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New data on *Perkinsus mediterraneus* in the Balearic Archipelago: locations and affected species

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ABSTRACT: *Perkinsus mediterraneus*, a protozoan parasite that can cause perkinsosis (marine mollusc disease), was first detected in oysters *Ostrea edulis* from Mahon (Minorca, Balearic Islands, Spain) in 2004. Several years later it was also found in Andratx Harbour (Majorca, Balearic Islands) and in the Gulf of Manfredonia (Adriatic coast of Italy) in oyster populations. Since 2007, *Perkinsus* surveys have been conducted in different localities and shellfish species in the Balearic Archipelago. In the present work, we found *P. mediterraneus* in the Balearic Islands infecting oyster and other shellfish species. We describe infection with *P. mediterraneus* for the first time in *Arca noae* and *Mimachlamys varia.* The detection was carried out using Ray's fluid thioglycolate medium (RFTM), histology and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methodologies. The internal transcribed spacer (ITS) region (including ITS1, 5.8S and ITS2) of *P. mediterraneus* ribosomal DNA was sequenced from infected bivalve gills (or from the body in *Chamelea gallina*) from Balearic Archipelago localities. Twelve haplotypes with a strong genetic similarity between them (97–100%) were observed in our samples. These data were completed with 12 more haplotypes from GenBank sequences. The phylogenetic relationship between Balearic *P. mediterraneus* haplotypes found in this study, those previously obtained in Mahon Harbour, and the *Perkinsus* spp. sequences available in GenBank clearly grouped the different *Perkinsus* spp. in distinct clades supported by strong bootstrap values. Moreover, these analyses detected different *P. mediterraneus* groups in *O. edulis* from Minorca Island. No abnormal mortalities or decline in populations were detected during the survey, except for *C. gallina*, which is also affected by *Marteilia refringens*.

KEY WORDS: *Perkinsus mediterraneus* · *Arca noae* · *Chamelea gallina* · *Mimachlamys varia* · *Ostrea edulis* · Balearic Islands · Internal transcribed spacer

INTRODUCTION

Perkinsosis is a marine mollusc disease caused by *Perkinsus* spp. protozoan parasites. It has been associated with elevated mortalities of commercially important molluscs in the Gulf of Mexico, along the Atlantic coast of North America (Andrews & Hewatt 1957, Mackin & Hopkins 1962, Burreson & Ragone Calvo 1996, Ford 1996, Soniat 1996), in British Columbia, Canada (Bower et al. 1998), along the Atlantic coast of SW Europe (Azevedo 1989), in the Mediterranean Sea (Da Ros & Canonzier 1985, Santmartí et al. 1995), in southern Australia (Lester & Davis 1981, Goggin & Lester 1995), and along the south and west coasts of Korea (Choi & Park 1997, Park & Choi 2001).

Several *Perkinsus* species have been found in molluscs, but to date, only 7 are recognized as valid. The first described species was *P. marinus* in USA in the

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1940s; it affects the American oyster *Crassostrea virginica* and induces severe mortalities (Ray 1966b). *P. olseni*, the second named species of *Perkinsus*, was described in 1981 in the abalone *Haliotis ruber* in Australia, and was also found to be responsible for important mortalities of the abalone *H. laevigata* (Lester & Davis 1981, Goggin & Lester 1995). It is also believed to occur in a wide variety of mollusc species from the Great Barrier Reef (Goggin & Lester 1995). In 1989, *P. atlanticus* was detected in the carpet shell clam *Ruditapes decussatus* following mass mortalities in Portugal (Azevedo 1989). Other clams and areas have also revealed the presence of these species (Villalba et al. 2004). In the meantime, molecular systematics demonstrated that *P. olseni* was a senior synonym of *P. atlanticus* (Murrell et al. 2002). Two other *Perkinsus* species were proved to be synonymous on the basis of molecular analysis: *P. chesapeaki*, a parasite of the soft-shell clam *Mya arenaria* (McLaughlin et al. 2000), and *P. andrewsi*, a parasite of the Baltic clam *Macoma balthica* (Coss et al. 2001). *P. qugwadi*, responsible for mortalities of the Japanese scallop *Patinopecten yessoensis* in Canada, is distinguished from the other species so far identified because of its distinct characteristics: it does not enlarge in Ray's fluid thioglycollate medium (RFTM) or stain blue-black with Lugol's iodine (no development in prezoosporangia); its zoospores can develop within the interstitial space of its living host; it can proliferate and be a pathogen at cold temperatures, and its molecular characteristics are considerably different from other species of *Perkinsus* (Bower et al. 1998, Blackbourn et al. 1998). Currently, its classification in the *Perkinsus* genus is controversial (Casas et al. 2002b). Other new *Perkinsus* species have been characterized: *P. mediterraneus* in the flat oyster *Ostrea edulis* from the Balearic Islands, Spain (Casas et al. 2004), *P. honshuensis* in Manila clam *R. philippinarum* from Japan (Dungan & Reece 2006), and *P. beihaiensis* in the oysters *C. hongkongensis* and *C. ariakensis* from southern China (Moss et al. 2008). In Mediterranean waters, only 2 species, *P. olseni* and *P. mediterraneus*, have been described (Casas et al. 2004, Abollo et al. 2006, Elandaloussi et al. 2009, Valencia 2010, Ramilo et al. 2010). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and *in situ* hybridization have been used for differential diagnosis between them.

In the present study, we report the presence of *P. mediterraneus* found in several mollusc species (*O. edulis*, *Mimachlamys varia*, *Chamelea gallina* and *Arca noae*) from the Balearic Islands, based on RFTM assay, histology, PCR-RFLP and analysis of internal transcribed spacer (ITS) sequences. The phylogenetic relationships between *P. mediterraneus* lineages from this area with other *Perkinsus* spp. were also established.

MATERIALS AND METHODS

Mollusc tissue samples

A total of 314 wild and cultivated European flat oysters *Ostrea edulis*, Noah's Ark shells *Arca noae*, striped Venus shells *Chamelea gallina*, cultivated variegated scallops *Mimachlamys varia* and mussels *Mytilus galloprovincialis* were collected from coastal locations in Minorca, Majorca and Ibiza-Formentera, Spain, in September from 2007 to 2013 (Table 1, Fig. 1). One gill from each individual was preserved in 95% (v/v) ethanol for DNA extraction and PCR analysis, except for *C. gallina*, in which half of the body was used, due to their small size. Visceral mass sections were also preserved for histological examination.

RFTM assays

For these assays, the other gill from each individual or a quarter of the *C. gallina* whole body was individually incubated in RFTM (Ray 1966a) supplemented with chloramphenicol and nystatin for a week at room temperature in the dark, to induce transformation of the parasite's trophozoites into hypnospores of *Perkinsus*. These samples were then partially crushed on a slide using a scalpel, stained with Lugol's iodine solution and observed under light microscopy to estimate the intensity of *Perkinsus* infection according to the following scale (Ray 1954, modified in Andrews & Hewatt 1957)–0 = null infection: no *Perkinsus* sp. detected in the whole slide (40×); 1 = very light infection: from 1 *Perkinsus* sp. hypnospore observed in the whole slide (40×); 2 = light infection: at least 1 *Perkinsus* sp. hypnospore observed in every one of 10 random fields scattered throughout the preparation (40×); 3 = moderate infection: at least 10 *Perkinsus* sp. hypnospores observed in every one of 10 random fields (40×), scattered throughout the preparation; 4 = heavy infection: at least 10 *Perkinsus* sp. hypnospores observed in every one of 10 random fields (100×), scattered throughout the preparation; and 5 = very heavy infection: at least 50 *Perkinsus* sp. hypnospores observed in every one of 10 random fields (100×), scattered throughout the preparation.

Table 1. Ray's fluid thioglycolate medium (RFTM) assay results obtained for *Perkinsus* infection. All bivalves (N = 314) were sampled in September except once in 2013. Mackin's scale was used to determine the degree of infection. The average value for each assay is represented. Oysters from Ibiza-Formentera were frozen, so RTFM results were obviously negative; however, PCR results indicated that 11 of them were infected, but no data were obtained concerning degree of infection. Size is mean ± SD

Location	Bivalve sp.	N	Year	Infected	Detection (%)	Degree of infection	Size (mm)
1 Mahon Harbour	*Ostrea edulis*[a]	32	2007	25	78.10	2.01	99.14 ± 11.64
2 Fornells Bay	*Ostrea edulis*	13	2010	8	61.50	0.77	86.52 ± 11.98
3 Andratx Harbour	*Ostrea edulis*	8	2011	5	62.50	1.37	74.66 ± 9.96
		6	2012	3	50.00	1.75	69.96 ± 18.55
		6	2013	4	66.70	0.67	87.40 ± 14.56
	Mimachlamys varia[a]	25	2011	15	60.00	0.87	34.52 ± 7.81
		25	2012	21	84.00	2.44	33.16 ± 4.71
		30	2013	25	83.30	2.4	41.97 ± 6.66
		14	2013[b]	14	100	2.36	34.49 ± 4.69
	Arca noae	20	2011	8	40.00	1.26	44.55 ± 5.15
		30	2012	16	53.30	0.8	50.29 ± 5.88
	Mytilus galloprovincialis[a]	30	2012	0	0	0	50.29 ± 5.88
4 Palmanova	*Ostrea edulis*	18	2010	5	27.80	1.14	90.67 ± 19.00
5 Porto Cristo	*Ostrea edulis*	15	2010	9	60.00	0.78	33.03 ± 4.23
6 Ibiza-Formentera	*Ostrea edulis*	18	2011	11[a]	61.10	No data	84.79 ± 33.83
7 Arenal Beach	*Chamelea gallina*	24	2012	6	25.00	0.25	20.40 ± 0.82

[a]Cultivated bivalves
[b]Sampled in October

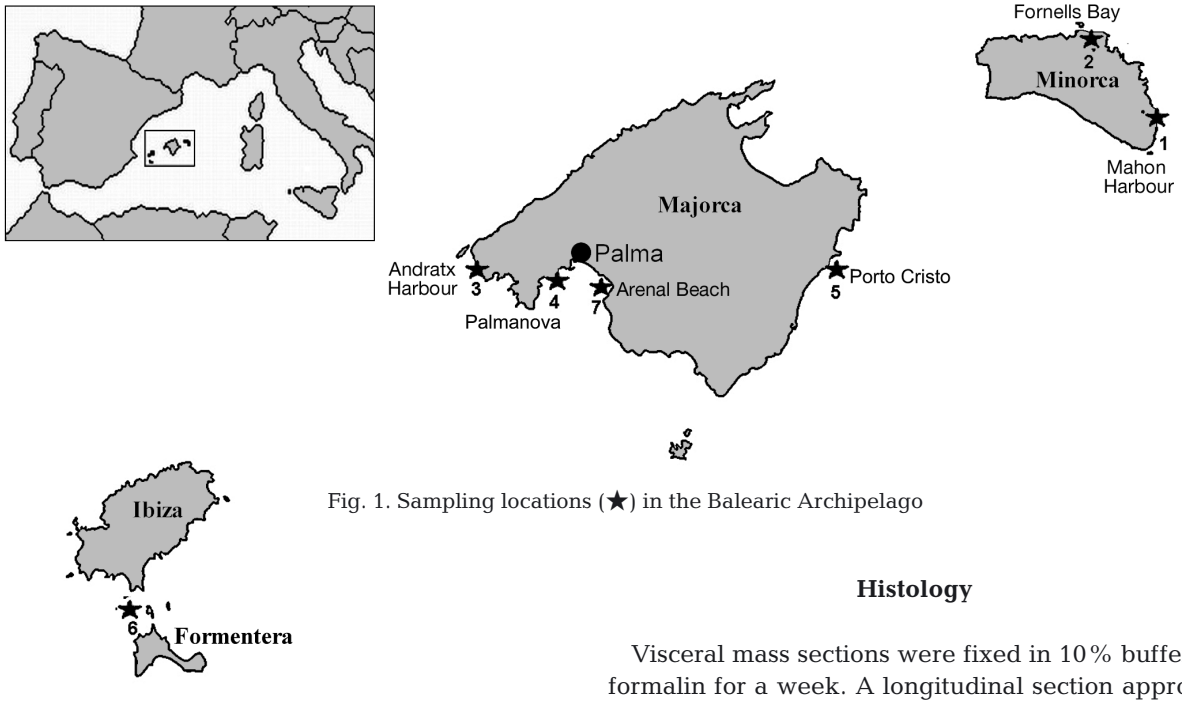


Fig. 1. Sampling locations (★) in the Balearic Archipelago

The percentage of detection of *Perkinsus* sp. was estimated as the percentage of infected molluscs in each sample. The mean intensity (degree of infection) was calculated as the average intensity among infected bivalves of each sample (Andrews & Hewatt 1957).

Histology

Visceral mass sections were fixed in 10% buffered formalin for a week. A longitudinal section approximately 5 mm thick was taken from the middle of the body including mantle, gonad, digestive gland, kidney and foot, cutting as is shown in Fig. 2 (Howard & Smith 2004). The section was dehydrated in ethanol series, embedded in paraplast, sectioned at 4 µm, and stained with haematoxylin & eosin for routine microscopic examination.

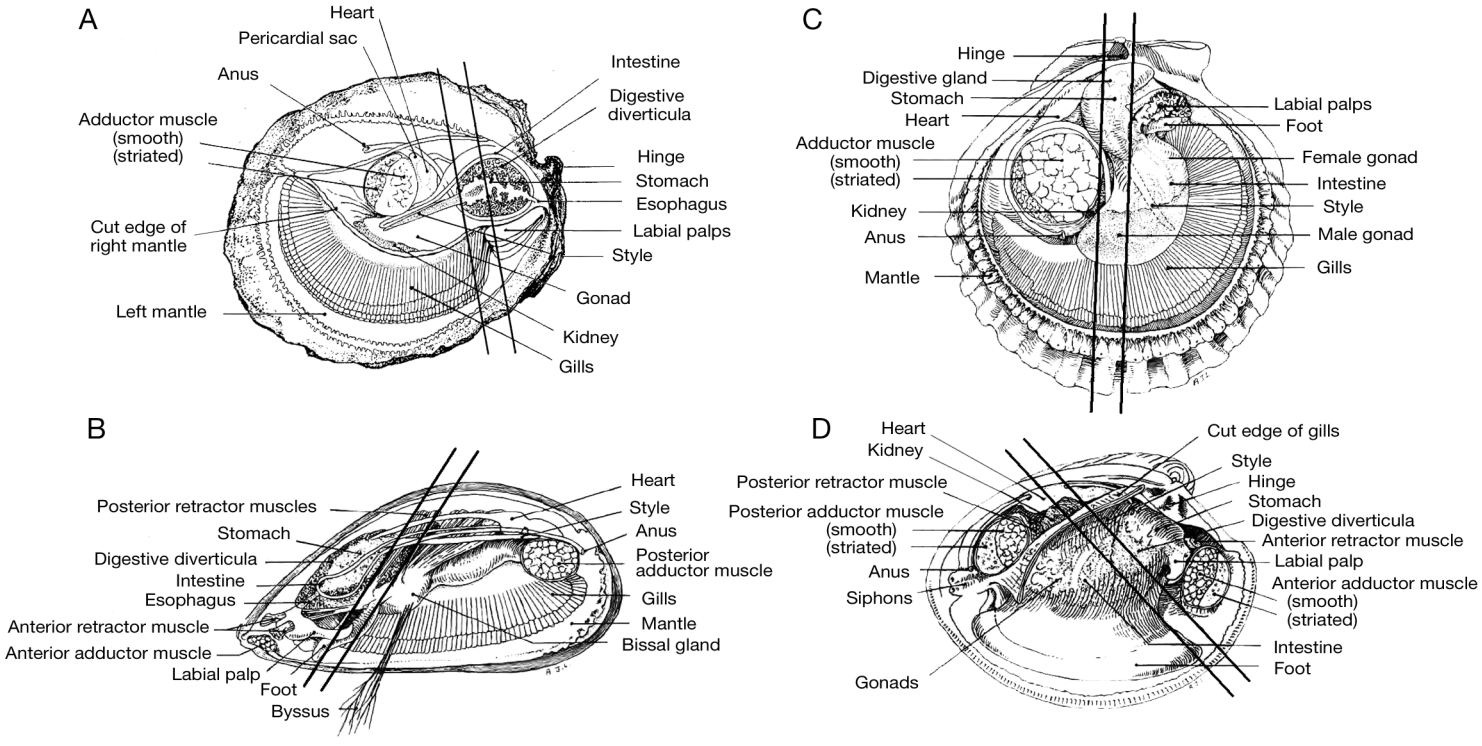


Fig. 2. Gross anatomy of (A) *Ostrea edulis*, (B) *Mytilus galloprovincialis*, (C) *Mimachlamys varia* and (D) *Chamalea gallina*. Bold parallel lines show location where cross-section should be taken. Illustration by J. A. Lippson, Bozman, MD (modified), in Howard & Smith (1983)

Genomic DNA extraction, PCR amplification and restriction analysis

DNA extraction was performed using Nucleospin reagents (Macherey-Nagel) according to the manufacturer's instructions. ITS regions of the ribosomal RNA (rRNA) gene (including ITS1, 5.8S and ITS2) were amplified by PCR using *Perkinsus*-specific ITS-region primers (Casas et al. 2002b, 2004): PerkITS85 (5′-CCG CTT TGT TTG GA/CTC CC-3′) and PerkITS750 (5′-ACA TCA GGC CTT CTA ATG ATG-3′).

PCR reactions were performed in a total volume of 20 µl containing 1 µl of genomic DNA, 10 µl of KAPA Taq Ready Mix DNA Polymerase (KapaBiosystems), 0.8 µl (20 mM) of each primer, and water to make up the final volume. The cycling protocol for the ITS region was 94°C for 5 min, 35 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 1 min; followed by 72°C for 10 min. Negative and positive (DNA of an oyster infected with *P. mediterraneus* from Andratx Harbour) controls were included.

The determination of the parasite species was first made using the PCR-RFLP approach (Abollo et al. 2006) mainly for diagnosis purposes. Restriction reaction of PCR product was carried out in a final volume of 20 µl containing 10 µl of DNA, 2 µl of enzyme buffer, 1 µl of restriction enzyme (first *Rsa*I and afterwards *Hin*fI) (Promega) and 7 µl of sterilized distilled water. Digestions were performed for 2 h at 37°C, and 20 min at 65°C to inactivate the enzymes. To visualize the PCR products and restriction patterns, 4 µl aliquots of PCR products and 4 µl of digested samples mixed with 1 µl loading buffer were subjected to electrophoresis through 2% agarose gel (Agarose Type I standard PB) stained with GelRed Nucleic Acid Gel Stain (Biotium) and run at 100 V for 25–30 min. A ladder (Roche molecular weight marker XIII or Norgen MiniSizer 50 bp DNA Ladder) was included as molecular weight marker. All PCR and restriction products were visualized under UV light.

Sequencing

Positive PCR products for *Perkinsus* were purified using Nucleospin Extract II PCR Clean-up Gel extraction (Macherey-Nagel) following the manufacturer's instructions and sequenced in both forward and reverse directions using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 3130XL.

Phylogenetic analysis

To determine the taxonomic affiliation of *Perkinsus* sp. isolated from shellfish, the resulting ITS 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 BioEdit 7.1.3.0 software package (Hall, 1999). Haplotype (Hd) and nucleotide diversity (π) were calculated using DnaSP 5 software (Librado & Rozas 2009).

Nucleotide sequences of the ITS region obtained in this study (N = 51) were submitted to GenBank with the accession numbers KJ569310 to KJ569360. The following sequences from GenBank (N = 53) were included in the phylogenetic analyses: *P. chesapeaki* AF102171, AY305326, AY876302, DQ370504 from *Macoma balthica* (USA); *P. olseni* AF140295 and AF369969 from *Ruditapes decussatus* (Spain), AF473840 from *R. philippinarum* (Korea), U07697 from *R. decussatus* (Australia), AF509333 from *R. decussatus* (Portugal), DQ194979 from *R. philippinarum* (Japan), EF204081 and EU293848 from *Astrovenus stutchburyi*, FJ481986 from *R. philippinarum* (Spain), and U07701 from *Haliotis laevigata* (Australia); *P. marinus* AY295184, U07700 and DQ370474 from *Crassostrea virginica* (USA), JQ266236 and JQ266240 from *C. corteziensis* (Mexico), and JX144357 from *C. rhizophorae* (Brazil); *P. mediterraneus* DQ370482–DQ370492 and AY487834–AY487843 from *O. edulis*, Mahon Harbour (Spain); *P. honshuensis* DQ516696–DQ516698, DQ516700 and DQ516702 from *R. philippinarum* (Japan); *P. beihaiensis* EU068080, EU068083, EU068090, EU068095 and EU068107 from *C. hongkongensis* (China); and *P. qugwadi* AF151528 from *Patinopecten yessoensis* (Canada). Phylogenetic trees were rooted against a dinoflagellate sequence *Dinophysis sacculus* (AY040583), since molecular data support a close affinity between the phylum Dinoflagellata and *Perkinsus* species (Reece et al. 1997, Siddall et al. 1997, de la Herrán et al. 2000, Murrell et al. 2002, Saldarriaga et al. 2003).

Neighbor-joining (NJ) analyses were conducted using MEGA v 5.05 software (Tamura et al. 2011) with 10 000 replicates for bootstrap values, and the cut-off value for the consensus tree was 60%. Genetic distance corresponding to the number of base substitutions per site by averaging over all our sequence pairs was estimated using the Tajima-Nei model (Tajima & Nei 1984) in MEGA. Bayesian inference was also performed using MrBayes version 3.2 (Huelsenbeck et al. 2001) with the best-fit model and parameters according to jModelTest v 2.1.4 (Darriba et al. 2012). A statistical parsimony network (with a 95% connection limit) was constructed using ITS sequences from all the *P. mediterraneus* by means of TCS v.1.21 (Clement et al. 2000) in order to examine the sequence relationships.

RESULTS

RFTM assays

A total of 314 bivalves were incubated in RFTM (Ray 1966a), and 164 were positive for *Perkinsus* infection. Examination with light microscopy of tissue samples after RFTM showed black or blue spheres measuring 7.9–183 µm (n = 844, mean size = 59.0 ± 40.3 µm) in diameter, typical *Perkinsus* sp. hypnospores, in *Ostrea edulis*, *Mimachlamys varia*, *Arca noae* and *Chamelea gallina* (Fig. 3).

The percentage of detection ranged between 0% (*Mytilus galloprovincialis*, Andratx Harbour) and 100% (*M. varia*, Andratx Harbour). The degree of infection varied from 0 (*M. galloprovincialis*, Andratx Harbour) to 2.44 (*M. varia*, Andratx Harbour) (Table 1).

Histology

Seventy-four histological preparations of the 164 RFTM positives were used for histological analyses because the other samples were lost (Table 2). Only very high to moderate infections were visible in the histological sections in *O. edulis* and *M. varia*. Infections were always visible in *A. noae*.

Histological examination of infected *O. edulis* revealed the presence of spherical trophozoites in the connective tissue. In slides from infected *A. noae* and *M. varia*, encapsulated multicellular stages (rosettes) were easily observed in connective tissue of many different organs, surrounded by haemocytes or by fibrous material enclosing *P. mediterraneus* trophozoites (Fig. 4). No trophozoites or rosettes were observed in slides from *C. gallina*.

PCR amplification and restriction analysis

Positive RFTM samples were subjected to PCR amplification. In all cases, the primer pair PerkITS

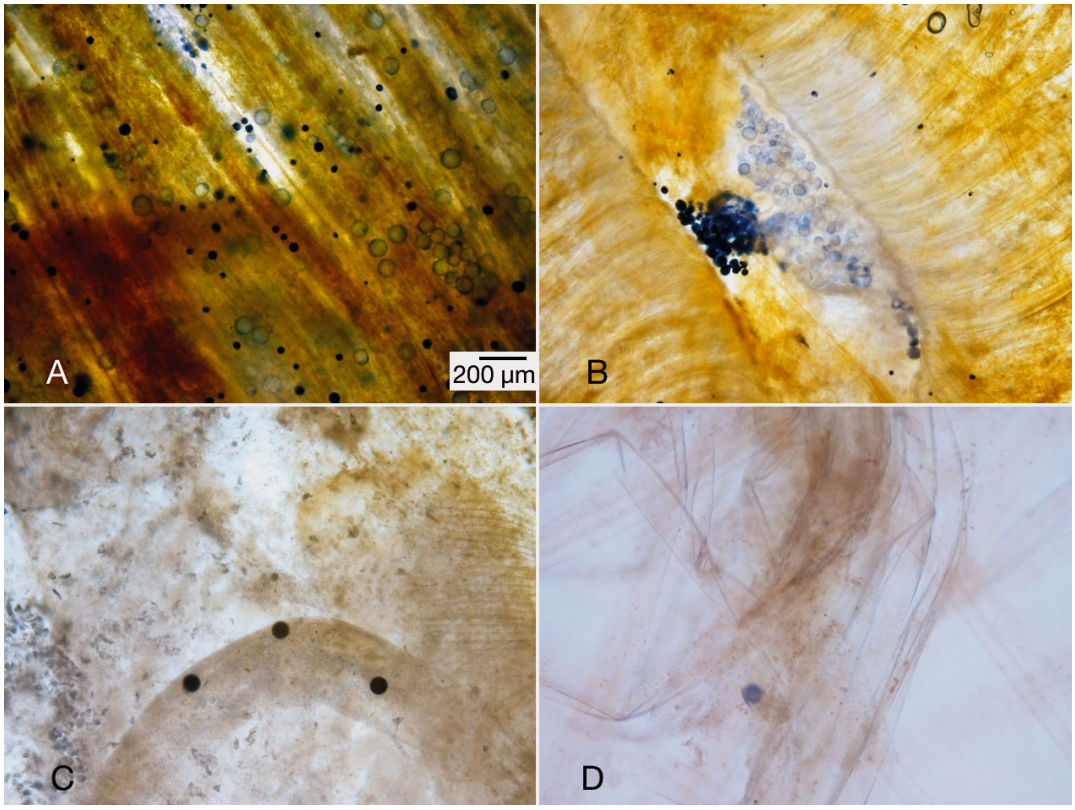


Fig. 3. Ray's fluid thioglycolate medium (RFTM) assay. (A) *Perkinsus* hypnospores in very heavy infection in *Ostrea edulis*; (B) *Perkinsus* hypnospores (trophozoites) in very heavy infection in *Mimachlamys varia*; (C) light infection in *Venus verrucosa*; (D) light infection in *Arca noae*. Scale bar applies to all panels

Table 2. Results of the histological survey. RTFM: Ray's fluid thioglycolate medium

Species	Location	RFTM positives	Histological negatives	Histological positives
Ostrea edulis	Palmanova	4	4	0
	Fornells Bay	8	7	1
	Andratx Harbour	8	6	2
Mimachlamys varia	Andratx Harbour	26	8	18
Arca noae	Andratx Harbour	22	10	12
Chamelea gallina	Arenal Beach	6	6	0
Total		74	41	33

yielded amplicons of approximately 700 bp of the ITS region (Fig. 5A). The amplified ITS fragments were digested and restriction patterns obtained with *Rsa*I enabled us to distinguish between *P. cheasapeaki*, *P. marinus* and *P. olseni*/*P. mediterraneus*. Two bands of approximately 193 and 74 bp plus a fragment of about 400–410 bp make up the pattern of *P. olseni*/*P. mediterraneus*. An additional *Hin*fI digestion allows the differentiation of *P. olseni* and *P. mediterraneus*; thus, 4 fragments of 192–179 (doublet) and 162–149 bp (doublet), typical of *P. mediterraneus*, can be observed (Fig. 5B).

Sequence analyses

We detected 12 different haplotypes (Table 3) in 51 individuals of *P. mediterraneus* sequenced in the present work (Hd = 0.646 ± 0.075 SD). The most frequent haplotype (named haplotype 03) was present in 30 individuals and was observed in all species and locations. Low nucleotide diversity (π) was detected (0.00171 ± 0.00029), with 8 polymorphic positions (all transitions): 300, 333, 374, 385, 409, 410, 492 and 574. The most frequent transition occurs in position 374 (G→A, 9 ind.), the second in 385 (T→C, 6 ind.) and the third in 574 (C→T, 5 ind.). The addition of the 21 sequences from GenBank (accession nos.

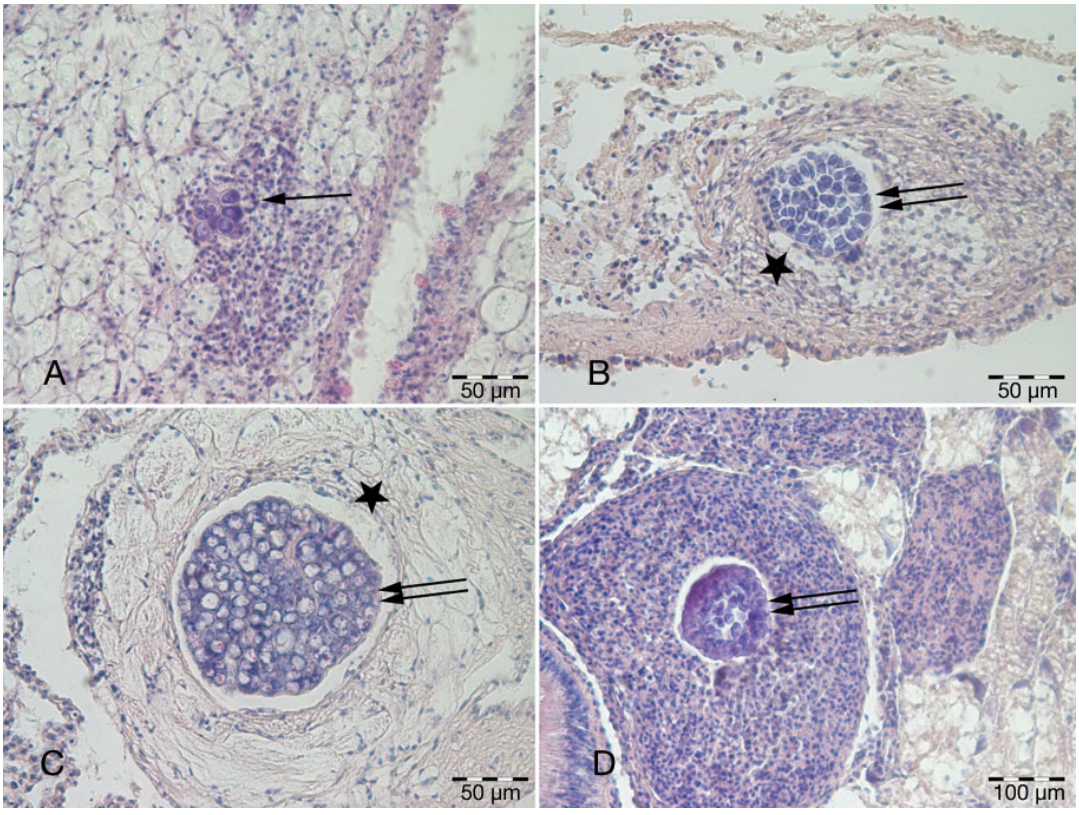
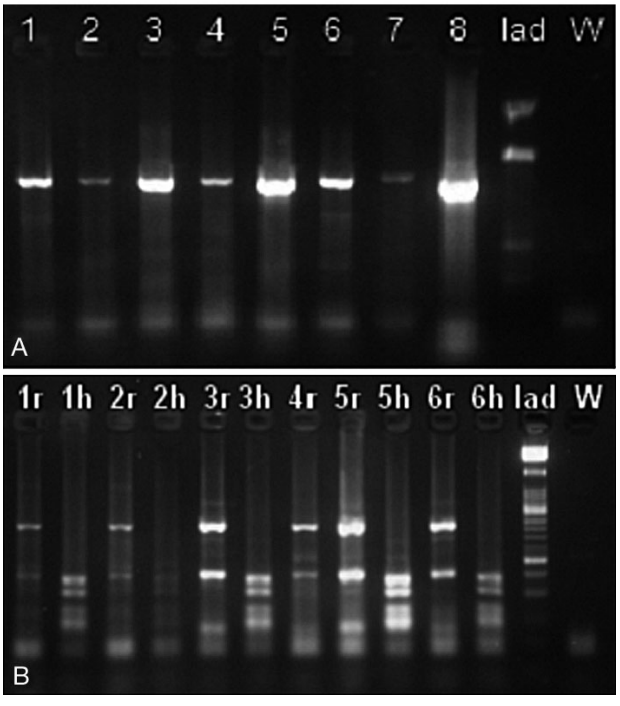


Fig. 4. (A) Trophozoites (arrow) of *Perkinsus mediterraneus* in *Ostrea edulis*; (B) multicellular stages of *P. mediterraneus* resulting from vegetative multiplication of mother cells with daughter cells in a rosette-like arrangement (cyst) in *Arca noae* (double arrow) surround by fibrous material enclosing the cyst (★); (C,D) cysts in *Mimachlamys varia* (double arrow). Star as in (B). Haematoxylin & eosin staining



DQ370482–DQ370492 and AY487834–AY487843), obtained previously in *O. edulis* from Mahon Harbour, makes it possible to complete the number of haplotypes found in the species to date. These sequences showed 15 different haplotypes, 3 of them in common with the one observed in the present study: haplotype 3 (observed in 5 of these 21 sequences) and haplotypes 6 and 7 (each observed in 1 ind.). The set of sequences showed a total of 20 polymorphic positions (18 transitions and 2 transversions): 49, 83, 94, 98, 242, 300, 333, 358, 371, 374, 385, 409, 410, 425, 448, 492, 563, 574, 611 and 619. The most frequent transition occurs in position 374 (G→A, 13 ind.), the second occurs in 385 (T→C, 11 ind.) and the third in position 333 (T→C, 8 ind.).

Fig. 5. (A) PCR patterns from oysters infected with *Perkinsus mediterraneus*. Lanes 1 to 7: Fornells Bay; Lane 8: positive control; lad: Roche molecular weight marker XIII; W: negative control. (B) Ray's fluid thioglycolate medium (RFLP) patterns digestion with *Rsa*I and *Hin*fI. Lane numbers correspond with (A) r: digestion with *Rsa*I; h: subsequent digestion with *Hin*fI

Table 3. Polymorphic nucleotide positions identified in the ITS1, 5.8S and ITS2 sequences of *Perkinsus mediterraneus* from shellfish collected in the Balearic Islands (*Ostrea edulis*, *Mimachlamys varia*, *Arca noae* and *Chamelea gallina*). The most frequent haplotype, 3, was used as the base. Transversions are marked as grey columns and transitions in white. Haplotypes 1 to 12 were obtained in this work; haplotypes 13 to 24 were obtained from GenBank data

Haplotype	ITS1				5.8S	ITS2															Total infected individuals	*O. edulis* Andratx Harbour	*O. edulis* Palmanova	*O. edulis* Fornells Bay	*O. edulis* Porto Cristo	*O. edulis* Ibiza-Formentera	*M. varia* Andratx Harbour	*A. noae* Andratx Harbour	*C. gallina* Arenal Beach	*O. edulis* Mahon Harbour (GenBank)
	49	83	94	98	242	300	333	358	371	374	385	409	410	425	448	492	563	574	611	619										
1										A	C	C									1	1								
2													T								1	1								
3	A	T	T	T	T	T	C	T	G	G	T	T	C	C	T	A	T	C	G	G	35	4	4	5	5	4	4	2	2	5
4												C									2		1				1			
5											C										2		1				1			
6												C						T			3	1		1						1
7						C	T									G					2				1					1
8										A											5	1			1	2			1	
9										A	C										2	1					1			
10																		T			3	1						2		
11							T				C										1	1								
12							T			A											1								1	
13	T														C						1									1
14										A	C		T								2									2
15		C				C	T									G			A		1									1
16						C	T									G			A		2									2
17						C	T	C								G					1									1
18				C																	1									1
19	T		C																		1									1
20									A			C								T	1									1
21	T				C				A								C				1									1
22	T									A	C		T							T	1									1
23	T										C										1									1
24	T									A	C		T	T							1									1

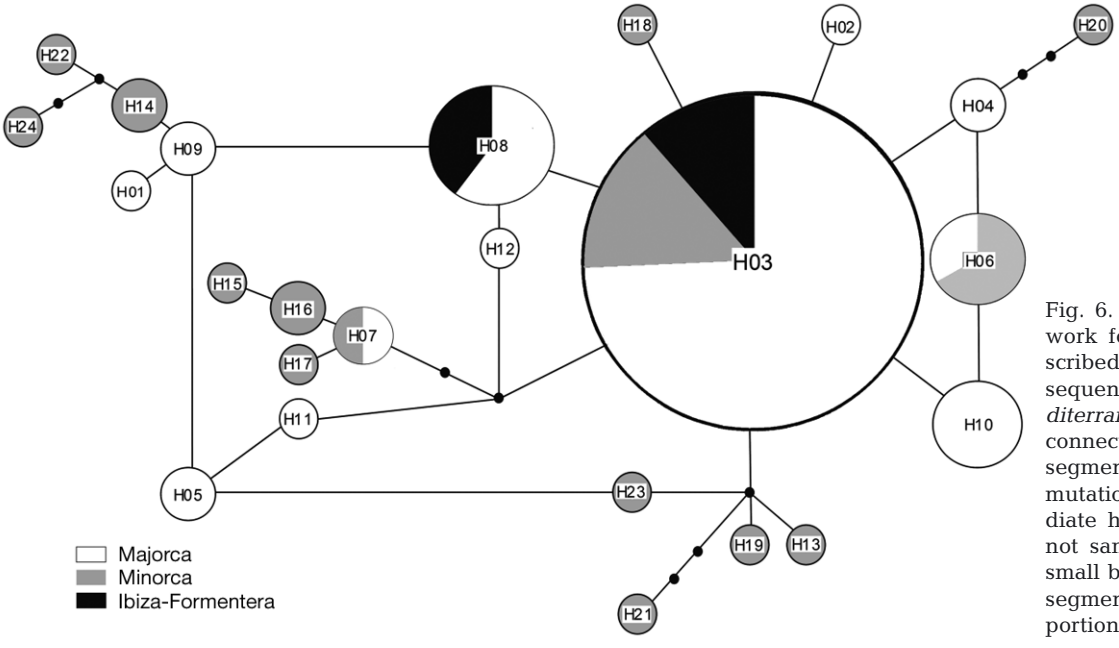


Fig. 6. TCS haplotype network for the internal transcribed (ITS) spacer region sequences in *Perkinsus mediterraneus* based on a 95% connection limit. Each line segment represents a single mutation. Inferred intermediate haplotypes that were not sampled are shown as small black dots on the line segments. Circle size is proportional to haplotype frequency

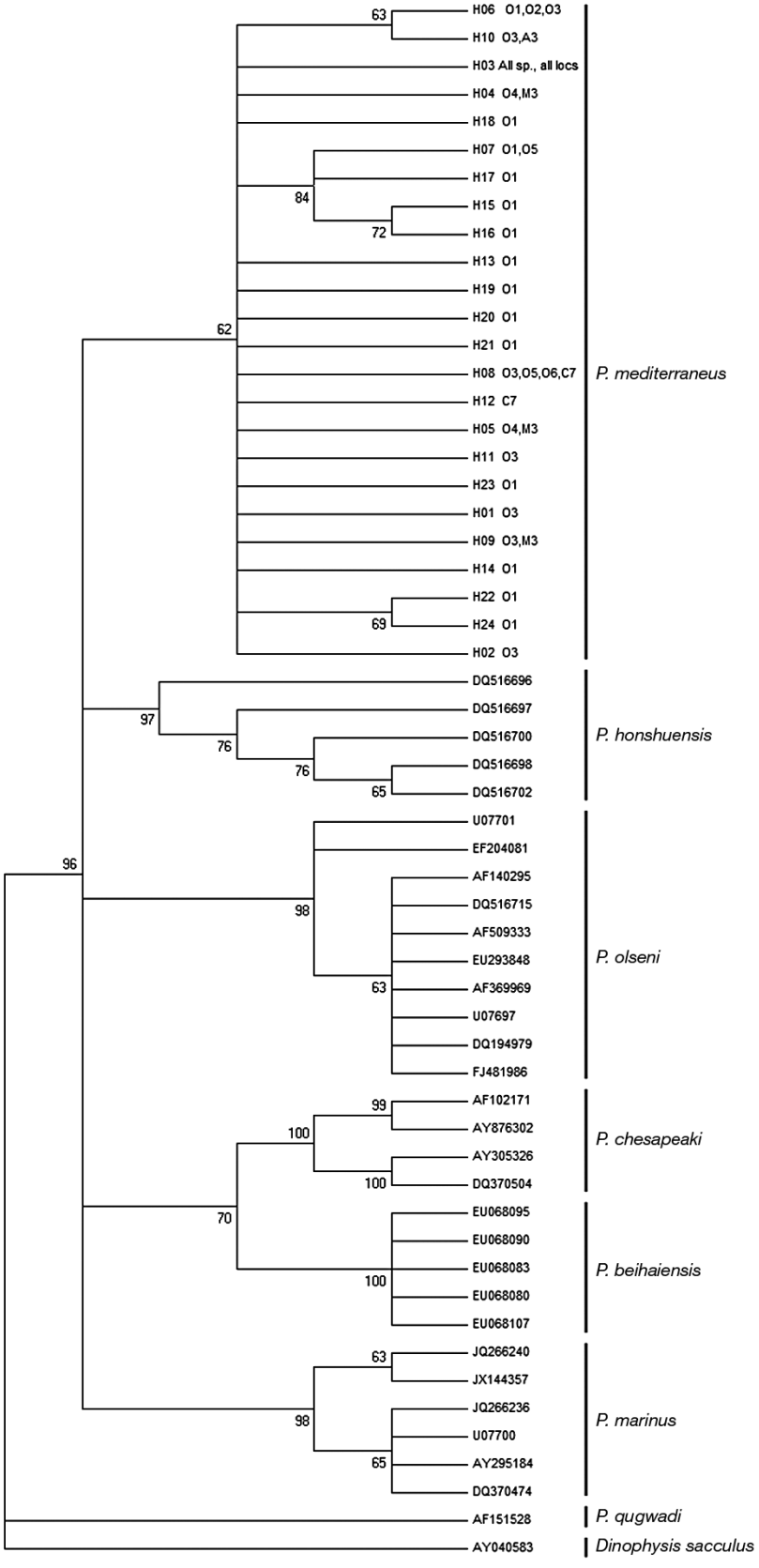


Fig. 7. Neighbor-joining tree resulting from the analysis of the internal transcribed (ITS) of *Perkinsus* spp. Bootstrap values >60% are given on branches. For *P. mediterraneus* (H01 to H24) haplotypes, letters indicate the shellfish species and numbers indicate the location. O: *O. edulis*; M: *Mimachlamys varia*; A: *Arca noae*; C: *Chamelea gallina*. 1: Mahon Harbour; 2: Fornells Bay; 3: Andratx Harbour; 4: Palmanova; 5: Porto Cristo; 6: Ibiza-Formentera; 7: Arenal Beach

All *Perkinsus* sp. sequences showed 97–100% identity with the DNA ITS region of *P. mediterraneus*. The results increased to 99–100% identity when the sequences with accession numbers EU-068099 and EU068097 (Casas et al. 2008) were removed, surely due to the fact that these sequences were obtained after several years of *in vitro* culture.

Fig. 6 shows the TCS graph of all of the *P. mediterraneus* sequences. Haplotype 3 (H03) occupies a central position. This haplotype appears in 35 individuals and is present in all mollusc species, locations and years surveyed. The other haplotypes derive from it with a maximum of 6 mutational steps. The second most frequent haplotype is H08, with 5 individuals. It appears only in *O. edulis* and *C. gallina* from Majorca and *O. edulis* from Ibiza-Formentera. In Majorca, we found H04, H05 and H09 in *O. edulis* and *M. varia*, H10 in *O. edulis* and *A. noae*, and H12 in *C. gallina*. The rest of the haplotypes are exclusive to *O. edulis*, including haplotypes 13 to 24 from Mahon Harbour (GenBank sequences), H01, H02 and H11 in Andratx Harbour (Majorca), and H06 and H07 in Majorca and Minorca. H15, H16, H17 and H07 (Mahon and Porto Cristo) are grouped together. Two other groups were observed in Minorca, one comprising H14, H22 and H24, and another comprising H13, H19 and H23 (together with H21, with 2 more mutational steps). The H18 and H20 haplotypes were found only in Minorca.

The NJ and Bayesian trees based on the ITS region sequences (Figs. 7 & 8) clearly grouped the different *Perkinsus* spp. (*P. olseni*, *P. marinus*, *P. mediterraneus*, *P. honshuensis*, *P. beihaiensis*, *P. chesapeaki* and *P. qugwadi*) into distinct clades supported

by strong bootstrap values (62–100%). Furthermore, within the *P. mediterraneus* clade, the 2 trees revealed the existence of the groups described in the TCS analysis. The groups H07, H15, H16 and H17, and H14, H22 and H24 are detected in both trees. NJ also detects a third group with haplotypes H06 and H10, but in the Bayesian tree the group is formed by H04, H06 and H20. The haplotypes H13, H19 and H23 (and also H21) are grouped only in the Bayesian tree.

DISCUSSION

The present results provide evidence that *Perkinsus mediterraneus* infects a variety of shellfish in the Balearic Archipelago, such as oyster, variegated scallop, Noah's Ark shell and striped *Venus* shell. No infections were detected in mussels, as found in other locations such as in St. Gilla Lagoon, Sardinia (Culurgioni et al. 2006).

Perkinsosis is a serious threat for clam cultures around the world, with high mortalities of shellfish worldwide. Nevertheless, despite the presence of *P. mediterraneus* in our samples, no deaths or population decline were recorded, as happens with *Perkinsus* spp. in *Ruditapes decussatus* and *Cerastoderma glaucum* (Culurgioni et al. 2006), with *P. olseni* and *P. cheasapeaki* in clams from France (Arzul et al. 2012), and with *P. marinus* in *Crassostrea corteziensis* in Mexico (Escobedo-Fregoso et al. 2013). Differences in the impact of *P. marinus* on the oyster *Crassostrea virginica* were associated with (1) variations in environmental factors (Oliver et al. 1998); (2) virulence among *P. marinus* genotypes (Bushek & Allen 1996); and (3) genetic variability among oyster stocks, which may lead to differences in resistance to *P. marinus* (Encomio et al. 2005). A combination of these factors could explain the resistance to infection by *P. mediterraneus* of the bivalves analyzed in this study.

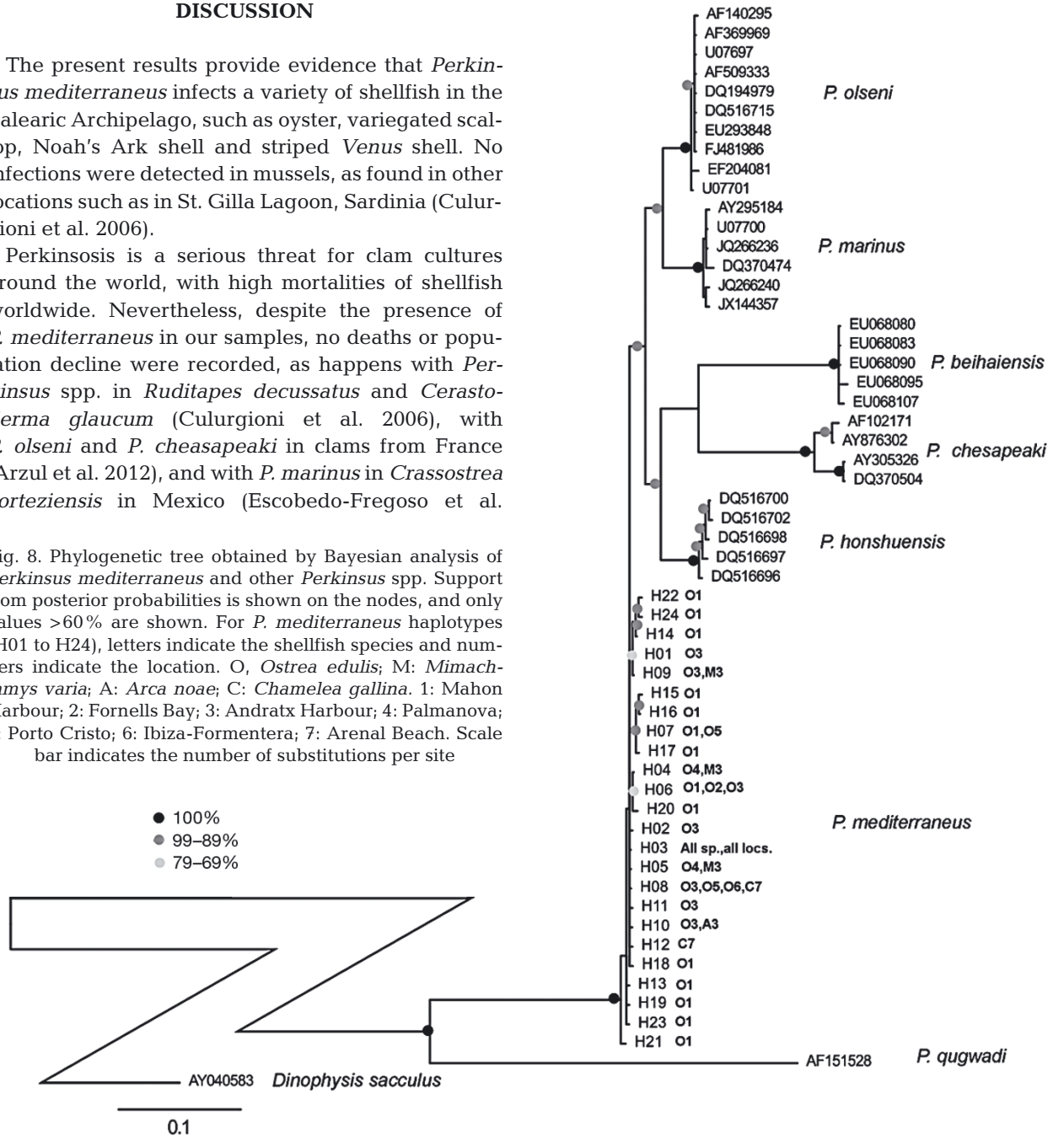


Fig. 8. Phylogenetic tree obtained by Bayesian analysis of *Perkinsus mediterraneus* and other *Perkinsus* spp. Support from posterior probabilities is shown on the nodes, and only values >60% are shown. For *P. mediterraneus* haplotypes (H01 to H24), letters indicate the shellfish species and numbers indicate the location. O, *Ostrea edulis*; M: *Mimachlamys varia*; A: *Arca noae*; C: *Chamelea gallina*. 1: Mahon Harbour; 2: Fornells Bay; 3: Andratx Harbour; 4: Palmanova; 5: Porto Cristo; 6: Ibiza-Formentera; 7: Arenal Beach. Scale bar indicates the number of substitutions per site

In this research, we report data regarding the percentage of detection of *P. mediterraneus* by RTFM and PCR-RFLP, which is similar to that of other *Perkinsus* spp. (Dungan et al. 2002, Culurgioni et al. 2006, Park et al. 2006, Sabry et al. 2009, da Silva et al. 2013, Escobedo-Fregoso et al. 2013). The maximum percentage of detection was 100% in *Mimachlamys varia* (October 2013) in Andratx Harbour. The minimum (excluding *Mytilus galloprovincialis* which seems to be not susceptible to infection in these locations) was 25% in *Chamelea gallina*, at Arenal Beach. Percentage of detection is expected to be higher in individuals with more than 1 yr of exposure to the pathogen (Andrews 1996, Burreson & Ragone Calvo 1996, Soniat 1996, Powell et al. 1997, Park et al. 1999, Park & Choi 2001, Villalba et al. 2005). The *C. gallina* collected in Arenal beach were suspected to be young, because their size was 20.4 ± 0.8 mm SD, corresponding approximately to 1 yr of age (Vizuete et al. 1993). This is probably the reason for the low percentage of detection and degree of infection in this population.

The detection of *Perkinsus* spp. infection is routinely carried out by the RFTM assay. The use of histological methods is not very reliable. The species specificity of histological methodology is very low, and sensitivity is good for moderate to heavy infections but low for low intensity infections, as has been shown in our samples (OIE 2013). For diagnostic purposes, *P. mediterraneus* could be detected by RFLPs. This methodology gives *P. mediterraneum*-like restriction profiles in all the investigated areas and bivalve species. Several studies have reported success with the use of the rDNA ITS region fragments in phylogenetic analyses for molecular diagnosis of *Perkinsus* species (Casas et al. 2002a, 2004, Brown et al. 2004, Burreson et al. 2005, Dungan & Reece 2006, Moss et al. 2008, Sabry et al. 2009, Sanil et al. 2012). In the present study, both molecular methods showed the same efficiency in the identification of *P. mediterraneus*. However, RFLP alone could not ascertain parasite identification, especially for a possible new member of the *Perkinsus* genus. Thus, sequencing of the ITS fragment has proven to be very useful in order to assign the *Perkinsus* species and for detecting genetic variability.

On the basis of the genetic variability observed, the topologies of the phylogenetic trees confirm a close relationship among all of the *Perkinsus* species, although each of them forms a monophyletic group. As showed in the literature, ITS sequences suggest that *P. mediterraneus* clusters closely to *P. honshuensis*, *P. olseni* and *P. marinus*. *P. qugwadi* is the most divergent, whereas *P. chesapeaki* and *P. beihaiensis*, the most similar species, are intermediate (Casas et al. 2002b, Villalba et al. 2004, Moss et al. 2008).

The pairwise distances between our 12 haplotypes was 0.40%. This value increased to 0.60% when the *P. mediterraneus* sequences from GenBank were also included. These values are in the range of the intraspecific variations observed within the currently accepted *Perkinsus* species (Moss et al. 2008). These results support the clonal life cycle that is typical of these parasites and has been described in the literature with other species such as *P. olseni* (Vilas et al. 2011). The 3 methods of phylogenetic inference used in this study, the TCS, NJ and Bayesian trees, jointly detected 3 different groups in *O. edulis* from Minorca that can be distinguished from the other conspecific lineages. This differentiation could be the result of several factors, such as environmental variation, different dates of detection and localities, movement of animals, human activity, etc. (Oliver et al. 1998, Bushek & Allen 1996, Encomio et al. 2005), but it is difficult to discern the importance of each factor. In any case, this differentiation indicates some type of isolation of these samples, and in this sense, we know that from 3 different *in vitro* culture of *P. mediterraneus*, namely PmG2, PmG3 and PmHt2, 11 sequences were obtained (GenBank accession nos. DQ370482–DQ370492), 4 of which corresponded with haplotypes H15, H16 and H17 (Abollo et al. 2006). It is possible that the *in vitro* conditions favoured the genetic differentiation (Dungan et al. 2002).

The ITS region of the ribosomal DNA has 3 different sequences (ITS1, 5.8S and ITS2). We found a greater variability in the ITS1 and ITS2 regions than in 5.8S. The latter only presents a transition in haplotype 21. We cannot discount that these differences in variability could also be a consequence of the importance of the well-conserved 5.8S sequence in the organisms in relation to ITS1 and ITS2.

In the present study we did not find co-infection with 2 species of *Perkinsus*, although it has been described: for example, *P. marinus* and *P. chesapeaki* in the oyster *C. virginica* from Chesapeake Bay (NE USA) (Pecher et al. 2008, Reece et al. 2008); *P. marinus* and *Perkinsus* spp. in *C. virginica* (NE USA) (Pecher et al. 2008); *P. olseni* and a *P. chesapeaki*-like parasite in *R. decussatus* from France (Arzul et al. 2012); and *P. olseni* and *P. mediterraneus* in *V. verrucosa* (Ramilo et al. 2010).

Regarding the impact of this parasite species on bivalves in the Balearic Islands, in 2004 anomalous mortality of *C. gallina* was detected at Arenal Beach. Histological analyses detected infection with *Mar-*

teilia refringens (endemic parasite in the Balearic Islands), although only with 4.3% percentage of detection. It was also confirmed by nested PCR and *in situ* hybridization (López-Flores et al. 2008). Nevertheless, due to the lack of the sensitivity of the histological methods (OIE 2013), we cannot discard a higher prevalence. We also found in *C. gallina*, in the same year, a *Perkinsus*-like organism later confirmed as *P. mediterraneus* (Moss et al. 2008). We did not detect co-infection by histology, although we cannot discard this possibility if more sensitive methods for *M. refringens* had been used. Therefore, the high mortality may be due to the simultaneous presence of *P. mediterraneus* and *M. refringens* in the natural bed of *C. gallina*.

No mortality and no population decline were reported in other locations investigated during the present study. However, considering that mortality has already been reported in association with *Perkinsus* spp., the intensity of their prevalence and infection in bivalve populations in the Balearic Islands should be monitored.

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