collected in Alfacs Bay was 790 bp in length (GenBank accession no. FJ626861).

A BLAST search on the ITS region sequence of our *Perkinsus* sp. isolate from *Ruditapes philippinarum* showed 100% identity with 100% coverage to many *P. olseni* sequences deposited in GenBank, including the *P. olseni* isolate previously detected in *R. decussatus* from the Ebro Delta (accession no. EU293848). NTS sequences from our isolate showed 81.8% similarity to Korean *P. olseni* (accession no. AF438150) isolated

from *R. philippinarum*, whereas the NTS sequence obtained from the *P. olseni* isolate previously detected in *R. decussatus* from the Ebro Delta showed 99.1% identity with *P. olseni* isolates from Galicia, Spain (accession no. AF140295) and Portugal (accession no. AF509333), both detected in *R. decussatus*. The degree of sequence divergence among various *Perkinsus* sp. lineages in the ITS region was relatively low when compared to that of the NTS region. Nevertheless, the percentage NTS sequence similarity among the *P. olseni* NTS regions was >80% whereas inter-species percent identity was <50% (Table 1).

The Bayesian, NJ and MP phylograms constructed based on the ITS region sequences clearly showed grouping of the various *Perkinsus* spp., namely *P. olseni*, *P. marinus*, *P. mediterraneus*, *P. honshuensis*, *P. beihaiensis*, *P. chesapeaki* and *P. qugwadi* in distinct clades supported by strong bootstrap values (78 to 100%) (Figs. 1 & 2). The Bayesian phylogram of the ITS-region sequences showed that *P. olseni* found in *Ruditapes decussatus* and *R. philippinarum* originating from Australia, Korea, Japan, Italy, Portugal and Spain formed a clade with a bootstrap value of 79% (Fig. 1). In Bayesian, parsimony and NJ phylogenetic analyses, the nucleotide sequences of the NTS region from various isolates of *P. olseni* showed close relationships between *P. olseni* isolates and various *Perkinsus* spp. isolates from Australia, New Zealand and Thailand (Fig. 3). Bayesian and MP analyses of the NTS sequences also showed that a group constituting *Perkinsus* spp. and *P. olseni* (GenBank accession no. AF438150) further split from the *P. olseni* isolates found in *R. decussatus* and *R. philippinarum*. The overall topologies of the trees generated from the Bayesian, NJ and MP analyses were similar for each of the NTS and ITS region sequences data sets.

DISCUSSION

The present results provide evidence that *Perkinsus olseni* infects the commercial clam *Ruditapes philippinarum* in the Ebro Delta. Our first attempts to PCR-amplify DNA from prezoosporangia obtained from infected Manila clams were unsuccessful and only the DNA that was further purified using the Geneclean Turbo kit gave amplicons in the PCR reactions. This discrepancy between PCR results has been already reported in several studies; it was hypothesised to result from the presence of PCR inhibitors, competitive inhibition or DNA variability within the primer sites (Robledo et al. 1999, McCoy et al. 2007, Audemard et al. 2008). For the present results, it is most likely that PCR inhibitors interfered in the PCR reaction, since the same extracted DNA could be amplified once it was

Table 1. Pairwise distance (below diagonal) and percent similarity (above diagonal) of nontranscribed spacer (NTS) rRNA sequences among *Perkinsus* spp. Species designation is indicated in parentheses (Po: *P. olseni*, Ps: *Perkinsus* sp., Pm: *P. marinus*, Pc: *P. chesapeaki*) following the GenBank accession number

Table ID	GenBank no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	FJ626860 (Po)		81.0	81.6	81.8	80.9	80.7	81.6	81.4	80.7	80.9	80.7	80.7	79.9	43.4	44.3	43.4	43.2
2	FJ626861 (Po)	0.183		99.1	98.6	97.7	97.5	99.1	98.8	97.5	97.7	97.5	97.3	96.6	43.7	47.2	43.7	42.9
3	AF140295 (Po)	0.183	0.003		99.5	98.6	98.4	100.0	99.7	98.4	98.6	98.4	98.2	97.5	44.0	47.2	44.0	43.2
4	AF438150 (Po)	0.179	0.005	0.003		98.2	97.9	99.5	99.3	97.9	98.2	97.9	97.7	97.1	44.0	47.2	44.0	43.2
5	AF466527 (Po)	0.193	0.018	0.016	0.018		99.3	98.6	98.4	99.7	100.0	99.7	99.5	98.8	44.0	46.7	44.0	43.0
6	AF466532 (Po)	0.196	0.021	0.018	0.021	0.008		98.4	98.2	99.1	99.3	99.1	98.8	98.2	43.8	47.0	43.8	43.2
7	AF509333 (Po)	0.183	0.003	0.000	0.003	0.016	0.018		99.7	98.4	98.6	98.4	98.2	97.5	44.0	47.2	44.0	43.2
8	AJ238400 (Po)	0.186	0.005	0.003	0.005	0.018	0.021	0.003		98.2	98.4	98.4	98.2	97.3	43.8	47.0	43.8	43.0
9	AF466533 (Ps)	0.196	0.021	0.018	0.021	0.003	0.010	0.018	0.021		99.7	100.0	99.3	98.6	43.8	47.0	43.8	42.7
10	AF466539 (Ps)	0.193	0.018	0.016	0.018	0.000	0.008	0.016	0.018	0.003		99.7	99.5	98.8	44.0	46.7	44.0	43.0
11	AF466538 (Ps)	0.196	0.021	0.018	0.021	0.003	0.010	0.018	0.021	0.000	0.003		99.3	98.6	43.8	47.0	43.8	42.7
12	AF466540 (Ps)	0.196	0.024	0.021	0.024	0.005	0.013	0.021	0.024	0.008	0.005	0.008		98.4	43.8	46.7	43.8	42.7
13	AF522321 (Ps)	0.206	0.029	0.026	0.029	0.010	0.018	0.026	0.029	0.013	0.010	0.013	0.016		43.2	46.3	43.2	42.1
14	AF252288 (Ps)	0.860	0.806	0.799	0.799	0.802	0.809	0.799	0.806	0.811	0.802	0.811	0.809	0.824		51.2	100.0	75.8
15	AF497479 (Pm)	0.854	0.720	0.721	0.721	0.737	0.728	0.721	0.728	0.729	0.737	0.729	0.735	0.753	0.637		51.2	49.4
16	AY305326 (Pc)	0.860	0.806	0.799	0.799	0.802	0.802	0.799	0.806	0.811	0.802	0.811	0.809	0.824	0.000	0.637		75.8
17	AF102171 (Pc)	0.874	0.837	0.830	0.830	0.842	0.831	0.830	0.837	0.852	0.842	0.852	0.850	0.865	0.274	0.670	0.274	

purified using the Geneclean Turbo kit. Thus, particular attention should be paid to the choice of the DNA extraction methods for PCR assays of *Perkinsus* spp. to minimise the effect of inhibitors, optimise DNA recovery and improve the sensitivity of the PCR assay.

Perkinsiosis is a serious threat to clam cultures around the world, with dramatic consequences in many areas. Whether and to what extent levels of virulence are influenced by the host species, environmental conditions or the existence of parasite genetic lineage is

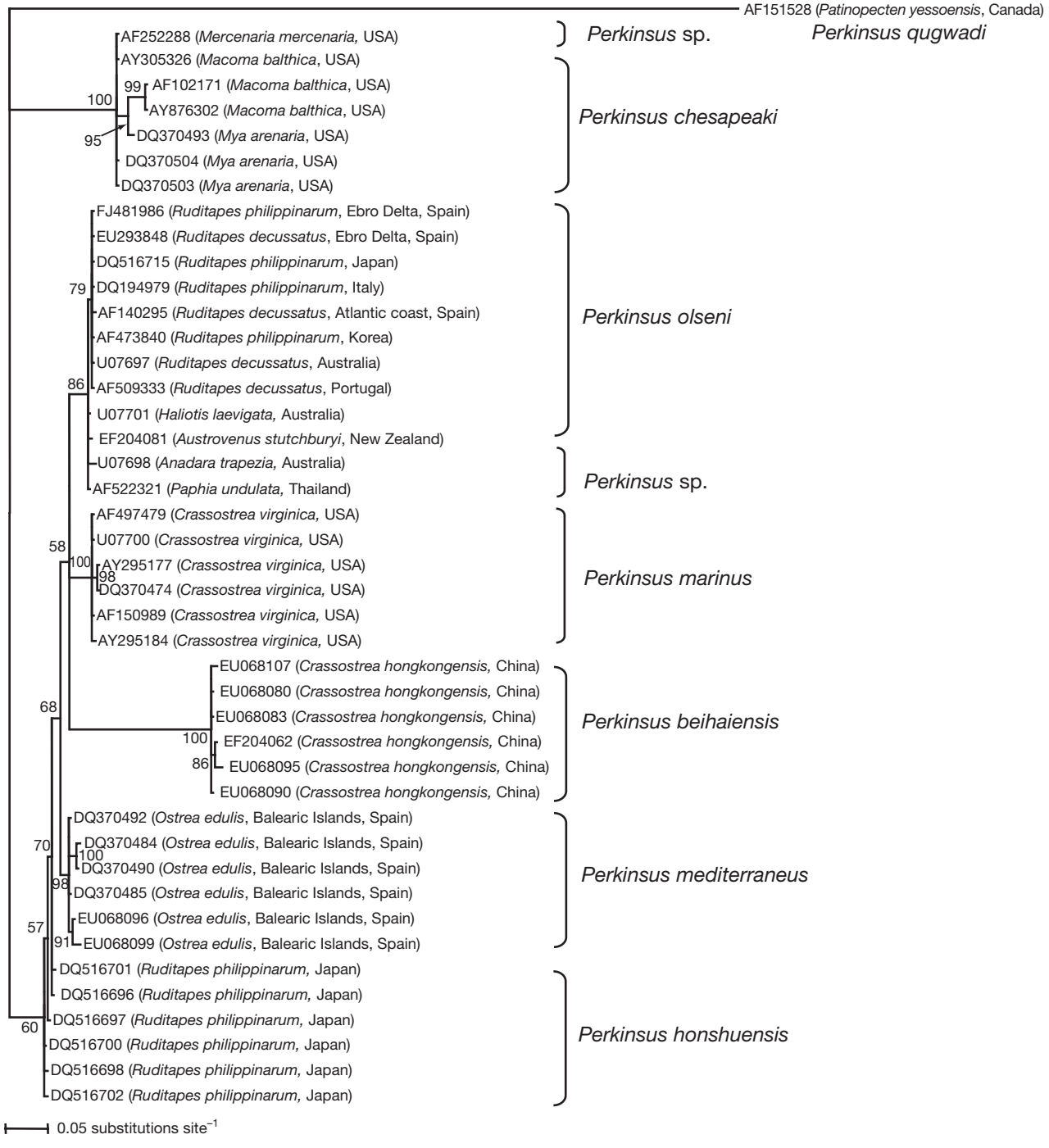

Fig. 1. Phylogenetic tree obtained by Bayesian analysis of *Perkinsus olseni* and other *Perkinsus* nuclear-encoded internal transcribed spacer (ITS) rDNA aligned sequences (including ITS1, 5.8S and ITS2). Support from posterior probabilities are shown on the nodes, and only values >50% are shown. Host and geographic location are indicated in parentheses following the GenBank accession number. Scale bar indicates the number of substitutions per site

still to be determined. Intra-species genetic variability among *Perkinsus* spp. is also likely to play a role in the intrinsic-virulence property of this parasite, and co-infections with multiple genetic strains within a single host have been found (Reece et al. 2001). However, despite the tremendous amount of genetic data now available, no link has been found between genetic strains and virulence. Most of the data available concern the rDNA sequences and in particular the ITS region. Analysis of the ITS region sequences is recognised as a robust tool for discrimination among *Perkinsus* spp. and has proven useful for phylogenetic studies and assessment of inter- and intra-specific genetic variability. In the present study, phylogenetic analysis based on the ITS region unequivocally placed our isolate within the *P. olseni* clade. Analysis of the NTS region confirmed the species identity of our isolate. The value of NTS as a taxonomic marker has not been established; however, sequence variability in the NTS region has been found to vary with geographic origin, at least for *P. marinus* and *P. chesapeaki* (Robledo et al. 1999, Coss et al. 2001). In the present study, the NTS

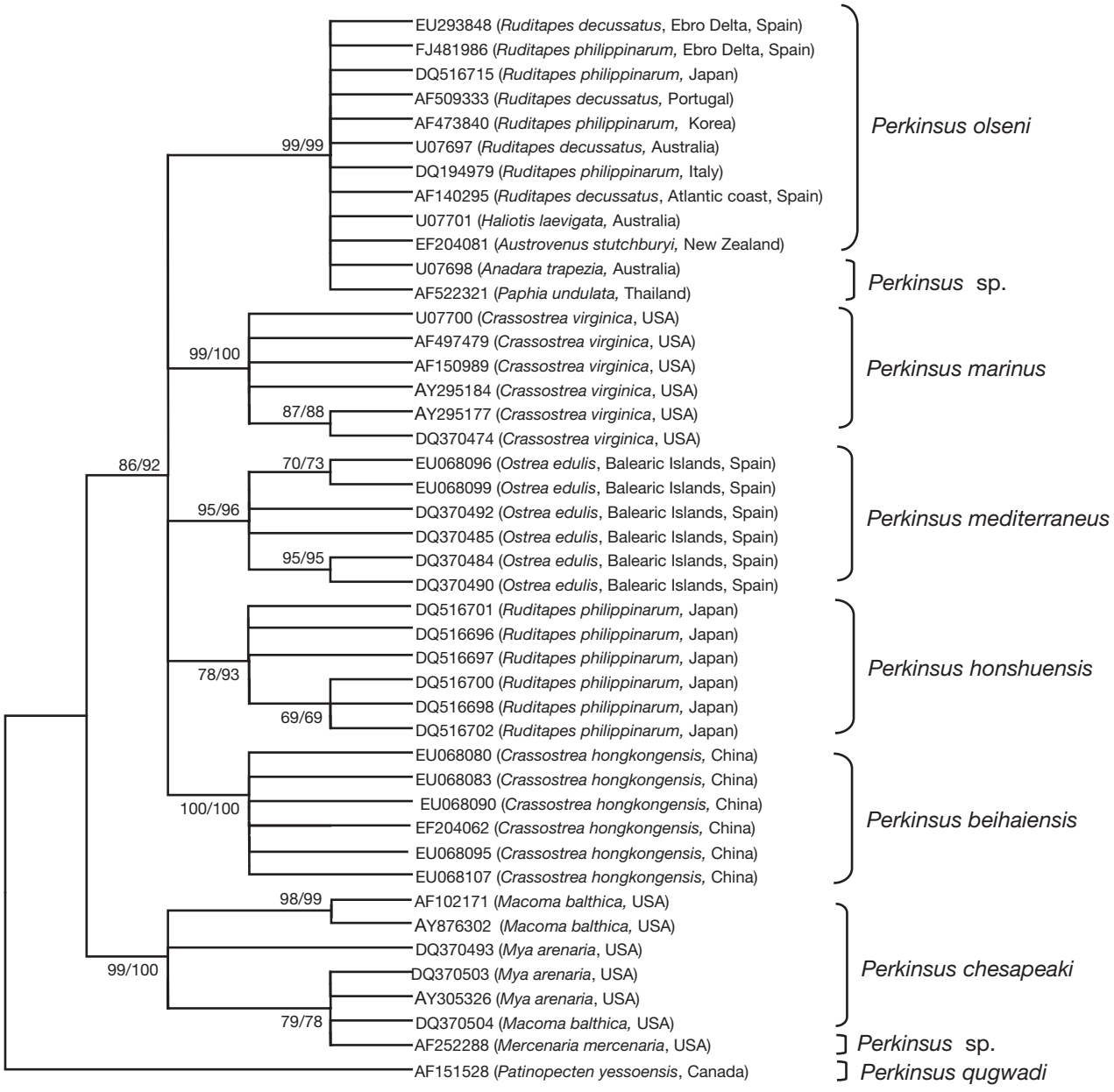

Fig. 2. Unrooted neighbour-joining (NJ) tree resulting from the analysis of the internal transcribed spacer region sequences of *Perkinsus* spp. Bootstrap values >50% are given on branches; first value is from NJ analysis, second value is from parsimony analysis. Host and geographic location are indicated in parentheses following the GenBank accession number

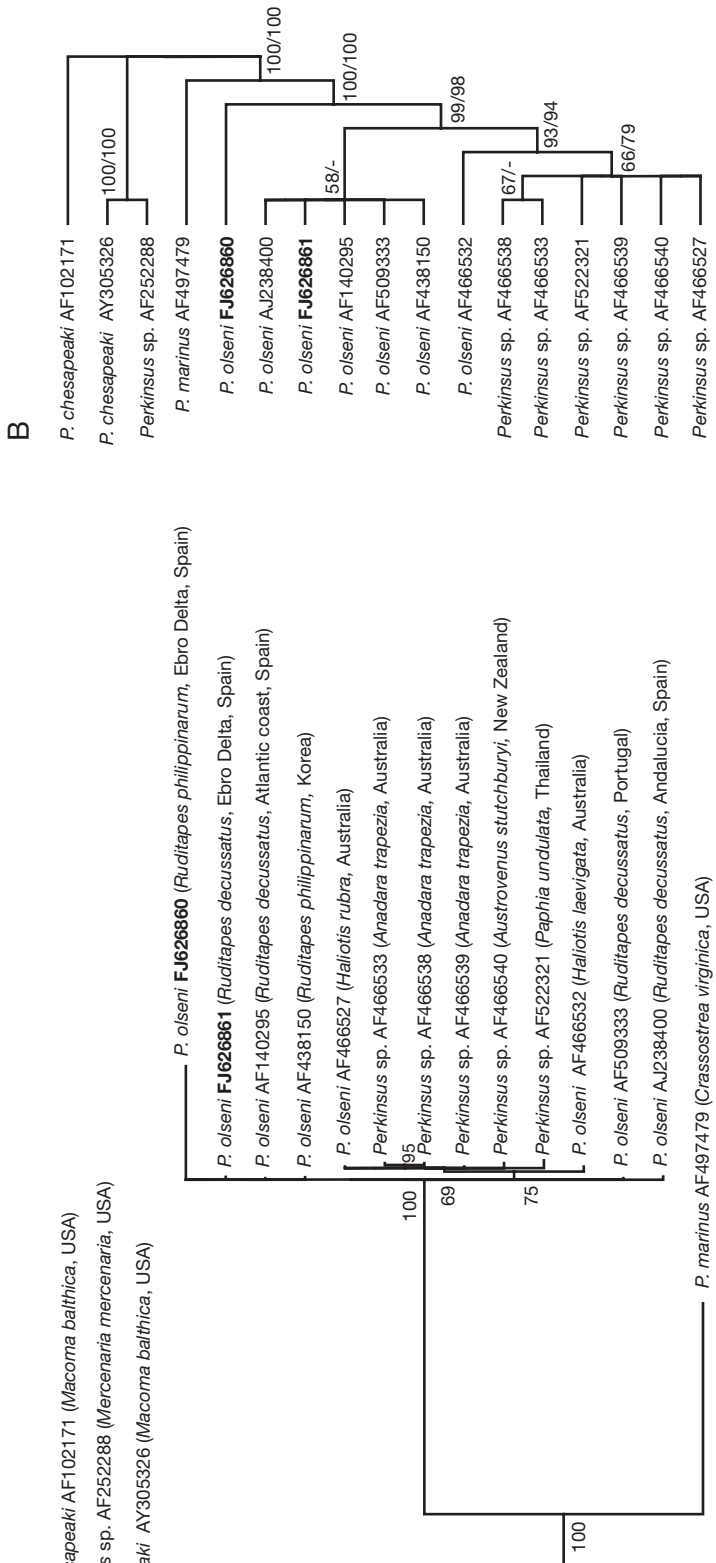

Fig. 3. Phylogenetic tree obtained by (A) Bayesian analysis and (B) maximum parsimony (MP) analysis of the nontranscribed spacer region sequences of *Perkinsus* spp. (A) Support from posterior probabilities are indicated on the nodes; only values >50% are shown. (B) Bootstrap values >50% from 1000 replicates are given on branches; first value is from MP analysis, second value is from neighbour-joining analysis. The sequences derived from the present study are highlighted in **bold**. Host and geographical location are indicated in parentheses following the GenBank accession number

sequence of our isolate showed the greatest similarity with a Korean *P. olseni* strain detected in Manila clams, whereas the NTS sequence of the isolate previously detected in *Ruditapes decussatus* from the Ebro Delta displayed higher similarities with isolates from Galicia and Portugal, both also detected in the carpet-shell clam. *P. olseni* is believed to have been introduced in Europe through the importation of *R. philippinarum* from Asia (Hine 2001). However, it cannot be excluded that *P. olseni* was already present in the native European clam *R. decussatus*. Whether the polymorphism observed in the NTS sequences of *P. olseni* found in *R. decussatus* and *R. philippinarum* from the same area indicates host adaptation of an alien strain of *P. olseni* or a European isolate of this parasite is largely unknown. High degrees of sequence variability of the NTS region in *P. marinus*, *P. chesapeaki* and *P. olseni* strains have already been reported (Robledo et al. 1999, Coss et al. 2001, Park et al. 2005). Coss et al. (2001) reported 3 NTS sequence types in a 290 bp NTS fragment in *P. chesapeaki* found in amplicons from both oysters and clams. A study of the geographic distribution of *P. marinus* genetic strains on the east coast of the USA revealed that the frequency of the 2 types of NTS sequences encountered varied with the geographic origin of the samples (Robledo et al. 1999). Nevertheless, in view of the present results, phylogenetic analysis seems to indicate that *P. olseni* isolates group together according to host rather than geographic origin.

As more of their NTS sequences become available, the phylogenetic relationships among *Perkinsus olseni* isolates will become clearer. Assessment of the genetic variability in *Perkinsus* spp. NTS sequences and/or other molecular markers will help to determine whether the variability is due to regional patterns or host preference of *P. olseni* strains.

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