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Characterization of actin genes in Bonamia ostreae and their application to phylogeny of the Haplosporidia

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Abstract:

Bonamia ostreae is a protozoan parasite which infects the European flat oyster Ostrea edulis, causing systemic infections and resulting in massive mortalities in populations of this valuable bivalve species. In this work, we have characterized B. ostreae actin genes and used their sequences for a phylogenetic analysis. Design of different primer sets was necessary to amplify the central coding region of actin genes of B. ostreae. Characterisation of the sequences and their amplification in different samples demonstrated the presence of two intragenomic actin genes in B. ostreae, without any intron. The phylogenetic analysis placed B. ostreae in a clade with Minchinia tapetis, Minchinia teredinis and Haplosporidium costale as its closest relatives, and demonstrated that the paralogous actin genes found in Bonamia resulted from a duplication of the original actin gene after the Bonamia origin.

Keywords: Bonamia ostreae, Ostrea edulis parasite, actin gene, molecular phylogeny.

INTRODUCTION

Bonamia ostreae is a small-size (2-3 μm) protozoan parasite, responsible for a bonamiosis, an haemocytic disease affecting European flat oyster *Ostrea edulis*. In Europe, it was first described in France (Pichot *et al.*, 1980) as the causative agent of serious mortalities in this native species. Since then, it has been responsible for a drastic decrease in *O. edulis* production in different farming areas along the European Atlantic coast (see review in Carnegie and Cochennec-Laureau 2004). It has also been detected in *O. edulis* populations on the Pacific and Atlantic coasts of North America (Carnegie and Cochennec-Laureau 2004; Marty *et al.*, 2006). Other known and characterized species of the genus are *B. exitiosa* from *O. chilensis* in New Zealand and Chile, *O. angasi* in Australia, *O. puelchana* in Argentina, and *Crassostrea ariakensis* in North Carolina, USA (Campalans *et al.*, 2000; Hine *et al.*, 2001; Burreson *et al.* 2004; Kroeck and Montes, 2005; Corbeil *et al.* 2006); *B. roughleyi* (previously known as *Mikrocytos roughleyi*; Carnegie and Cochennec-Laureau, 2004) in *Saccostrea glomerata* in Australia (Farley *et al.*, 1988); and *B. perspora* in *Ostreola equestris* in North Carolina, USA (Carnegie *et al.* 2006).

Recent isolation of the gene coding for the small subunit of the ribosomal RNA in *Bonamia ostreae* (18S or SSU rDNA) has clarified phylogenetic affinities of *Bonamia* species. Thus, after different taxonomic affiliations, analysis of SSU rRNA placed *Bonamia* within the Haplosporidia (Carnegie *et al.*, 2000; Reece *et al.*, 2004). Genes coding for proteins provide the possibility of performing phylogenetic analyses under a different evolutionary rate compared to those governing ribosomal genes. In this respect, actin has the advantage of being an ubiquitous protein in eukaryotic cells and one of the most conserved throughout evolution, from yeast to human (Sheterline and Sparrow, 1994). Actin is a cytoskeletal protein involved in cellular functions like maintaining cell morphology, cell motility and division, and intracellular transport (Sheterline and Sparrow, 1994). Actin proteins are encoded by a multigene family in all organisms examined so far, except in yeast and in some alveolata where they are encoded by one single gene (Hightower and Meagher, 1986; Reece *et al.*, 1997; Zhou *et al.*, 2006). In other protozoans, like *Plasmodium falciparum, Entamoeba histolytica,* several species of foraminifera and the haplosporidians *Haplosporidium louisiana, Minchinia chitonis, Minchinia teredinis* and *Minchinia tapetis,* two to four actin genes have been described (Wesseling *et al.*, 1988; Huber *et al.*, 1987; Pawlowski *et al.*, 1999;

Reece *et al.*, 2004). The gene family differs in size among these organisms, and the number and location of introns within actin genes are variable (Hightower and Meagher, 1986; Reece *et al.*, 2004; Flakowski *et al.*, 2006).

This study reports, through the use of nucleotide and amino acid sequence analyses, the identification of at least two actin genes within *Bonamia ostreae*. Phylogenetic analyses were carried out to assess orthologous/paralogous relationships of the sequences and to infer the phylogenetic position of *B. ostreae* within the Haplosporidia based on the actin gene sequences.

MATERIALS AND METHODS

Samples and DNA extraction

Infected oysters (*Ostrea edulis*) were obtained by experimental infections. Healthy animals were injected with *Bonamia ostreae* cells previously purified (see below) from naturally infected oysters from Quiberon bay, Brittany (France) and maintained in a laboratory tank. This procedure allowed us to obtain highly infected oysters which were used for parasite purification. In parallel, non injected oysters were used as negative control. Infections were confirmed by microscopic examination of tissue imprints and by PCR assay based on the 18S rDNA gene of the parasite, using primers Bo and BoAs (Cochennec *et al.*, 2000).

Parasites were purified as described previously (Cochennec *et al*., 2000). About 50 x 10^6 cells could be obtained from each highly infected oyster. Cells were centrifuged at 12000 g and pellets from several purifications were pooled and conserved at –20ºC.

In addition, two samples (gill tissues fixed in 95% ethanol) of *O. chilensis* from New Zealand infected with *B. exitiosa* were used as controls.

Genomic DNA from purified parasites and oyster gills was extracted according to Winnepenninckx *et al*. (1993) using 2 x 10^8 parasite cells or 100 mg of oyster tissue. Integrity and quantity of DNA was measured by electrophoresis on agarose gel stained with ethidium bromide and spectrophotometry respectively.

Amplification of actin genes by PCR

Different samples and sets of degenerate primers (four forward primers were combined with five reverse ones) were used to amplify *Bonamia ostreae* actin gene fragments. Two of these primer pairs were used previously for the amplification of actin genes in dinoflagellata and haplosporidia species (Reece *et al.*, 1997; 2004), and in a rhizopod species (Longet *et al.*, 2004). The remaining primers tested have been designed for this work based on alignment of conserved regions of actin protein (GenBank accession numbers: AY450412, AY450407, AY450414, AY450416, AJ132374, AJ32375, J01016, D50839, AF057161, M86241, U84287, TGU10429, M19871, M19871). An available oyster actin sequence (AAB81845) was also included to prevent amplification of the host gene. DNA from a non-infected *Ostrea edulis* was used as control.

Amplification products were separated on 1% agarose gels stained with ethidium bromide. Testing of all primers combinations showed that one forward primer (F) and two reverse primers (R1 and R2) allowed amplification of two putative *B. ostreae* sequences (Fig. 1, panel A). The reaction was carried out in a volume of 50 μl with 2 mM Mg_2SO_4, 0.2 mM of each dNTP, 1 μM of each primer, 1.5 U of *Taq* polymerase (New England Biolabs) and 5-10 ng of DNA. Thermal cycling was 95 ºC for 1 min, 40 cycles of 95 ºC for 1 min, 50 ºC for 1 min and 65 ºC for 1 min, followed by 65 ºC for 10 min.

Amplified products were cloned using TOPO vector system (Invitrogen) and the nucleotide sequences were determined using an ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems) following the manufacturer’s recommendations. Sequences were identified by comparison with those included in the GenBank and EMBL databases using BLAST algorithm (Altschul *et al.*, 1997).

Primer pairs BostAct1F/BostAct1R and BostAct2F/BostAct2R were designed for independent amplification of Actin1 and Actin2 genes of *B. ostreae* based on the cloned sequences (Fig. 1, panel A). Actin genes sequences from *Ostrea edulis* and *Ostrea chilensis* obtained during this work (recorded in GenBank with accession numbers AM410916, AM410917 and AM410918) were aligned with *B. ostreae* sequences to ensure specificity of the primers for *B. ostreae* actins. DNA from a non-infected *Ostrea edulis* and DNA from an *O. chilensis* highly infected with *Bonamia exitiosa* were used as controls. Amplification reactions were performed as described previously but increasing annealing and extension temperatures from 50 to 55 ºC and 65 to 72 ºC respectively. Identification of each sequence type, Actin1 and Actin2, was done by digestion of PCR product (PCR-RFLP) with the restriction enzyme *Bst*UI (Promega).

The reactions consisted in 16 μl of PCR product, 2 μl 10x Buffer and 5 U of enzyme in a final volume of 20 μl.

Sequence and phylogenetic analyses

Phylogenetic analyses of the actin genes were performed using both nucleotide and amino acid sequences. Sequences used in the phylogenetic analyses from haplosporidian species were downloaded from GenBank (*Haplosporidium costale*, AY450407; *H. louisiana,* AY450411; *H. nelsoni,* AY450412; *Minchinia chitonis*, AY450414; *M. tapetis*, AY450417; *M. terenidis*, AY450420; *Urosporidium crescens*, AY450422). Sequences from foraminiferan species *Reticulomyxa filosa* (AJ132374), *Ammonia* sp. (AJ132372) and *Allogromia* sp. (AJ132370) and the Cercozoa *Cercomonas* sp. (AF363534) were chosen as outgroup according to the results obtained by Reece *et al.* (2004). Multiple sequence alignment was done using the program MEGALIN of the DNAstar package (LASERGEN) by Clustal method and revised by eye. Phylogenetic analyses were performed with the program PAUP* version 4.0b10 (Swofford, 2003) using the maximum parsimony criterion. Parsimony analysis involved heuristic searches. Data matrices were subjected to 1000 replicates of random sequence additions using tree bisection-reconnection branch-swapping. In the protein analysis the characters were considered as unordered states with equal weight, while in the nucleotide analysis the third nucleotide of each codon was downweighted. Gaps were treated as missing data and the ambiguous regions in the alignment were deleted from the analysis. The starting tree was obtained by stepwise addition. The characters were optimized by accelerated transformation. Finally, 1,000 bootstrap replicates (Felsenstein, 1985) with 10 heuristic searches were performed to assess internal support for nodes.

RESULTS

Sequence characterisation

Two major actin gene fragments, different in size, could be obtained by combining one forward with two reverse primers in different PCR reactions using DNA of purified *Bonamia ostreae*. They were 869 bp and 784 bp in length. The shorter one was similar to the larger one except on the 3’ end 85 nucleotides (Fig. 1, panel B). Comparison with sequences included in GenBank and EMBL data bases by BLAST allowed to identify partial actin DNA fragments, corresponding to central region of the gene. These sequences were named Actin1 (BoAct1, 869 bp) and Actin2 (BoAct2, 784 bp).

Three cloned sequences per actin sequence type were obtained (Fig. 2A). The GenBank Accession numbers are AM410919-AM410921 and AM410922-AM410924. Excluding primer sites, the sequence to be compared was 745 bp in length. Intra-type variability between clones was low, and estimated at five nucleotides between Actin1 sequences and one between Actin2 sequences (Fig. 2A). Three of the five sites corresponded to non-synonymous changes with respect to deduced Actin1 amino acid sequence (TCT coding S to CCT coding P, GAT coding D to GAA coding E, and ATG coding M to TTG coding L at positions 451, 474 and 700 respectively). The variable site of Actin2 partial sequences corresponded also to a non-synonymous change (ATC coding I to GTC coding V at position 403) (Fig. 2B). Nucleotide sequence type 1 and type 2 (Actin1 and Actin2) demonstrated 83.7% of identity. Variable positions were mainly located in the second half of the sequences (Fig. 2A). These variable positions (108 in total) usually corresponded to the third position of the codons (58) while 33 and 17 variable sites corresponded to the second and the first codon positions respectively. No gene intron was identified when sequences were aligned with homologous actin genes.

Encoded amino acid sequences of these two actin gene fragments (289 and 261 amino acid residues for Actin1 and Actin2 respectively) showed a full length open reading frame and demonstrated 87.2% of sequence identity. The first 121 amino acid residues were identical between the sequences and corresponded to identical nucleotide sequences (Fig. 2B).

In order to determine if sequence types, Actin1 and Actin2, corresponding to actin genes belong to *Bonamia ostreae* genome, a variable region of nucleotide sequences was used to design primers for amplification of Actin1 and Actin2 sequences in different isolates (BostAct1F/BostAct1R and BostAct2F/BostAct2R respectively, see Fig. 2A). Both primer pairs amplified the expected 264 bp product when DNA of the parasite purified from four different naturally infected oysters (*O. edulis*) was used as template. The different sequence types were determined by digestion of PCR products with the restriction enzyme *Bst*UI (PCR-RFLP). Actin2 sequences had no recognition site for this enzyme while Actin1 sequences contained two of them, yielding, after digestion of the 264 bp amplicon, three fragments of 13, 83 and 168 bp. Figure 3 shows differential band patterns obtained by PCR-RFLP. Both types of sequences could be easily identified in the four different DNAs from *B. ostreae* purified cells, while no amplification was obtained for oyster DNA either healthy *Ostrea edulis* or *O. chilensis* infected with *B. exitiosa*.

Phylogeny based on actin genes

Alignment of all 17 actin gene nucleotide sequences (all *Bonamia ostreae* actin gene sequences, seven of haplosporidian, and the four outgroup sequences) resulted in a 1113 bp matrix (after excluding ambiguous regions). Of the 1113 characters 416 were parsimony-informative (285 in the ingroup). Phylogenetic analysis based on nucleotide sequences produced three most-parsimonious trees of 3377 steps in length (CI: 0.585; RI: 0.595; HI: 0.415), which differ only in the relationships between the Actin2 sequences of *Bonamia ostreae*. Figure 4A shows the strict consensus tree which coincides with one of the most-parsimonious trees. The tree shows that *Bonamia ostreae* Actin1 and Actin2 were paralogous sequences to each other, and orthologous to the remaining haplosporidian actin gene sequences (Fig. 4A). *B. ostreae* actin genes clustered together with 100% bootstrap support (Fig. 4A), and this grouping remained when the multiple haplosporidian paralogous sequences described by Reece and co-workers (Reece *et al.*, 2004) were included in an overall analysis (data not shown). *B. ostreae* resulted a sister clade to that formed by *Minchinia tapetis* and *M. teredinis and Haplosporidium costale*. Both *Haplosporidium* and *Minchinia* genera resulted paraphyletic. *Urosporidum crecens* was the basal species of the haplosporidians.

Alignment of all 17 protein sequences resulted in a 370 amino acid matrix (excluding an ambiguous region), from which 84 were parsimony-informative (65 in the ingroup). Phylogenetic analysis using the protein sequences yielded four most-parsimonious trees of 329 steps in length (CI: 0.796; RI: 0.765; HI: 0.204), which differ only in the relationships between the Actin2 sequences of *B. ostreae,* and also in the relationships between *H. costale*, *M. tapetis* and *M. teredinis*. Strict consensus tree is shown in Figure 4B. The main difference of this tree with respect to the nucleotide tree (Fig. 4A) was the closer relationships of *B. ostreae* with *H. nelsoni* instead of the clade consisting of *M. tapetis, M. teredinis* and *H. costale*.

DISCUSSION

Two actin genes in the genome of Bonamia ostreae

Amplification by PCR using “universal” primers of unknown genes from non-cultivable eukaryotic parasites like *Bonamia ostreae* is a difficult aim. Conserved or “universal” primers are mostly designed from higher organism sequences and frequently amplified host DNA (always present in the samples) instead of parasite DNA. Combination of conserved primers and primers designed using sequences from related organisms, and the use of DNA from *Ostrea edulis* and from *O. chilensis* infected with *B. exitiosa* as separate controls, allowed us to amplify actin genes in *B. ostreae*. Characterization of amplified products allowed the identification of two sequence types encoding two proteins. Both types were identical at the amino terminal end. Variable positions were mainly located at third codon positions with decreasing proportions at second and first codon positions. Deduced amino acid sequences of Actin1 and Actin2 were about 13% divergent, a relatively high value for actin proteins encoded by genes from the same species (Hightower and Meagher, 1986). Nevertheless, higher divergence values could be observed between both actin paralogs described in species of the related foraminiferan phylum, 16% in *Reticulomyxa filosa*, 17% in *Ammonia* sp and 18% in *Allogromia* sp (personal obs. based on sequences analysed by Pawlowski *et al.*, 1999). In the apicomplexa *Plasmodium falciparum*, actin protein sequences showed 21% sequence divergence and were considered extremely divergent (Wesseling *et al.*, 1988).

All together these data suggested that two paralogous actin genes are present in the genome of *Bonamia ostreae*. The absence of amplification from non-infected oyster and a sister species (*B. exitiosa*) DNAs, the comparison with *O. edulis* sequences and the amplification by PCR of both sequence types in different *B. ostreae* samples from different origins demonstrated that both types of sequences corresponded to *B. ostreae* genes and not to actin genes from different species (the host, other parasites, algae, etc.), coding for one actin protein each. The two actin genes fragments of *Bonamia ostreae* reported in this study represent the first protein-encoding sequences obtained for any *Bonamia* species. Isolation and characterization of new genes or loci of its genome is of great interest in order to create a molecular data base for this parasite as exist for other protozoans useful for further research.

Actin Phylogeny

PCR amplification and sequencing analysis suggested that there were at least two actin genes in the genome of *Bonamia ostreae* and phylogenetic analysis supported this assumption. Both types of actin genes sequences obtained in this study (Actin1 and Actin2) grouped together on the basis of nucleotide and amino acid sequences when trees were inferred using orthologous sequences from species belonging to the genera *Haplosporidium, Minchinia* and *Urosporidium,* as well as when their paralogous sequences were included in the analysis. This result suggests that the two Actin1 and Actin2 nucleotide sequences are the result of a gene duplication event (resulting in two paralogous loci) in *B. ostreae* after differentiation from the common ancestor. Within haplosporidians, multiple paralogs are present in species of the genus *Minchinia*, while *Haplosporidium* genus includes species, like *H. nelsoni* and *H. costale* in which only a single type of actin gene has been amplified by PCR until now. Many of these sequences were found to contain introns, and genes with introns as well as single intronless actin genes were establish to be orthologous (Reece *et al.*, 2004). In *Bonamia ostreae* at least two paralogous actin genes without introns exist. Gain of introns in actin sequences throughout the evolution has been described in the group of foraminifera (Flakowski *et al.*, 2006), and this could be applied to the haplosporidian sequences to explain the existence of paralogous genes in some species containing different number of introns.

Actin phylogeny based on amino acid sequences placed *Haplosporidium nelsoni* as the closest species to *Bonamia ostreae*, in a sister clade to that formed by *Haplosporidium costale, Minchinia tapetis* and *Minchinia teredinis*. In the nucleotide based tree, this topology is reversed since *B. ostreae* is more closely related to the clade of *H. costale-M. tapetis* and *M. teredinis* than to *H. nelsoni*, although the bootstrap support was low. *Minchinia* and *Haplosporidium* resulted as paraphyletic genera in both analysis. Our results are similar to those previously obtained using actin gene sequences (Reece *et al.*, 2004). Phylogeny based on small subunit of ribosomal gene performed by these authors allowed to include more haplosporidian species in the analysis and therefore they described more precisely the ingroup relationships. The genus *Bonamia* showed closest relationship to the genus *Minchinia*, which resulted monophyletic on the basis of ribosomal gene sequence. Characterisation of actin genes in the remaining haplosporidian species and subsequent analysis may clarify the discrepancies between actin and 18S phylogenies concerning the relationships between *Bonamia, Minchinia and Haplosporidium* genera, and also provide a wider knowledge of relationships within the group.

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REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein data base search programs. *Nucleic Acids Research* **25**, 3389-3402. doi: 10.1093/nar/25.17.3389.

Burreson, E. M., Stokes, N. A., Carnegie, R. B. and Bishop, M. J. (2004) *Bonamia* sp. (Haplosporidia) found in nonnative oysters *Crassostrea ariakensis* in Bogue Sound, North Carolina. *Journal of Aquatic Animal Health* **16**, 1-9. doi: 10.1577/H03-008.1.

Campalans, M., Rojas, P. and González, M. (2000) Haemocytic parasitosis in the farmed oyster *Tiostrea chilensis*. *Bulletin of the European Association of Fish Pathologists* **20**, 31-33.

Carnegie, R. B., Barber, B. J., Culloty, S. C., Figueras, A. J. and Distel, D. L. (2000) Development of a PCR assay for detection of the oyster pathogen *Bonamia ostreae* and support for its inclusion in the Haplosporidia. *Diseases of Aquatic Organisms* **42**, 199-206.

Carnegie, R. B. and Cochennec-Laureau, N. (2004) Microcell parasites of oysters: Recent insights and future trends. *Aquatic Living Resources* **17**, 519-528. doi: 10.1051/alr:2004055.

Carnegie, R. B., Burreson, E. M., Hine, M., Stokes, N. A., Audemard, C., Bishop, M. J. and Peterson, C. H. (2006) *Bonamia perspora* n. sp. (Haplosporidia), a parasite of the oyster *Ostreola equestris*, is the first *Bonamia* species known to produce spores. *The Journal of Eukaryotic Microbiology* **53**, 232-245. doi: 10.1111/j.1550-7408.2006.00100.X.

Cochennec, N., Le Roux, F., Berthe, F. and Gerard, A. (2000) Detection of *Bonamia ostreae* based on small subunit ribosomal probe. *Journal of Invertebrate Pathology* **76**, 26-32. doi: 10.1006/jipa.2000.4939.

Corbeil, S., Arzul, I., Diggles, B., Heasman, M., Chollet, B., Berthe, F. C. J. and Crane, M. St. J. (2006) Development of a TaqMan PCR assay for the detection of *Bonamia* species. *Diseases of Aquatic Organisms* **71**, 75-80.

Farley, C. A., Wolf, P. H. and Elston, R. (1988) A long-term study of “microcell” disease in oysters with a description of a new genus, *Mikrocytos* (g. n.), and two new species, *Mikrocytos mackini* (sp. n.) and *Mikrocytos roughleyi* (sp. n.). *Fishery Bulletin* **86**, 581-593.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783-791.

Flakowski, J., Bolivar, I., Fahrni, J. and Pawlowski, J. (2006) Tempo and mode of spliceosomal intron evolution in actin of Foraminifera. *Journal of Molecular Evolution* **63**, 30-41. doi: 10.1007/s00239-005-0061-z.

Hightower, R. C. and Meagher, R. B. (1986) The molecular evolution of actin. *Genetics* **114**, 315-332.

Hine, P. M., Cochennec-Laureau, N. and Berthe, F. C. (2001) *Bonamia exitiosa* n. sp. (Haplosporidia) infecting flat oysters, *Ostrea chilensis*, in New Zealand. *Diseases of Aquatic Organisms* **47**, 63-72.

Huber, M., Garfinkel, L., Gitler, C., Mirelman, D., Revel, M. and Rozenblatt, S. (1987) *Entamoeba histolytica*: cloning and characterization of actin cDNA. *Molecular and Biochemical Parasitology* **24**, 227-235. doi: 10.1016/0166-6851(87)90154-X.

Kroeck, M. A. and Montes, J. (2005) Occurrence of the haemocyte parasite *Bonamia* sp. in flat oysters *Ostrea puelchana* farmed in San Antonio Bay (Argentina). *Diseases of Aquatic Organisms* **63**, 231-235.

Longet, D., Burki, F., Flakowski, J., Berney, C., Polet, S., Fahrni, J. and Pawlowski, J. (2004) Multigene evidence for close evolutionary relations between *Gromia* and Foraminifera. *Acta Protozoologica* **43**, 303-311.

Marty, G. D., Bower S. M., Clarke, K. R., Meyer, G., Lowe, G., Osborn, A. L., Chow, E. P., Hannah, H., Byrne, S., Sojonky, K. and Robinson, J. H. (2006) Histopatology and real time PCR assay for detection of *Bonamia ostrea* in *Ostrea edulis* cultured in western Canada. Aquaculture **261**, 33-42. doi: 10.1016/j.aquaculture.2006.07.024.

Pawlowski, J., Bolivar, I., Fahrni, J., de Vargas, C. and Bowser, S. S. (1999) Naked foraminiferans revealed. *Nature* 399, 27. doi: 10.1038/19881.

Pichot, Y., Comps, M., Tigé, G., Grizel, H. and Rabouin, M. A. (1980) Recherche sur *Bonamia ostreae* gen. n., sp. n., parasite nouveau de l'huître plate *Ostrea edulis* L. *Revue des Travaux de l'Institut des Pêches Maritimes* **43**, 131-140.

Reece, K., Siddall, M. E., Burreson, E. M., Graves, and J. E. (1997). Phylogenetic analysis of *Perkinsus* based on actin gene sequences. *Journal of Parasitology* **83**, 417-423.

Reece, K., Siddall, M. E., Stokes, N. A. and Burreson, E. M. (2004) Molecular phylogeny of the Haplosporidia based on two independent gene sequences. *Journal of Parasitology* **90**, 1111-1122. doi: 10.1645/GE-102R.

Sheterline, P. and Sparrow, J. C. (1994) Actin. *Protein profile* **1**, 1-121.

Swofford, D. L. (2003) PAUP*. Phylogenetic analysis using parsimony (*and other methods), version 4. Sinauer Associates, Sunderland, Massachusetts.

Wesseling, J. G., Smits, M. A. and Schoenmakers, J. G. G. (1988) Extremely diverged actin proteins in *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* **30**, 143-154. doi: 10.1016/0166-6851(88)90107-7.

Winnepenninckx, B., Backeljau, T. and de Wachter, R. (1993) Extraction of high molecular weigh DNA from molluscs. *Trends in Genetics* **9**, 407. doi: 10.1016/0168-9525(93)90102-N.

Zhou, J., Huang, B., Suzuki, H., Fujisaki, K., Igarashi, I. and Xuan, X. (2006) Isolation and identification of an actin gene from *Babesia gibsoni*. *Journal of Parasitology* **92**, 208-210. doi: 10.1645/GE-577R2.1.

Fig. 1. Primer description (A) and schematic representation of the genes that encode for actin in *Bonamia ostreae* (B). Arrows indicate approximate locations of the primers used for PCR amplifications. Primers BostAct1F/R and BostAct2F/R, designed after amplification of Actin1 with primers F/R1 and Actin 2 with primers F/R2, amplify a 264 bp fragment of Actin1 and Actin2 genes.

Fig. 2. Sequences alignment of *Bonamia ostreae* actin genes 1 and 2. (A) Nucleotide sequences corresponding to cloned actin sequences Type 1 (BostAct1) and Type 2 (BostAct2). Last numeric values indicate the recombinant *E. coli* clones. Dots (.) indicate identical bases. Locations of degenerate forward primer (position 1-17) and reverse ones (763-784 and 850-869) are underlined. Locations of specific primers for each actin gene type (BostAct1F and 2F, position 454-472, and BostAct1R and 2R, position 697-717) are highlighted in grey. Recognition sites for restriction enzyme *Bst*UI (CG^CG) in Type 1 sequences are showed in bold and underlined (cut sites: positions 537 and 705). (B) Inferred amino acid sequences corresponding to protein Actin 1 and Actin 2. Dots (.) indicate identical residue.

Fig. 3. Detection of a polymorphism involving *Bst*UI restriction sites in *Bonamia ostreae* actin genes sequences. DNA from four different *B. ostreae* samples (purified parasite) independently amplified with primers BostAct1F/R (Actin Type 1) and BostAct2F/R (Actin Type 2) yielded amplicons 264 bp in length. After digestion with *Bst*UI, two RFLP profiles were observed: a no-digested 264 bp band in Actin2 sequences (lanes 6 to 9) and bands of 168 + 83 bp for Actin1 sequences (lanes 2 to 5; the third expected band of 13 bp can not be observed in the gel). Lanes 1 and 10

correspond to the molecular weigth marker (Smart Ladder SF, Eurogentec). Negative controls were non-infected *Ostrea edulis* DNA (lane 11), DNA from *O. chilensis* highly infected with *B. exitiosa* (lane 12) and no-DNA water control (lane 13).

Fig. 4. Parsimony analysis based on actin gene sequences showing the paralogy of *Bonamia ostreae* sequences obtained in this study. Numbers at nodes indicate percentage support of 1000 bootstrap replicates (bootstrap support values below 50 are not presented). Phylogeny was performed using (A) nucleotide sequences and (B) translated amino acid sequences.

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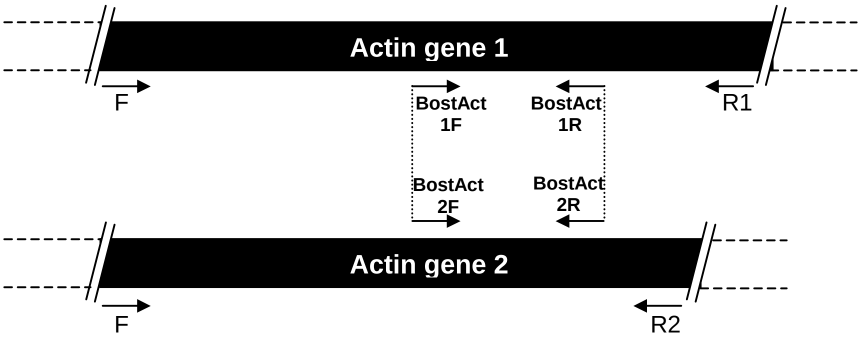
Bonamia ostreae actin gene

Fig. 1

(A)

Primer	*Sequence (5’-3’)*	*Reference*
F	AACTGGGAYGAYATGGA	Longet *et al.*, 2004
R1	GGWCCDGATTCATCRTAYTC	Longet *et al.*, 2004
R2	CNSWRTAYTTYCTYTCNGGN	Designed for this study
BostAct1F	GCTTCGACCGAAAGTTCCG	Designed for this study
BostAct1R	TTCAGACTGCACGCGCATATC	Designed for this study
BostAct2F	TCTTCCCAATCTTGTTCGA	Designed for this study
BostAct2R	CTCCTTCCGTAGACGTTCATT	Designed for this study

(B)



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(A)

```
BoAct1_1       AAC TGG GAT GAC ATG GAG AAG ATC TGG CAC CAC AGC TTC TTC AAC GAA CTG AGA GTC GCC   60
BoAct1_2       ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_26      ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_34      ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_45      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

BoAct1_1       CCC GAA GAG CAC CCC GTC CTC CTC ACC GAG GCA CCG CTC AAT CCC AAA GCC AAT CGC GAG  120
BoAct1_2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_26      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_34      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_45      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

BoAct1_1       AAG ATG ACT CAG ATC ATG TTC GAG ACC TTC AAC ACC CCC GCG ATG TAC GTC GCC ATC CAA  180
BoAct1_2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_26      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_34      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_45      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

BoAct1_1       GCG GTC CTC TCC CTC TAC GCG TCC GGA AGA ACC ACC GGA ATC GTC TTG GAC TCC GGA GAT  240
BoAct1_2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_26      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_34      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_45      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

BoAct1_1       GGC GTC AGC CAC ACC GTC CCG ATC TAC GAA GGA TAT GCC CTT CCT CAC GCC ATT CTG AGA  300
BoAct1_2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_26      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_34      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_45      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

BoAct1_1       TTG GAC ATC GCC GGA AGA GAT CTC ACC GAC TAC TTG ATC AAA ATC CTC ACC GAG CGC GGC  360
BoAct1_2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_26      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_34      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_45      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

BoAct1_1       CAC AGC TTC ACC ACC ACC GCC GAA CGC GAA ATC GTC CGC GAC ATC AAA GAA ACG CTT TGC  420
BoAct1_2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_26      ... .A. ... ... ... ... ... ... ... ... ... ... ... ... G.. ... ... ... ... ...
BoAct2_34      ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_45      ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

BoAct1_1       TAC TGC GCA AAG GAT TTC GAA GCA GAA ATG TCT GCT TCG ACT GAA AGT TCC GAA ATC GAC  480
BoAct1_2       ... ..T ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ..T ... ...
BoAct1_14      ... ..T ... ... ... ... ... ... ... ... C.. ... ... ..C ... ... ... ..T ... ...
BoAct2_26      ... ... ... .CT ... ... ... ... ... ... G.C T.. ..C CAA TCT T.. ..G A.G ... ..G
BoAct2_34      ... ... ... .CT ... ... ... ... ... ... G.C T.. ..C CAA TCT T.. ..G A.G ... ..G
BoAct2_45      ... ... ... .CT ... ... ... ... ... ... G.C T.. ..C CAA TCT T.. ..G A.G ... ..G

BoAct1_1       AAG TCG TAC GAA CTC CCC GAT GGA CAA ACT TTG ACC ATC GGA AAC GAG CGT TTC CGC GTC  540
BoAct1_2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_26      ... AAC ... ... ..T ..A ... ... ... ..A G.. ..G ... ..G ..T ..A ..C ..T ..A ..T
BoAct2_34      ... AAC ... ... ..T ..A ... ... ... ..A G.. ..G ... ..G ..T ..A ..C ..T ..A ..T
BoAct2_45      ... AAC ... ... ..T ..A ... ... ... ..A G.. ..G ... ..G ..T ..A ..C ..T ..A ..T

BoAct1_1       CCC GAA GTA CTG TTC AAT CCT TCT GCT ATC GGA AGC GAG TCA TCG GGA ATC CAC AAA TTG  600
BoAct1_2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_26      A.A ... ..T T.. ... C.G ... AG. TTG ... ... ..T ... ..T GAA ..G G.G ... ... C..
BoAct2_34      A.A ... ..T T.. ... C.G ... AG. TTG ... ... ..T ... ..T GAA ..G G.G ... ... C..
BoAct2_45      A.A ... ..T T.. ... C.G ... AG. TTG ... ... ..T ... ..T GAA ..G G.G ... ... C..

BoAct1_1       GCC CAC ACC TCG ATT ATG AAG TGC GAC ATT GAT ATC AGA AAA GAC CTC TTC GCC AAC ATC  660
BoAct1_2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_26      A.G TT. .AT ... ..A ... ..A ..T ... G.C ..C ... ..G ... ..T ..T .AT T.G ..T ..T
BoAct2_34      A.G TT. .AT ... ..A ... ..A ..T ... G.C ..C ... ..G ... ..T ..T .AT T.G ..T ..T
BoAct2_45      A.G TT. .AT ... ..A ... ..A ..T ... G.C ..C ... ..G ... ..T ..T .AT T.G ..T ..T

BoAct1_1       GTT TTG TCT GGA GGA TCC ACA ATG TTC CCC AAT ATC GAT TTG CGC GTG CAG TCT GAA ATT  720
BoAct1_2       ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ...
BoAct2_26      ..C A.. ..G ..C ... AG. ..G ... ... GAA C.. ... A.. GAA ..T C.A .G. AAG ..G ...
BoAct2_34      ..C A.. ..G ..C ... AG. ..G ... ... GAA C.. ... A.. GAA ..T C.A .G. AAG ..G ...
BoAct2_45      ..C A.. ..G ..C ... AG. ..G ... ... GAA C.. ... A.. GAA ..T C.A .G. AAG ..G ...

BoAct1_1       TCT TTG CTG GCT CCT TCT TCC ATC GAA GTC AAG GTG ATC TGT CCT CCT GAG CGC AAA TAT  780
BoAct1_2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
BoAct2_26      ..G A.. A.. ... ..A ... ..G ..A A.. A.. ..C ... ... GC. ..A ... ... A.A ... ..C
BoAct2_34      ..G A.. A.. ... ..A ... ..G ..A A.. A.. ..C ... ... GC. ... ..A ... A.G ... ..C
BoAct2_45      ..G A.. A.. ... ..A ... ..G ..A A.. A.. ..C ... ... GC. ..G ... ... A.A ... ..C

BoAct1_1       TCC GTT TGG ATC GGA GGA TCT ATT TTG TCA TCT CTG TCT ACG TTC CAG CAG ATG TGG ATC  840
BoAct1_2       ... ... ... ... ..G ... ... ... C.. ... ... ... ... ... ... ... ... ... ... ...
BoAct1_14      ... ... ... ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ... ... ...
BoAct2_26      A.. .-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
BoAct2_34      AGA .-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
BoAct2_45      A.. .-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

BoAct1_1       ACC AAA GAC GAG TAT GAT GAA TCT GGT CC                                            869
BoAct1_2       ... ... ... ..A ... ... ... ... ... ..
BoAct1_14      ... ... ... ..A ... ... ... ... ... ..
BoAct2_26      --- --- --- --- --- --- --- --- --- --
BoAct2_34      --- --- --- --- --- --- --- --- --- --
BoAct2_45      --- --- --- --- --- --- --- --- --- --
```

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Bonamia ostreae actin gene

Fig. 2B

(B)

```
Bonamia ostreae Actine1      NWDDMEKIWHHSFFNELRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNTPAMYVAIQ   60
Bonamia ostreae Actine2      ............................................................

Bonamia ostreae Actine1      AVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLDIAGRDLTDYLIKILTERG  120
Bonamia ostreae Actine2      ............................................................

Bonamia ostreae Actine1      HSFTTTAEREIVRDIKETLCYCAKDFEAEMSASTESSEIDKSYELPDGQTLTIGNERFRV  180
Bonamia ostreae Actine2      .N............I........T......AS.QSC.K.E.N........V.........

Bonamia ostreae Actine1      PEVLFNPSAIGSESSGIHKLAHTSIMKCDIDIRKDLFANIVLSGGSTMFPNIDLRVQSEI  240
Bonamia ostreae Actine2      T....Q..L.....E.V...TFN......V......YS...M.......EH.NE.LRK..

Bonamia ostreae Actine1      SLLAPSSIEVKVICPPERKYSVWIGGSILSSLSTFQQMWITKDEYDESG            289
Bonamia ostreae Actine2      .MM.....KIN..A......T----------------------------
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Bonamia ostreae actin gene

Fig. 3

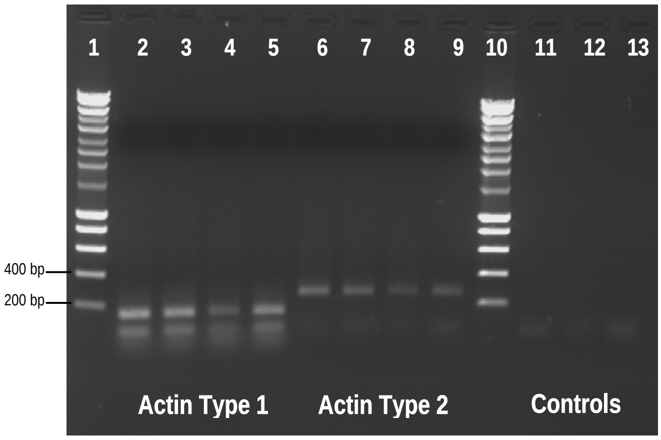

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Fig. 4