

Molecular characterization of the ribosomal RNA gene region of *Perkinsus atlanticus*: its use in phylogenetic analysis and as a target for a molecular diagnosis

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SUMMARY

Due to their widespread distribution and virulence, protozoan species of the genus *Perkinsus* are especially worrisome parasites for shellfish farmers. In the present paper, we investigate the organization and the structural features of the nuclear ribosomal genes of *Perkinsus atlanticus* as well as the use of DNA sequence information from this region for phylogenetic analyses. This information has been useful, further, for the development of a diagnostic test based on the amplification by the polymerase chain reaction (PCR) technique. We have isolated a high-copy DNA sequence in this species, and, after its characterization, we have determined that it corresponds to the ribosomal RNA (rRNA) genes 28S–5S–18S and the intergenic spacers. By comparing the complete sequence of the 5S rRNA gene and a partial sequence of the 18S rRNA gene of *P. atlanticus* with the sequences of those genes in other Alveolates, we have found additional support for the hypothesis that *Perkinsus* is more closely related to species of Dinoflagellata than to species of Apicomplexa. The intergenic spacer sequence between the 5S and the 18S rRNA genes was used to design a pair of primers to be used as a PCR-based diagnostic test. †‡

Key words: *Perkinsus atlanticus*, ribosomal genes, molecular diagnosis, phylogenetic analysis.

INTRODUCTION

Protozoan species of the genus *Perkinsus* have historically been associated with massive molluscan mortality (Andrews & Hewatt, 1957; Da Ros & Canzonier, 1985; Chagot *et al.* 1987). Genetic analyses of these species have been useful for both basic and applied research. The taxonomic status of *Perkinsus* spp. has been controversial in that they were classified initially as fungal species of the genus *Dermocystidium* and then included successively with the Ascomycetales, the Entomorphorales and Saprolegniales (Mackin, 1951; Ray, 1954; Perkins & Menzel, 1967). Currently, the species of *Perkinsus* are classified as Alveolata protozoan (Cavalier-Smith, 1993; Goggin & Barker, 1993; Ragan *et al.* 1996; Van de Peer, Van der Auwera & De Wachter, 1996). However, within the Alveolata these parasites were at first considered Apicomplexans (Cavalier-

Smith, 1993; Ragan *et al.* 1996; Van de Peer *et al.* 1996), but recent data support their inclusion in the Dinoflagellata (Siddall *et al.* 1997).

In any case, because of their widespread distribution and virulence, *Perkinsus* species are especially worrying parasites for shellfish farmers. At present, 5 species have been described in the genus *Perkinsus* (*P. marinus*, *P. olseni*, *P. karlssoni*, *P. atlanticus* and *P. qugwadi*), infecting over 67 molluscan species around the world (Perkins, 1993; Blackbourn, Bower & Meyer, 1998). *Perkinsus atlanticus*, one of the main parasites found in clams, was first described in *Ruditapes decussatus* from the Algarve (Portugal), where heavy mortality occurred (Azevedo, 1989). Later a parasite similar to *P. atlanticus* was also found in *R. decussatus*, *R. philippinarum*, *Venerupis aurea* and *V. pullastra* from the Huelva coast (S. W. Spain, near the Algarve) (Navas *et al.* 1992). The capacity of this parasite to infect different species (Rodríguez, Godoy & Navas, 1994) makes it especially interesting from an epizootic standpoint. Current diagnostic methods to detect *Perkinsus* involve either staining with lugol after incubation of host tissue in fluid thioglycollate medium or the conventional paraffin histological techniques. Both approaches require several days of development and have limited efficiency (Rodríguez & Navas, 1995). Recently, Marsh, Gauthier & Vasta

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† The nucleotide sequence data reported in this paper are available in the EMBL database with the accession number AJ238400.

‡ The commercial use of the diagnostic test presented here is protected by a patent. This patent is pending upon the application number P9801511.

(1995) have developed a molecular test based on the use of the polymerase chain reaction (PCR) technique to detect *P. marinus* within the tissues of the oyster *Crassostrea virginica*. These researchers characterized the intergenic spacer of the ribosomal genes and designed primers for the amplification of 307 bp of this region. We tested these primers for the detection of *P. atlanticus* DNA by PCR in *R. decussatus* samples from the south-western European coasts, but without success.

Therefore, we were prompted to characterize a specific region of the genome of *P. atlanticus* with the aim of developing a molecular diagnostic test for use in shellfish farms. We isolated a high-copy DNA sequence of this species and, after its characterization, demonstrated that it corresponds to a fragment of the intergenic region of the nuclear ribosomal RNA (rRNA) genes of the species. Further characterization of the complete intergenic region between the 28S and the 18S rRNA genes revealed 2 informative aspects. First, a special organization of the ribosomal genes was found, in that the 5S rRNA gene proved to be linked to the ribosomal unit. Second, the DNA sequence of the intergenic region of *P. atlanticus* was quite unique, with very low sequence similarity to the same region in the related species *P. marinus*. In the present paper, we examine the organization and the structural features of the ribosomal genes of *P. atlanticus* and use the DNA sequence information from this region for phylogenetic analysis. In addition, we present a diagnostic test using primers for the PCR technique that amplify a 554 bp DNA fragment of the intergenic spacer of *P. atlanticus*.

MATERIALS AND METHODS

DNA isolation

Pre-sporangia of *Perkinsus atlanticus* were obtained from heavily infected clam gills previously cultured for 5 days at 28 °C in thioglycollate medium supplemented with 20 g NaCl/ml and penicillin G (20 U/ml) and streptomycin sulfate (40 µg/ml). The tissues were trypsinized (0.25% in sterile seawater, 28 °C, 1 h) and pre-sporangia were isolated by filtering through 200–100 µm mesh, harvesting on 20 µm mesh and cleaning with sterile seawater. Zoospores were purified from cultured pre-sporangia after 5 days, at 28 °C in sterile seawater with penicillin/streptomycin. *P. atlanticus* genomic DNA was extracted using a standard method involving CTAB digestion with proteinase K and SDS, extraction with phenol/chloroform and precipitation with ethanol (Winnepenninckx, Backeljau & De Wachter, 1993). *R. decussatus* genomic DNA was extracted using the same method from infected and uninfected samples.

Cloning and sequencing of a high-copy DNA sequence

Genomic DNA from *P. atlanticus* was digested with 7 restriction enzymes (*Bam*HI, *Hae*III, *Hinf*I, *Eco*R I, *Sac*I, *Hind*III and *Dra*I) individually. Digestion of genomic DNA with *Dra*I restriction enzyme and separation of the generated fragments on 0.9% agarose gels revealed a prominent band. For the molecular cloning of the fragments from this band, 10 µg of *Dra*I digested DNA from *P. atlanticus* was electrophoresed on 3% Nu-Sieve GTG agarose and stained with ethidium bromide. The prominent band of about 730 bp was excised from the gel and melted at 65 °C for 10 min. DNA in the band was purified by extraction with phenol and chloroform, precipitated with ethanol, and ligated to the *Hind*II blunt site of pUC19. The ligation mixture was used to transform competent cells of *E. coli* DH5- α , and the recombinant clones were detected as white colonies in LB plates containing ampicillin, X-gal, and IPTG (Sambrook, Fritsch & Maniatis, 1989). Bacteria containing the fragment of interest were selected after screening with labelled DNA (see below) obtained from the same band used for cloning. Four recombinant plasmids containing *Dra*I sequences (PK14, PK39, PK63 and PK101) were used for further analysis and as templates for sequencing by the dideoxynucleotide chain termination method of Sanger, Nicklen & Coulson (1977). Sequencing products were analysed by an automated laser fluorescent DNA sequencer (Pharmacia). The same method was followed for the sequencing of the intergenic region ribosomal genes 18S and 28S of *P. atlanticus* (see below).

Quantification of the high-copy DNA sequence

To estimate the amount of the *Dra*I sequences in the genome of *P. atlanticus*, a *Dra*I digest of total DNA was subjected to electrophoresis in 0.9% agarose, stained with ethidium bromide, and photographed. From the integrated area under the different peaks in a densitometric scan of the photograph, we estimated the percentage of the genome of *P. atlanticus* which corresponds to the *Dra*I sequences.

Southern blotting and DNA hybridization

Genomic DNA from *P. atlanticus* was digested with different restriction enzymes at 5 U/µg DNA, fractionated in 0.9% agarose gel in 0.5 × TBE buffer (1 × TBE = 0.09 M Tris-borate, 0.002 M EDTA), and transferred to a Hybond N⁺ nylon filter (Sambrook *et al.* 1989). For Southern-blot hybridization, we used the plasmid insert of the PK14 clone (containing a 730 bp *Dra*I fragment) as a probe and we used the non-radioactive chemiluminescence

5' GTAAGTATGA GAGTAGGCTT ACGATCCACT GAGATTCATC ACGTGTGCG CCGATTGTCTCCTCCGTCT CTA CTCTCCCAT CTACGGACTT CCAAAGAAAAG 100
 ATGGATTACT CCGCACTCAG GAGACTTGGA GCGCAGCGAT GCGGTGTGCG GCAGAGTTGG ACTGCCCCAG AGGGTCTTAC TAGTGAGCAG AAGTGAGATG 200
 GAAAGTCTTG ACTTGTGGC GAAATTTGGT CCACTTGGTC GATTTTGTAT TGCCGAGGTC CGTGATGACG TTGCATTTTG GACAACCTCA GAGTGGCCAG 300
CGGCCATACT GCGTCGAATA CACCGGATCC CATCCGAAT CCGA**AGTAA** **GCGGCG**CAAG GCCCGGATAG **TACTGGGG**TG GGGGACCGCC CGGGAAGTCC 400
 TTAGGGTGTCT GCTGGCTTCT **TTTTAAATCG** CACTCATGGC TTGTGCATGC GTGCAAGCCC CCGGAGCCCC CTGGACAAT GTTATCCCAG CTC AACACAG 500
 AGCAACAGTG CTATGGCAAG TAGTCCACTA GAGAGCCAAG TCGACAATCT CTACAACATT GTCCAAGGGG GAAAGGGGGG CGCCTTGGCG AAGTTGACTT 600
 GCAGGCGAGG GTAAAAGATG CTATGGTTGG TTGCGGACCA AGTTCGCGGT GTGGGTCATC ATTATCGAGG TCTGTGGTGA CGATGGACTA GTTTTTAGGG 700
 ATTTCCCGGA GGTGTCACCA CGGACCCCC AACTTTGGCG ACGGGGGGTA CTC AATTTTA AGTAAAATT AAGTAAAATT TACTTAAAAT TCAGGTTTTT 800
 GGGTGCSCAA AGTTGAGGTG GTGACTGGT ACACGAAAAT TTTAAAAAG AGAGATATTA AAAAAATAT TATATTTTCT GTGTCACCGT **GACCAGTC** 900
ACCACAGGGC **GTAATTTCC** GGGAAATTT CAGATTTTCC GAAAAAATTG CATTTTGGGG TAAATAGTGT CCGTCAGAAT TTTGCCAAAG GACTGTCGTG 1000
 ATGTCAGAGT TCCCAAATG AGGGTTTTG GACATCGCTC TGAATCGCT AACGGCGTTT CAGATTTCCG ACTTTTCGAC ATATTCGGG TATTTGATAG 1100
 CTGCCAAATC GGTGAGCGT GAATATTCCA ATATTTCAA GGAT**ATATGA** **TATCGCGAGA** TATCATTGGA TTTCATGGGG TTTTGTATTA GTACCCGCTC 1200
 ATCGTGGGAA AGTCGGGTGA ATTTATTCAA CCCGCAAATC TAATCAAGAT TTGCATGATG CAGCGACTGA CCGGGG**TGAG** **TGTAGCAGCT** GTTCTACGGC 1300
 TTGCTACGCA GACCTATCGT GTTAGTAGTT GCGACTCTTG GCGTGAACCG GAAGACCGGA CCTCGCTTTC GACTATTCAT TCCGATGAAT ATGAGATGTC 1400
 AAGGGTATCG CTTCTGCGCA TATTTAGTGA **TCATCAGAGC** ACGTAC**AC** TTCAGTATAT CCTCGGATAC ACAGAAGCTC GCAAGCATTG CATGATGCAA 1500
 TCACCTGGTT GATCCTGCCA GTAGTCATAT GCTTGTCTCA AAGATTAAGC CATGCATGTC TAAGTATAAG CTT -3' 1573

Fig. 1. Nucleotide sequence of the intergenic region of the nuclear ribosomal genes of *Perkinsus atlanticus* obtained by PCR, using primers that anneal to conservative sequences in the 28S and in the 18S ribosomal genes (see Materials and Methods section). The 3' end of the 28S gene (position 1 to 57), the 5S gene linked to this locus (position 295 to 419) and the 5' end of the 18S gene (position 1503 to 1573) are boxed. The underlined sequence corresponds to the 730 bp sequence initially obtained by conventional cloning (clones PK14, PK39, PK63 and PK101) after digesting the genomic DNA with the restriction enzyme *DraI* (see text). The sequence was identical for all clones. Bold italics text indicates the different conserved motifs that we have identified within the 5S ribosomal RNA genes: the termination signal of transcription for RNA polymerase III, at the transcriptional initiation site for the RNA polymerase I and within the ETS sequence (see text). Finally, the boxed-arrows indicate the primers designed for the development of the diagnostic test for the species proposed here.

method (ECL, Amersham) to label the probe as previously described by Garrido-Ramos *et al.* (1995). Also, to analyse the hybridization patterns of the *DraI* sequences to *P. atlanticus* genomic DNA restricted with several enzymes, we compared these patterns with those obtained with a ribosomal DNA probe (labelled following the same procedure). Inserts of recombinant plasmids HM123 and HM456 (Cortadas & Pavon, 1982) were used as probes. HM123 contains a 4.8 kb fragment of the *Xenopus laevis* rDNA with most of the 28S sequence and the ITS, whereas HM456 contained a 6.6 kb fragment which contains most of the 18S sequence and the IGS (both plasmids were kindly provided by Dr I. G. R. Ruiz of Sao Paulo, Brazil).

PCR amplification of the intergenic region of the nuclear ribosomal RNA genes

PCR-amplification of the intergenic region between the nuclear ribosomal genes of *P. atlanticus* was carried out using primers complementary to conserved regions near the 3' end of the 28S rRNA gene (5'-GTAAGTATGAGAGTAGGCTT-3') and the 5' end of the 18S rRNA gene (5'-ACTGGCAGGATCAACCAGGT-3'). One hundred nanograms of DNA from *P. atlanticus* were used as a template. PCR reaction was performed with *Taq* polymerase

with 40 cycles of 95 °C for 90 s, 54 °C for 120 s and 72 °C for 180 s. The amplified product was sequenced and the sequence was deposited in the EMBL database under the accession number AJ23-8400.

Phylogenetic inference

The phylogeny of the Alveolata was analysed by comparing the sequence data obtained for the 5S rDNA gene of *P. atlanticus* to the 5S rDNA gene sequences from other alveolates including *Cryptosporidium parvum*, *Eimeria tenella*, *Toxoplasma gondii* (belonging to Apicomplexa), *Cryptothecodinium cohnii* (Dinoflagellata), *Paramecium tetraurelia* and *Tetrahymena thermophila* (Ciliophora), with *Trypanosoma rangeli* (Euglenozoa) used as a protozoan outgroup (Cavalier-Smith, 1993). In addition, the phylogenetic relationships of these species were analysed by using the combined data set of the 5S rDNA gene and the first 70 nucleotides of the 3' end of the 18S rDNA gene. The sequences for these genes of the species mentioned above were obtained from the GenBank database (see accession numbers in the legend to the Fig. 4). Multiple alignments were first performed by computer program CLUSTAL W 1.7 (Thompson, Higgins & Gibson, 1997). Neighbour-joining and parsimony phylogenetic analyses were

performed using the MEGA package (Kumar, Tamura & Nei, 1993). Genetic distances were calculated according to the Jukes–Cantor method.

Development of a molecular diagnostic test for Perkinsus atlanticus

The presence of the parasite *P. atlanticus* in the host *Ruditapes decussatus* was detected by PCR-amplification of a 554 bp fragment of the *P. atlanticus* nuclear ribosomal DNA intergenic region by the use of a pair of primers named PK1 and PK2 with the sequences: 5'-ACCAGTCACCACAGGGCGTAAAT-3' (PK1) and 5'-GTAGCGTGCTCTGATGATCACT-3' (PK2). The design of these primers was based on the DNA sequence data obtained in this study and amplify a region located within the spacer sequence between the ribosomal genes 5S and 18S (see Fig. 1). PCR thermal parameters were 35 cycles of 94 °C for 120 s, 58 °C for 180 s, and 72 °C for 120 s. All amplifications were done in 10 mM Tris–HCl (pH 8.3), 5 mM NH₄Cl, 50 mM KCl, 2 mM MgCl₂, 0.2 mM of each dNTP and 1.25 U *Taq* DNA polymerase. To test the sensitivity of this diagnostic test, we used the PCR assay for the detection of *P. atlanticus* in infected clams of *R. decussatus* with different levels of infection ranging from level 5 (maximum) to the level 1 (minimum) according to the Mackin's scale (Mackin, 1962).

RESULTS

Isolation of a specific high-copy DNA sequence of Perkinsus atlanticus

Genomic DNA from *P. atlanticus* was digested with the 7 restriction enzymes indicated in the Materials and Methods section. Digestion of genomic DNA with *Dra*I restriction enzyme and separation of the generated fragments on agarose gels, revealed a prominent band of about 730 bp (not shown). This band was isolated and cloned, and from the recombinant clones obtained, 4 of those cross-hybridizing with an aliquot of the same isolated band were selected for further analysis (PK14, PK39, PK63 and PK101). Southern-blot hybridization using these clones as a probe revealed a restriction pattern similar to that observed after hybridization using as a probe the ribosomal unit of *Xenopus laevis* (not shown), indicating that the cloned sequences could correspond to a fragment of the ribosomal RNA gene region. Further sequence analysis of the 4 clones demonstrated that these sequences corresponded to a 730 bp fragment of the nuclear ribosomal unit of *P. atlanticus* (Fig. 1). A search of the EMBL/GenBank sequence databases revealed that the 70 bp of the 3' end of the cloned fragments matched the 5' end of the 18S ribosomal gene (Fig. 1). However, the search failed to identify any significant similarity of the

upstream sequence (660 bp) with any other sequence deposited in the databases. That region is assumed to be part of the ribosomal intergenic spacer upstream from the 18S gene (Fig. 2).

The method of isolating this fragment reveals that the ribosomal unit of *P. atlanticus* may be repeated a great number of times. In fact, we calculated the percentage of ribosomal units in the *P. atlanticus* genome as an estimate of the amount of the *Dra*I sequences in the genome of that species. According to the procedure indicated in the Materials and Methods section, the repeat unit amounted to 5% of the total genomic DNA.

Characterization of the ribosomal intergenic region of P. atlanticus

To characterize the intergenic ribosomal RNA gene region we cloned a fragment amplified by PCR using primers that anneal to conserved sequences of the 18S and 28S genes (Fig. 2). The sequence of the 1573 bp PCR product is shown in Fig. 1. This sequence included the 3' end of the 28S gene (57 bp), an intergenic spacer of 238 bp, the complete sequence (124 bp) of the 5S rRNA gene (which in this case is linked to the other ribosomal RNA genes), a second intergenic spacer of 1084 bp, which included the initial cloned fragment (730 bp), and the 5' end of the 18S gene (70 bp). Downstream from the 28S rRNA gene, the region coding for 5S rRNA was determined by comparisons and alignments with the 5S rDNA sequences from other Alveolata species. The transcription direction of the 5S rRNA gene in *P. atlanticus* was found to be the same as that of the 18S and 28S rRNA genes. In addition, conserved features of the 5S gene were found, such as a TTTTTT termination site for RNA polymerase III in the 3' end of the gene or conserved internal sequence motifs. These motifs corresponded to the internal control region (ICR) (Ciliberto *et al.* 1983). This ICR consists of 2 boxes (Box A and Box C). Box A, located between +50 and +60 within the *P. atlanticus* 5S rDNA coding region, shows 100% sequence similarity with the same region in other protozoa, such as *Cryptosporidium parvum* (Taghi-Kilani, Remacha-Moreno & Wenman, 1994) and 73% homology with the same region of *Xenopus borealis* (Ciliberto *et al.* 1983). Box C spans positions +71 to +83 and shows 84.61% and 76.92% sequence similarity with the same region in *C. parvum* (Taghi-Kilani *et al.* 1994) and in *X. borealis* (Ciliberto *et al.* 1983), respectively. The deduced 5S rRNA could be folded according to the secondary structure model proposed by De Wachter, Chen & VanDenberghe (1982) (not shown). This structure was compared to other secondary structures for 5S rRNAs, such as that of *Toxoplasma gondii* (Guay *et al.* 1992), *C. parvum* or *X. laevis* (Taghi-Kilani *et al.* 1994).

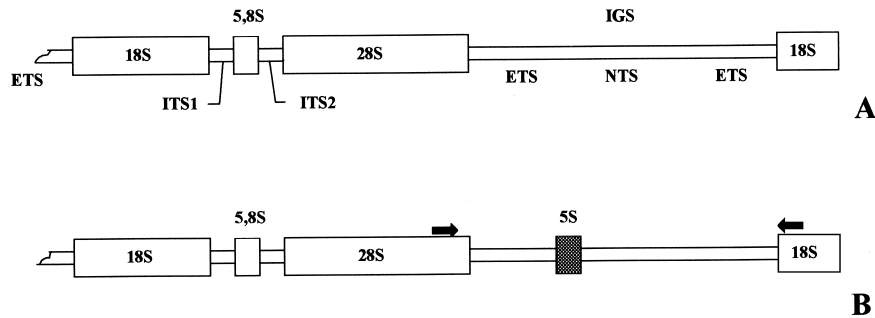


Fig. 2. Schematic representation of the nuclear ribosomal DNA repeating unit. The figure shows the most common organization of the ribosomal genes (A) and the less common organization in which the 5S gene is linked to the 18S–5.8S–28S repeating unit (B). **IGS**, Inter Genic Spacer; **ETS**, External-Transcribed Spacer; **NTS**, Non-Transcribed Spacer; **ITS**, Internal-Transcribed Spacer. Arrows indicate the region from which the primers were designed for the amplification of the IGS sequences from *Perkinsus atlanticus*. The sequence of the PCR product using these primers is given in Fig. 1.

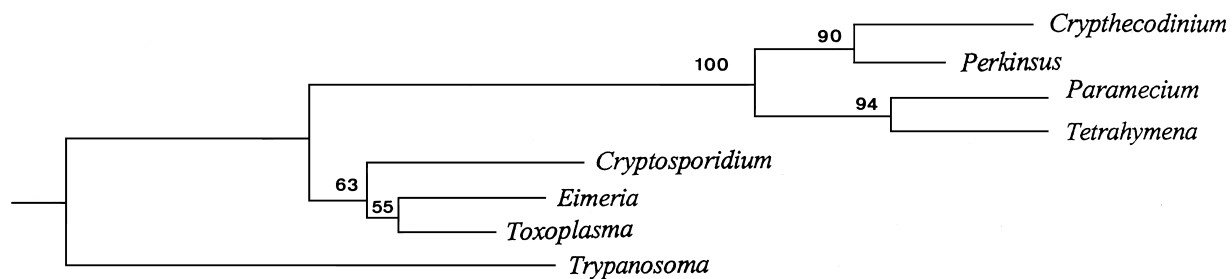


Fig. 3. Phylogenetic tree of the species of Alveolata as deduced from their 5S and 18S sequences using the neighbour-joining phylogenetic inference method. The tree was rooted by using as an outgroup species the Euglenozoa protozoan *Trypanosoma rangeli*. Numbers are bootstrapping values for the level of support for individual nodes. The sequences of these genes for the different species used here were obtained from the GenBank database under the Accession numbers: *Cryptosporidium parvum* (L20049 and X64341), *Eimeria tenella* (M86547 and U67121), *Toxoplasma gondii* (M63161 and L24381), these species belonging to Apicomplexa, *Cryptothecodinium cohnii* (M25115 and M64245) of Dinophyceae, *Paramaecium tetraurelia* (J01872 and X03772) and *Tetrahymena thermophila* (J01893 and AF060822) of Ciliophora and *Trypanosoma rangeli* (X62675 and AJ009160) as a protozoan outgroup.

With respect to the intergenic regions, a FASTA search of the EMBL/GenBank sequence databases revealed that the intergenic spacers demonstrated no homology with other intergenic sequences deposited in the databases. We found only 45% homology between the region from 431 to 888 positions (second intergenic spacer) and the 307 bp region of the intergenic spacer of *Perkinsus marinus* targeted by Marsh *et al.* (1995) for the molecular diagnostic test for this species. We conclude then, that the region described by these authors must be located within the spacer between 5S and 18S genes. Although no significant similarities could be found between the spacers of *P. atlanticus* and those of other species, it is important to mention that some characteristic features of this region are conserved in the protozoan species. Thus, it is noteworthy the existence of 2 motifs conserved in other species: TATATGATA and TGAGTGTA (see Fig. 1).

Phylogenetic position of *Perkinsus* among Alveolata

To obtain support for the recent revision of the systematic position of *Perkinsus* within Alveolata, we

used the sequence information gained from the 5S gene and from the 5S gene plus the 5' end of the 18S gene (190 bp in total) of the species mentioned in the Materials and Methods section. Using the data set based on the aligned 5S rDNA gene sequences, the neighbour-joining and the parsimony inference methods displayed a phylogenetic tree in which *P. atlanticus* appeared more closely related to *Cryptothecodinium cohnii* (Dinoflagellata) than to any other species analysed, but the bootstrapping support was low (not shown). The same association was obtained after using the combined set of data (5S gene plus 18S gene). Both inference methods displayed the same phylogenetic tree. The neighbour-joining phylogenetic tree obtained is shown in the Fig. 3. The inferred phylogenetic tree supports (more than 90% of 100 bootstrapped trees per node, with 5000 replicates) 3 separate clades within Alveolata, one composed of species ascribed to Ciliophora, one leading to Apicomplexa and another composed of Dinoflagellata species and *Perkinsus*. These data suggest that this parasite appears to be closely related to *C. cohnii*, forming a separate clade (Dinoflagellata) from the Apicomplexan species.

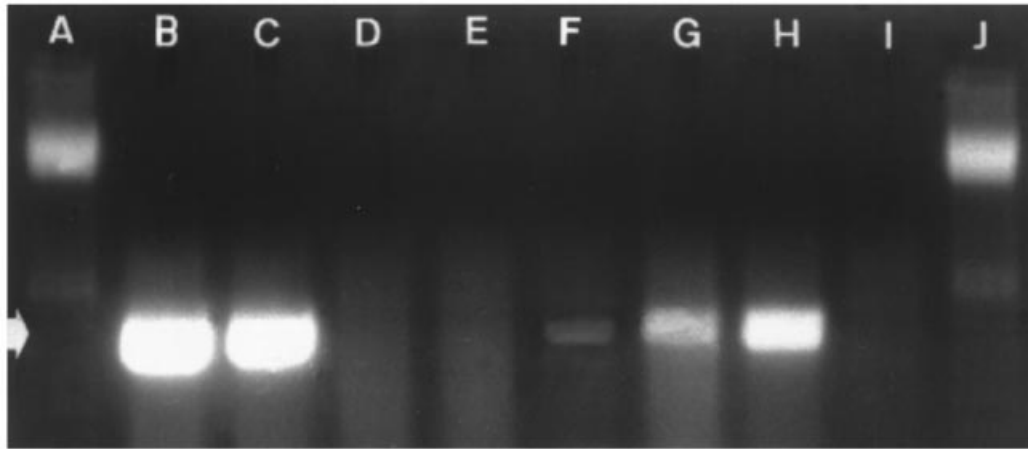


Fig. 4. Efficiency and sensitivity of the diagnostic test proposed here for the detection of *Perkinsus atlanticus* within the tissues of *Ruditapes decussatus*. Using the primers PK1 and PK2 described in the text and, as templates, samples of DNA extracted from different clams, we conclude that the presence of the parasite in infected clams can be detected by this PCR assay. The presence of the parasite is revealed by the resulting PCR product of 554 bp (arrow indicates that size). Lanes A and J, are molecular weight markers. The DNA samples in lanes B and C were the positive controls: genomic DNA from *P. atlanticus* and the cloned fragment of 730 bp from the ribosomal genes of these species. The DNA samples in lanes D and E were from uninfected clams. The DNA samples of the lanes F, G and H, were from infected clams. The lane I is a negative control. As can be seen, the quantity of the amplified product correlated with the infection level of the clams (levels 1, 3 and 5, respectively, of the Mackin's scale; see text).

Development of a molecular test for diagnosing P. atlanticus infections

We initially tried to use the primer pairs designed by Marsh *et al.* (1995) for the detection of *P. marinus*, to detect *P. atlanticus* infections. However, this primer set was not useful for the detection of *P. atlanticus* DNA in the DNA isolation from infected clams. In fact, these primers were not even effective for amplification of the expected PCR product when using the pure DNA of *P. atlanticus* as a template. Thus, we designed 2 primers (PK1 and PK2) for the amplification of a DNA fragment located within the spacer between 5S and 18S genes (positions 894 to 1446) of *P. atlanticus* (see Fig. 1). These primers amplify a PCR product of 554 bp when the DNA of the parasite is used as a template. The accuracy of these primers for the development of a diagnostic test was first confirmed by Southern-blot hybridizations. With the PCR product as a probe, filters containing DNA of different clam species demonstrated that the probe does not cross-hybridize with the *Dra*I digested DNA of uninfected clams (not shown). Consequently, we used the primers to test the presence of *P. atlanticus* in its molluscan host *R. decussatus*. These primers amplified the expected 554 bp product using as template the DNA obtained from infected clams, but not when DNA from uninfected clams was used (Fig. 4). The intensity of the amplified DNA in the gel (i.e. the quantity of amplified product) correlated with the level of infection of the cultures from which the clams were analysed. As can be seen in Fig. 4, highly infected clams (level 5 according to the Mackin scale; Mackin

(1962)) displayed the greater DNA band intensity on the gel, while that intensity gradually decreased as the level of infection decreased (levels 3 and 1, respectively, of the Mackin scale).

DISCUSSION

We have examined the organization of the ribosomal genes of *P. atlanticus* and have used the DNA sequence information obtained to make phylogenetic inferences within the group Alveolata. This sequence information has also been useful for the development of a diagnostic test based on amplification by the PCR technique, using primers which amplify a DNA fragment of 554 bp from the intergenic spacer of *P. atlanticus*.

In most eukaryotes, the nuclear rDNA genes are typically arranged as a 5'-28S-ETS-18S-ITS1-5.8S-ITS2-28S-3' transcription unit organized in tandem repeats separated by a non-transcribed spacer (NTS) region, NTS region plus the external transcribed spacer (ETS) regions forming the intergenic spacer (IGS). In most eukaryotes, 5S rDNA genes are found at another location on the nuclear genome. We determined that in the case of *P. atlanticus* the 5S ribosomal genes are located between the 28S and 18S ribosomal genes, closely linked to the 28S gene (238 bp between the two genes). This organization can be found in some fungi and algae species (reviewed by Kawai *et al.* 1995) as well as in some protozoa. Among this latter group, while some Apicomplexa such as *Toxoplasma* (Guay *et al.* 1992) present this organization of ribosomal DNA, others such as the Apicomplexa *Plasmodium* (Shippen-

Lentz & Vezza, 1988) and *Cryptosporidium* (Taghi-Kilani *et al.* 1994) or the ciliates *Euplotes* and *Tetrahymena* (Pederson *et al.* 1984) have genomes with the 5S rDNA unlinked to the other rRNA genes. With respect to the genus *Perkinsus*, Marsh *et al.* (1995) determined that *P. marinus* has the 5S rRNA genes linked to the 28S–18S genes. Here, we confirm with a second species, *P. atlanticus*, that in the genus *Perkinsus* the nuclear ribosomal loci are clustered together. Several data support the functionality of these 5S genes in *P. atlanticus*. First, the genome of *P. atlanticus* shows no evidence of unlinked 5S genes, as demonstrated by means of Southern-blot hybridization by the using of a 5S gene probe. Second, characteristic features of the gene such as the secondary structure of the deduced 5S rRNA and the presence of RNA polymerase III initiation and termination signals for transcription are conserved in *P. atlanticus*. However, we do not know whether the conserved motifs found in the *P. atlanticus* 5S rRNA encoding gene may function as transcriptional regulating signals for the RNA polymerase III, although the preservation of these sequence motifs supports such an interpretation.

Repetitive sequences evolve by concerted evolution; that is, sequence differences between repetitive units of a species are lower than differences between units of different species. Molecular drive forces (Dover, 1982), such as unequal crossing-over or gene conversion are responsible for intraspecific sequence homogenization and interspecific sequence divergence. Thus, consensus sequences for one repetitive family of sequences can be representative (i.e. diagnostic) of each species. As a locus composed of coding and non-coding sequences, regions of the ribosomal loci are subjected to different selective constraints. rDNA genes evolve at a low rate, while the intergenic spacers evolve at a faster rate. Because of its universal occurrence and conservative rate of evolution, ribosomal RNA appears to be one of the most useful molecules for the resolution of phylogenetic relationships at high hierarchical levels. To determine the phylogenetic position of *Perkinsus* among Alveolata, the sequence of the 5S rDNA gene of *P. atlanticus* was compared with the same sequences of other protozoan species in order to gain some phylogenetic clues to the taxonomical position of *Perkinsus*. After an initial inclusion of *Perkinsus* in different orders of fungi (Mackin, 1951; Ray, 1954; Perkins & Menzel, 1967), this genus has been securely included among the protozoan Alveolata. However, within Alveolata the placing of *Perkinsus* within the Apicomplexa (Cavalier-Smith, 1993; Ragan *et al.* 1996; Van de Peer *et al.* 1996) has been rebutted recently. Reece *et al.* (1997) and Siddall *et al.* (1997) have concluded that *Perkinsus* spp. are more closely related to Dinoflagellata, than to Apicomplexa. Therefore, species of *Perkinsus* could be included within Dinoflagellata, as suggested by

our data as well as those of other authors (Goggin & Barker, 1993; Reece *et al.* 1997; Siddall *et al.* 1997). Further support for this view is provided by the fact that we determined that the ribosomal genes of *P. atlanticus* comprise 5% of the genome. There is a general conclusion that Apicomplexa species have a low copy number of both 5S and ribosomal DNA (4–5 copies; Taghi-Kilani *et al.* 1994; Piper, Bankier & Dear, 1998). On the other hand, the phylogenetic trees based on the 5S sequences and in the 5S plus 18S 5' end sequences suggest the existence of 3 clades within Alveolata: Dinoflagellata, Apicomplexa and Ciliophora. This is consistent with the recently proposed view of Gajadhar *et al.* (1991) and Cavalier-Smith (1993).

Finally, we analysed and used the intergenic spacer (IGS) of the ribosomal genes. Among the eukaryotic sequences, repetitive DNAs such as satellite DNA or intergenic spacers of the ribosomal genes, because of their high rate of evolution, have proved to be valuable tools for disclosing phylogenetic relationships between closely related species (Pleyte, Duncan & Phillips, 1992; Bena *et al.* 1998; Garrido-Ramos *et al.* 1999), genetic identification of species (Garrido-Ramos *et al.* 1997) and molecular diagnosis (Meredith *et al.* 1991; Bachmann *et al.* 1993). We have characterized the region of the IGS of *P. atlanticus* with the aim to design a diagnostic test for this parasite. Our analysis reveals the absence of significant sequence conservation of this region with respect to the same region in other species. In fact, the data support the lack of sequence conservation between species of even the same genera such as *P. marinus* and *P. atlanticus*. Within the IGS, the most 3' sequences constitute the 5'-ETS of the ribosomal unit. The length of the entire ETS is highly variable among different species (Bena *et al.* 1998). This length is marked by the position occupied by the promoter flanking the RNA polymerase I transcription initiation site. According to Cordesse *et al.* (1993), the ETS preceding the 18S rDNA gene of *P. atlanticus* could have a length of approximately 350 bp, based on the position occupied by a motif (TATATGATA) which is conserved at the transcription initiation site. Within this region (ETS), we have detected the presence of a sequence highly conserved among different organisms (TGAGT-GTA) including fungi, plants and animals (Bena *et al.* 1998). Although the ETS of *P. atlanticus* has the eukaryotic ETS conserved motif described by Bena *et al.* (1998), the absence of sequence conservation of the rest of the ETS is characteristic with respect to known ETSs of other species, as revealed by a FASTA search in the GenBank database. In fact, this is the norm, as reflected by the fact that the ETS show a gradual decrease of nucleotide sequence conservation upstream from the 18S gene among different species even of the same genera (Kato *et al.* 1990; Tucci *et al.* 1994). Moreover, the same lack of

sequence conservation is expected for the NTS, as we find in this case for species of *Perkinsus*. Therefore, this region can be used as a molecular marker for species identification. Further, if the species is a disease-causing parasite, the marker is a good target for a molecular diagnosis (Bachmann *et al.* 1993).

We designed a pair of primers to be used in the PCR technique for the amplification of a fragment of 550 bp from the IGS of *P. atlanticus*. After demonstrating that the primers amplify this region using as template the DNA of *P. atlanticus*, we tested the usefulness of the primers to detect the presence of the parasite among tissues of infected clams belonging to the species *R. decussatus*. These primers amplified the expected 554 bp product when the DNA obtained from infected clams was used, but not when DNA from uninfected clams was used. The results demonstrate that the test is effective and also that the intensity of the product (i.e. quantity amplified) is proportional to the degree of infection of the clams used. In fact, the PCR diagnostic test detects levels of infection as low as the level 1 of the Mackin scale.

Therefore, we have developed a diagnostic test for this species that has proved to be fast, reliable, efficient and sensitive. This test could be used with different applications such as; a quantitative and non-destructive diagnostic method (haemolymph analysis); the selection of resistant individuals; studies on the progression of the illness; a quick and reliable detection method of the presence/absence of the parasite in total samples (stock control).

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