

# Whole-genome amplification: a useful approach to characterize new genes in unculturable protozoan parasites such as *Bonamia exitiosa*

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## SUMMARY

*Bonamia exitiosa* is an intracellular parasite (Haplosporidia) that has been associated with mass mortalities in oyster populations in the Southern hemisphere. This parasite was recently detected in the Northern hemisphere including Europe. Some representatives of the *Bonamia* genus have not been well categorized yet due to the lack of genomic information. In the present work, we have applied Whole-Genome Amplification (WGA) technique in order to characterize the actin gene in the unculturable protozoan *B. exitiosa*. This is the first protein coding gene described in this species. Molecular analysis revealed that *B. exitiosa* actin is more similar to *Bonamia ostreae* actin gene-1. Actin phylogeny placed the *Bonamia* sp. infected oysters in the same clade where the herein described *B. exitiosa* actin resolved, offering novel information about the classification of the genus. Our results showed that WGA methodology is a promising and valuable technique to be applied to unculturable protozoans whose genomic material is limited.

Key words: *Bonamia exitiosa*, *Bonamia ostreae*, actin, Haplosporidia, Whole-Genome Amplification.

## INTRODUCTION

The protozoan parasites of the genus *Bonamia* (Haplosporidia) are intra-haemocytic parasites of several oyster species mainly of the *Ostrea* genus. In Europe, *Bonamia ostreae* infects the native flat oyster *Ostrea edulis* causing mass mortalities in natural beds associated with important economic losses (Meuriot and Grizel, 1984). Other representative of the genus, *Bonamia exitiosa*, was firstly observed in several areas of the Southern hemisphere. In New Zealand, *B. exitiosa* was described to infect *Ostrea chilensis* haemocytes (Hine *et al.* 2001) and in Australia this parasite was detected in the oyster *Ostrea angasi* (Corbeil *et al.* 2006). *Bonamia exitiosa* has been described as the causative agent of devastated mortality events in these areas (Doonan *et al.* 1994; Cranfield *et al.* 2005). This parasite is included in the list of exotic notifiable diseases of the Directive 2006/088/EC and in the Aquatic Animal Health Code (OIE, 2013). However, the notification of *B. exitiosa* in *O. edulis* from Spain (Abollo *et al.* 2008) and its detection in *Crassostrea ariakensis* from North Carolina (Burreson *et al.* 2004) support the apparent worldwide distribution of this parasite.

*Bonamia exitiosa* has been observed infecting *Ostrea stentina* oysters from Tunisia (Hill *et al.* 2010; Carnegie *et al.*, 2014) and *O. edulis* from different European countries including Spain (Abollo *et al.* 2008, Carrasco *et al.* 2012), Italy (Narcisi *et al.* 2010), France (Arzul *et al.* 2010) and the UK (Lonshaw *et al.* 2013) questioning its impact on native flat oyster populations.

Some difficulties have been found in classifying some of the representatives of the *Bonamia* group. In some cases, the species affiliation remains still unresolved and the classification attains to genus level such as *Bonamia* spp. infecting *O. chilensis* from Chile (Campalans *et al.* 2000; Lohrmann *et al.* 2009).

Molecular information based on functional genes might offer very valuable information to clarify the taxonomic classification of the group. However these genes are scarce in protozoans. Recent advances in sequencing methodologies such as Next Generation Sequencing (NGS) have allowed the annotation of an important number of genes of the protozoan *Mikrocytos mackini* (Burki *et al.* 2013). The inclusion of these functional genes in phylogenomics analyses clarified the phylogenetic position of this organism among rhizarian. Regarding *B. ostreae*, two actin genes and the HSP90 gene are the unique functional genes described to date and also placed *Bonamia* representatives among haplosporidians (López-Flores *et al.* 2007, Prado-Alvarez *et al.* 2013). Actin genes are

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Table 1. Table showing the origin of the oyster samples analysed

Country	Location (Year of collection)	Specie	Provided by/reference
Turkey	Ayvalik (2009)	<i>Ostrea edulis</i>	Boronova Veterinary Control and Research Institute
United Kingdom	Cornwall (2011)	<i>Ostrea edulis</i>	English National Reference Laboratory
France	Thau (2008)	<i>Ostrea edulis</i>	French Research Institute for Exploitation of the sea
	Quiberon (2010)	<i>Ostrea edulis</i>	French Research Institute for Exploitation of the sea
	Bouin (2010)	<i>Ostrea edulis</i>	French Research Institute for Exploitation of the sea
	Corse (2009)	<i>Ostrea edulis</i>	French Research Institute for Exploitation of the sea
Italy	Pellestrina Island, Venize (2011)	<i>Ostrea edulis</i>	Italian National Reference Laboratory
Spain	Galicia, Ria de Arousa (2007)	<i>Ostrea edulis</i>	Spanish National Reference Laboratory
Tunisia	Monastir Bay (2009)	<i>Ostrea stentina</i>	Institut National Agronomique de Tunisie
Australia	Merimbula estuary, NSW (2002)	<i>Ostrea angasi</i>	The Commonwealth Scientific and Industrial Research Organization (Corbeil <i>et al.</i> 2006)
New Zealand	Southern Island (1999)	<i>Ostrea chilensis</i>	Investigation and Diagnostic Centre Biosecurity New Zealand
Chile	Chiloe Islands (2010)	<i>Ostrea chilensis</i>	Escuela de Ciencias del Mar, Catolic University Valparaiso

highly conserved, broadly distributed and are widely used in phylogenetic studies of protozoans (Leander and Keeling, 2004; López-Flores *et al.* 2007; Burki *et al.* 2010). However, the reconstruction of the evolution of haplosporidians has been a complicated task due to the lack of enough genomic information. The difficulty in culturing many of these organisms, such as *B. exitiosa*, hampers the obtaining of proper genomic material.

Amplification of actin gene was attempted on genomic material from *B. exitiosa* infected oysters and also from purified parasites without success. In the present work we applied Whole-Genome Amplification (WGA), a genomic approach especially effective when the amount of DNA is limited. The process consists of the amplification of the entire genome based on primer extension yielding a high quality DNA suitable for genotyping, hybridization, cloning and sequencing. This method allowed us to characterize the actin gene of *B. exitiosa* using for first time pure genomic material extracted from purified parasites. The phylogenetic position of *Bonamia* sp. infected oysters was also studied using the new actin gene described.

#### MATERIALS AND METHODS

##### *Bonamia exitiosa* purification

Heart imprints of *O. edulis* oysters collected from Corsica (France) in August of 2009 were visualized by light microscopy for *Bonamia* sp. detection. Following the criteria of Robert *et al.* (2009), the

most infected oysters were selected for parasite purification (Mialhe *et al.* 1988). *Bonamia exitiosa* infection was confirmed by histological analysis performed on infected oysters sections. The parasite was also sequenced in oysters found positive by restriction fragment length polymorphism (RFLP) analysis with *Hae* II and *Bgl*I on *O. edulis* genomic DNA. Parasites were counted on Malassez chamber. A total of  $7 \times 10^6$  parasites were obtained from four heavy infected oysters. Cells were centrifuged and saved frozen on 96% ethanol.

##### Gill tissues collection

Small pieces of gill tissues of *O. edulis* from Turkey, the UK, France, Italy and Spain; *O. stentina* from Tunisia, *O. angasi* from Australia and *O. chilensis* from New Zealand and Chile were analysed (Table 1) and maintained in 96% ethanol at 4 °C for further molecular analysis.

##### Genomic DNA extraction from gills and *Bonamia* sp. infection detection

Genomic DNA was extracted from gill tissue (25 mg) using the QIAamp DNA Mini Kit (Qiagen). DNA from gill tissues were adjusted to 100 ng  $\mu\text{L}^{-1}$  and used as a template to detect *Bonamia* sp. infection by polymerase chain reaction (PCR) using BO/BOAS primers according to Cochenne-Laureau *et al.* (2000). Posterior RFLP analysis with *Hae* II and *Bgl*I (Hine *et al.* 2001;

Table 2. List of primers used in the study

Primer	Sequence (5'–3')
BO (Cochennec-Laureau <i>et al.</i> 2000)	CATTTAATTGGTTCGGGCCGC
BOAS (Cochennec-Laureau <i>et al.</i> 2000)	CTGATCGTCTTCGATCCCC
Act-degF (Longet <i>et al.</i> 2004)	AACTGGAYGAYATGGA
Act-degR (Longet <i>et al.</i> 2004)	GGWCCDGATTTCATCRTAYTC
BeActI-F	TCCGGGACATCAAAGAAAAC
BeActI-R	ATCGAGTCGTACGCGAGTCT
TOPO-F	GACCATGATTACGCCAAGC
TOPO-R	CCCAGTCACGACGTTG

Cochennec-Laureau *et al.* 2003) were assayed on positive amplicons to confirm *B. ostreae* or *B. exitiosa* infection.

#### *Amplification of B. exitiosa genomic DNA using WGA method*

Genomic DNA was extracted from purified parasites ( $7 \times 10^6$  cells) using the QIAamp DNA Mini Kit (Qiagen). DNA was adjusted to  $10 \text{ ng } \mu\text{L}^{-1}$  and was amplified using the Illustra GenomePhi V2 Amplification Kit (GE Healthcare), a method based on isothermal strand displacement. Briefly, DNA (10 ng) and sample buffer containing random hexamers primers were heat-denatured at  $95^\circ\text{C}$  for 3 min and cooled on ice. A master-mix containing Phi29 DNA polymerase, additional random hexamers, nucleotides, salts and buffers were added to the mix and isothermal amplification was performed at  $30^\circ\text{C}$  for 1.5 h. After amplification, the enzyme was inactivated at  $65^\circ\text{C}$  for 10 min and the obtained DNA was cooled on ice and quantified.

#### *Actin gene extension from amplified genomic DNA from B. exitiosa*

Degenerate primers for actin amplification in Rhizopods (Longet *et al.* 2004) were tested in Whole-Genome amplified DNA of *B. exitiosa* purified cells (Table 2). The reaction was carried out in a volume of  $50 \mu\text{L}$  with 2 mM of each dNTP, 2.5 units of Taq polymerase (New England Biolabs) using 300 ng of amplified DNA as template. Thermal cycling was  $94^\circ\text{C}$  for 5 min, 40 cycles of  $94^\circ\text{C}$  for 1 min of denaturing,  $55^\circ\text{C}$  for 1 min of annealing and  $72^\circ\text{C}$  for 2 min of extension, followed by 10 min of final extension at  $72^\circ\text{C}$ . *Bonamia ostreae* DNA from infected oysters and distilled water were used as positive and negative controls, respectively.

#### *Amplification of actin gene from gills of Bonamia sp. infected oysters*

Specific primers (BeActI-F/BeActI-R, Table 2) for *B. exitiosa* actin amplification were designed in a

region with low similarity to *B. ostreae* actin sequences. These primers amplified a product of 220 pairs of bases and were used to detect *B. exitiosa* actin on genomic DNA from gills of *Bonamia* sp. infected oysters (Table 1). PCR reactions were performed in a volume of  $25 \mu\text{L}$  containing 2 mM nucleotides, 1.5 units of Taq polymerase (New England Biolabs) and 100 ng of genomic material. Thermal cycling was  $94^\circ\text{C}$  for 5 min, 30 cycles of  $94^\circ\text{C}$  for 1 min of denaturing,  $60^\circ\text{C}$  for 1 min of annealing and  $72^\circ\text{C}$  for 2 min of extension, followed by 10 min of final extension at  $72^\circ\text{C}$ .

#### *Cloning and sequence analysis*

PCR products obtained from the amplification of *B. exitiosa* actin in Whole-Genome amplified DNA from *B. exitiosa* (867 pb) and DNA from *Bonamia* sp. infected oysters (220 pb) were directly cloned and transformed on Top 10F competent bacteria using TOPO TA Cloning kit (Invitrogen). Clones of the expected size were selected and plasmid DNA were sequenced from both ends with TOPO-F and TOPO-R primers (Table 2) using the BigDye terminator Cycle Sequencing Ready Reaction Kit and a 3100 Avant Genetic analyser ABI Prism sequencer (Applied Biosystem). Raw chromatograms were analysed using Chromas 231 software (Technelysium). Sequence assembly, translation, multiple sequence alignment and searches of homology were performed using ExPaSy tools (<http://us.expasy.org/tools>), ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) and GenBank databases using Blast algorithm (<http://ncbi.nlm.nih.gov/blast/>).

#### *Phylogenetic analysis*

Nucleotide and amino acid sequences used to construct phylogenetic trees and pairwise analysis were downloaded from the GenBank database or were obtained from this study (Amino acid sequences: BoAct1-1 (CAL69233.1), BoAct1-2 (CAL69234.1), BoAct1-14 (CAL69228.1), BoAct2-26 (CAL69235.1), BoAct2-34 (CAL69230.1), BoAct2-45 (CAL69236.1); Nucleotide sequences: *B. ostreae* actin 1

(AM410919.1), *B. ostreae* actin 2 (AM410922.1), *Haplosporidium nelsoni* (AY450412.1), *Minchinia tapetis* (AY450418.1), *Minchinia terebinis* (AY450421.1), *Haplosporidium costale* (AY450407.1), *Minchinia terebinis* (AY450420.1), *Haplosporidium louisiana* (AY450409.1), *Minchinia chitonis* (AY450415.1), *Urosporidium crescens* (AY450422.1), *Allogromia* sp. actin 1 (AJ132370.1), *Reticulomyxa filosa* actin 1 (AJ132374.1), *Ammonia* sp. actin 1 (AJ132372.1), *Allogromia* sp. actin 2 (AJ132371.1), *Reticulomyxa filosa* actin 2 (AJ132375.1) and *Ammonia* sp. actin 2 (AJ132373.1). Multiple sequence alignments were performed using Clustal W (Thompson *et al.* 1997). Distance matrixes and phylogenetic trees were conducted using the Neighbour-Joining method under MEGA5 software (Tamura *et al.* 2011). Statistical confidence of the inferred phylogenetic relationships was assessed by bootstraps of 1000 and 10 000 replicates.

## RESULTS

### *Amplification of genomic DNA from B. exitiosa purified parasites by WGA*

An initial DNA extraction procedure on a sample of purified parasites containing  $7 \times 10^6$  cells yielded a genomic DNA sample with a concentration of  $0.143 \mu\text{g} \mu\text{L}^{-1}$  and 260/280 ratio of 2.14. A subsample of 10 ng of genomic DNA was amplified using the Illustra GenomiPhi V2 Amplification Kit (GE Healthcare) by the method of isothermal strand displacement. After 1.5 h of incubation, the Whole-Genome amplified DNA was more than 5-fold higher concentrated obtaining a final concentration of  $0.745 \mu\text{g} \mu\text{L}^{-1}$ . The obtained genomic DNA had proper quality with an A260/A280 ratio of 1.68 and a size higher than 50 kb with minimum smearing after verification on an agarose gel (0.6%).

### *Characterization of B. exitiosa actin gene*

Degenerate primers previously designed to amplify actin gene (Longet *et al.* 2004) were tested in Whole-Genome amplified DNA sample from *B. exitiosa* purified parasites (Table 2). The reaction yielded an amplification product of 867 nucleotides that encoded for a 289 amino acid sequence (Fig. 1). Four clones from one PCR product were forward and reverse sequenced and analysed. No introns were detected in the sequence. Among the three substitutions found in 1 of the 4 clones' analysis, one at position 62 corresponded to a non-synonymous change where the consensus codon GAA (Guanine-Adenine-Adenine) appeared replaced by GTA (Guanine-Thymine-Adenine) coding for Valine instead of Glutamic Acid. Maximum identity value, using Blast tools, reached 89% of homology with *B. ostreae* actin gene-1. The amplified fragment comprised a fraction of the characteristic actin domain including the

corresponding binding sites for ATP (Adenosine Triphosphate) (Fig. 1, in grey) and the barbed-end binding proteins gelsolin and profiling motives (Fig. 1, underlined). The new *B. exitiosa* actin gene was named BeAct and deposited on the GenBank database with the accession number KM073107.

Multiple alignment performed on BeAct amino acid sequence with *B. ostreae* actin sequences (BoAct1 and BoAct2) revealed the position of 211 conserved residues among the 7 actin sequences analysed (Fig. 2). The sequence was highly conserved up to residue 121. BeAct sequence had 26 distinctive positions (Fig. 2, underlined), 44 identities with BoAct1 and 6 identities with BoAct2 (Fig. 2, in grey). Compared with BoAct1, one deletion and one insertion were found in BeAct at position 1 and 290, respectively. Amino acid residue at position 158 showed identity only with one sequence of BoAct1.

Pairwise distance matrix between *B. exitiosa* and *B. ostreae* actin sequences were conducted using Maximum Composite Likelihood model (Table 3). The analysis involved 8 nucleotide sequences composing a final dataset of 614 positions. Results showed less evolutionary divergence between BeAct and BoAct1 (average distance of 0.153) than between BeAct and BoAct2 (average distance of 0.174). Evolutionary divergence among BeAct and each of BoAct sequences were higher than the distance between BoAct1 and BoAct2 (0.140). *Ostrea edulis* actin was also included in the analysis showing fewer differences (0.298) with *B. exitiosa* than with *B. ostreae* sequences (0.315 with BoAct1 and 0.325 with BoAct2).

### *Phylogenetic analyses based on B. exitiosa actin gene*

The Neighbour-Joining method was used to infer the phylogenetic relationship of *B. exitiosa* actin among haplosporidian representatives (Fig. 3). The analysis involved 17 nucleotide sequences considering representatives of the genus *Bonamia*, *Minchinia*, *Haplosporidium* and *Urosporidium*. *Allogromia* sp., *Reticulomyxa filosa* and *Ammonia* sp. were considered as outgroup. After removal of all ambiguous positions the final data set had a total of 427 informative positions. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches. *Bonamia exitiosa* actin grouped with *B. ostreae* actin gene-1 with a bootstrap value of 75%. *Haplosporidium nelsoni* resolved in the same clusters as *Bonamia* representatives. In sister clades grouped *Minchinia* and *Haplosporidium* representatives and *Urosporidium crescens*.

### *Molecular and phylogenetic characterization of actin sequences obtained from Bonamia sp. infected oysters*

Primers BeActI-F/BeActI-R (Table 2) were designed on *B. exitiosa* actin sequence to amplify

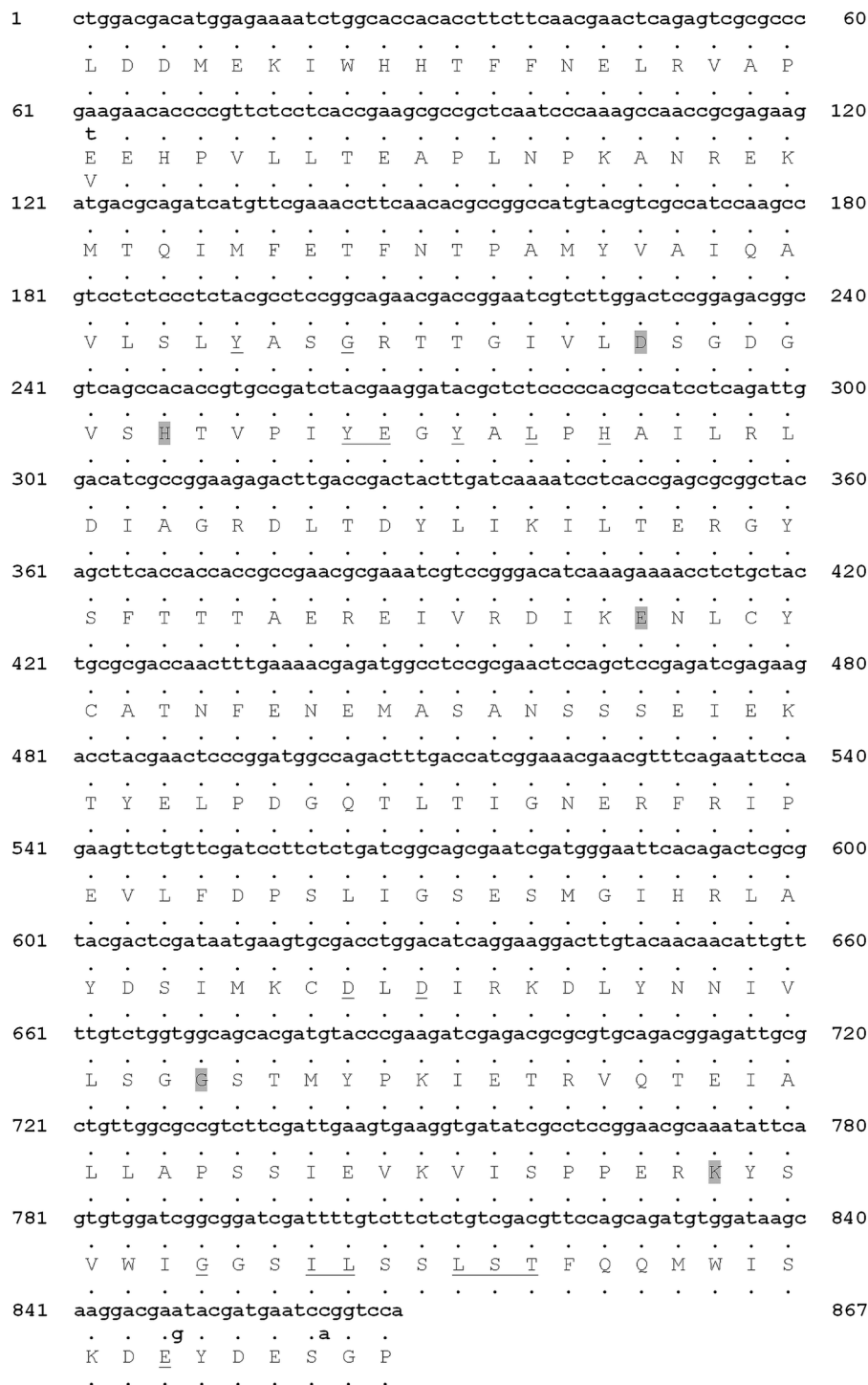


Fig. 1. Nucleotide sequence and deduced amino acid sequence of *Bonamia exitosa* actin (BeAct) Identical bases found in the alignment of the 4 clones are indicated with dots. Residues involved in ATP binding are indicated in grey. Residues involved in gelsolin and profiling recognition are underlined.

the most divergent region compared with *B. ostreae* actin genes. The specific primers yielded a product of 220 nucleotides in genomic DNA obtained from gill tissues of *Bonamia* sp. infected oysters. Several oyster species from different locations were analysed to amplify *B. exitosa* actin (Table 1). *Bonamia exitosa* infection was previously confirmed by studying the RFLP on PCR products obtained using BO-BOAS primers and sequencing (Cochennec *et al.*

2000; Hine *et al.* 2001). Table 4 summarizes the total number of actin sequences obtained after analysis of 40 different clones. A single actin sequence was obtained in *O. stentina* from Tunisia, *O. edulis* from Turkey, the UK, France-Atlantic coast and Italy and *O. chilensis* from Chile after the analysis of 2, 4, 3, 8, 3 and 1 clones, respectively. Two different actin sequences were obtained in *O. chilensis* from New Zealand and *O. edulis* from France-

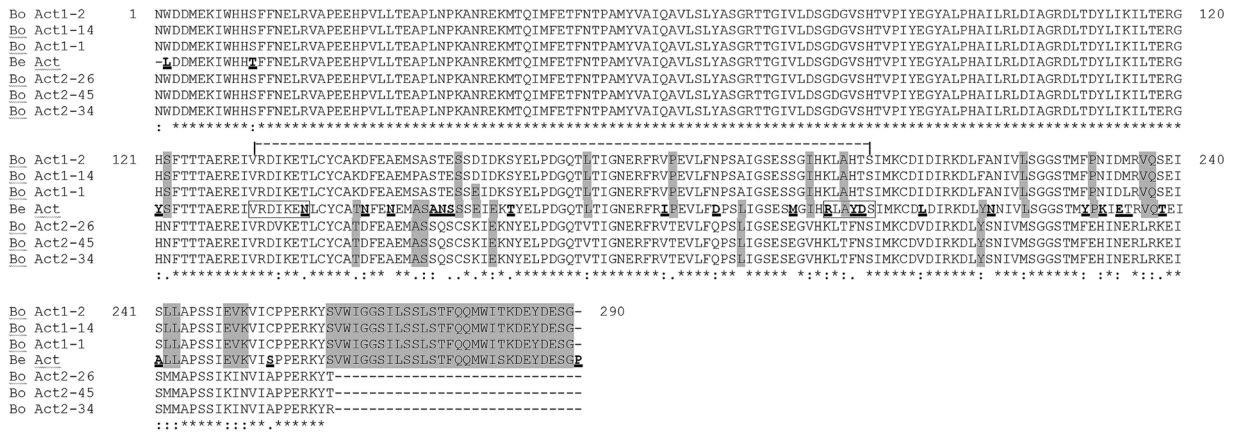


Fig. 2. Multiple alignment of actin amino acid sequence of *Bonamia exitiosa* with the two actin genes of *Bonamia ostreae*. Distinctive residues found in *Bonamia exitiosa* actin (BeAct) are underlined and the coincidences with each of *B. ostreae* actin genes are marked in grey. Squared amino acid show the location of the specific primers (BeActI-F and BeActI-R) and dot lines show the length of the amplified fragment.

Table 3. Pairwise distance between *Bonamia exitiosa*, *Bonamia ostreae* and *Ostrea edulis* actin sequences (Maximum Composite Likelihood model)

	BoAct1-1	BoAct1-2	BoAct1-14	BoAct2-28	BoAct2-34	BoAct2-45	OeAct
BoAct1-1							0.317
BoAct1-2	0.005						0.315
BoAct1-14	0.006	0.001					0.315
BoAct2-28	0.141	0.142	0.142				0.327
BoAct2-34	0.139	0.141	0.141	0.001			0.325
BoAct2-45	0.139	0.141	0.141	0.001	0.000		0.325
BeAct	0.154	0.152	0.152	0.175	0.173	0.173	0.298
	<b>0.153</b>			<b>0.174</b>			

Mediterranean coast in 5 and 6 clones, respectively. Three actin sequences were obtained in 3 clones of *O. angasi* samples from Australia and four different actin sequences were obtained in the analysis of 5 clones in one *O. edulis* sample from Spain. These sequences were deposited on the GenBank database (Table 4).

Multiple nucleotide alignment analysis including 18 different actin sequences obtained from *Bonamia* sp. infected oysters revealed 100% identity between the actin sequence from *B. exitiosa* purified parasites and sequences from *O. chilensis* from New Zealand (clone 1), *O. angasi* from Australia (clone 3), *O. edulis* from France (Atlantic and Mediterranean coasts, clone 2), the UK, Italy and Turkey and *O. stentina* from Tunisia (Fig. 4A). Among the 17 mismatches found in the alignment, 6 were specific to Chilean and Spanish clones. *Bonamia exitiosa* actin sequence from *O. chilensis* from New Zealand (clone 2) and *O. angasi* from Australia (clone 2) shared one different nucleotide compared with the other sequences. Remaining discrepancies were found in sequences from *O. angasi* oysters from Australia (clone 1) and *O. edulis* from France-Mediterranean coast (clone 1). These residues resulted in 12 non-synonymous positions in

the deduced amino acid sequences. Among them, 7 were observed in sequences obtained in *O. edulis* from Spain and *O. chilensis* from Chile.

The evolutionary relationship between actin sequences from different samples was inferred by the Neighbour-Joining method (Fig. 4B). The tree is drawn to scale according to the evolutionary distance (substitutions per site). The analysis involved 23 nucleotide sequences. The final dataset consisted in 217 positions after removing non-informative gaps and missing data. Actin sequences of infected *O. chilensis* from New Zealand, *O. angasi* from Australia and *O. edulis* from Turkey, Tunisia, France (Atlantic and Mediterranean coasts), the UK and Italy grouped with *B. exitiosa* actin sequence with a bootstrap value of 96%. The four different sequences obtained in infected *O. edulis* from Spain and *O. chilensis* from Chilean sample resolved in different branches close to the group containing *B. exitiosa* actin sequence from purified parasites. *Bonamia ostreae* actin sequences grouped in two different sister taxa into *Bonamia* genus clade, being *B. ostreae* actin gene-1 closer to *B. exitiosa* clade.

The pairwise distance of BeAct, BoAct1 and BoAct2 compared with sequences obtained in

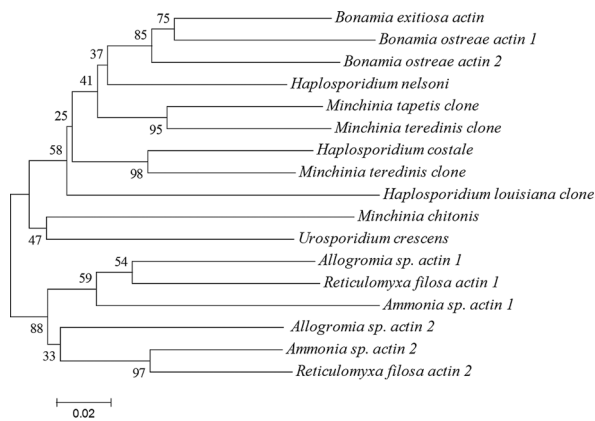


Fig. 3. Neighbour-Joining tree of the nucleotide sequences showing the phylogenetic relationships among actin sequences from haplosporidian representatives. Bootstrap of 10 000 repetitions.

infected oysters from different origins is shown in Table 5. No difference was observed among actin sequences obtained in *O. chilensis* from New Zealand (clone 1), *O. angasi* from Australia (clone 3) and *O. edulis* from Tunisia, Turkey, UK, Italy and France (Atlantic coast and Mediterranean coast clone 2). Low dissimilarity, up to 0.009, were obtained with actin sequences from infected *O. chilensis* from New Zealand (clone 2), *O. angasi* from Australia (clones 1 and 2) and *O. edulis* from France-Mediterranean coast (clone 1). The highest divergence was observed between BoAct and the Spanish and Chilean sequences. Overall divergence of BoAct2 was higher than BoAct1. Actin sequences obtained on infected *O. angasi* from Australia (clone 2) and those obtained on infected *O. edulis* from Spain (clones 1 and 4) were the most divergent to BoAct1 and BoAct2, respectively.

## DISCUSSION

*Bonamia exitiosa* was firstly detected in the Southern Hemisphere causing important mortalities in cultured oysters (Doonan *et al.* 1994; Cranfield *et al.* 2005). This parasite was recently detected in European waters however the magnitude of its effect and the reason of its presence in native oyster *O. edulis* are still unknown. Several difficulties have been found in the classification of some representatives of the genus *Bonamia* leading in some cases to unresolved categorizations. In this sense, beyond morphological descriptions through histological studies, the genomic characterization has become an indispensable tool to completely catalogue these species (Hill *et al.* 2010). Genomic information offers valuable data that allows homology searching in public databases and also inferring the phylogenetic evolution of a group. However, in some cases, as occurs in unculturable protozoans, the obtaining of genomic material becomes an arduous challenge.

In this study, we have amplified 6-folds the concentration of the initial genomic DNA obtained from *B. exitiosa* purified parasites using WGA by isothermal strand displacement with the GenomiPhi V2 DNA Amplification Kit. Among the methods of WGA described to date, the multiple displacement amplification technique was described to be better in genome coverage (Spits *et al.* 2006; Nelson 2014). Moreover, the GenomiPhi V2 DNA Amplification Kit had higher success rate and higher concentration of high molecular weight DNA compared with similar methods (Bouazid *et al.* 2010). WGA has been recently applied in human pathogens obtaining satisfactory results (Carret *et al.* 2005; Morrison *et al.* 2007; McLean *et al.* 2013; Seth-Smith *et al.* 2013). However, to our knowledge this is the first time that this method was tested in a marine mollusc parasite. Using the amplified genomic material as a template and the degenerate primers previously designed by Longet *et al.* (2004) we succeeded in the amplification of actin in *B. exitiosa*. This achievement might be related to the use of purified and uncontaminated cells. Since the amplification is unspecific, high relative concentration of exogenous DNA, such as bacteria or host cells in our case, could favour the generation of undesirable products (Pan *et al.* 2008).

We obtained a fragment of 867 nucleotides that corresponded to the characteristic actin domain found in actin-related proteins (Schutt *et al.* 1993; Sheterline *et al.* 1995). Conserved binding sites were found in the amplified fragment including ATP binding sites and gelsolin and profilin binding sites involved in capping the barbed end of actin polymers and their regulation (Korn *et al.* 1987; Schafer and Cooper, 1995).

Actin is generally encoded by a multi-gene family in Eukaryotes (Fairbrother *et al.* 1998; Fyrberg *et al.* 1980; Schwartz and Rotblum, 1981). The number of actin genes is highly variable in protozoan and increases in multicellular organisms (Sehring *et al.* 2007). Multiple paralogous genes were characterized in several haplosporidians including *H. louisiana*, *M. chitonis*, *M. teredinis* and *M. tapetis* (Reece *et al.* 2004). However, other representatives of the group such as *H. costale* and *H. nelsoni* as well as *U. crescens* have a single actin gene described to date. Regarding *Bonamia* genus, two actin genes were described in *B. ostreae* (López-Flores *et al.* 2007) and sequence analysis presented herein might suggest that *B. exitiosa* possesses one actin gene. However further works are required to conclude about the number of actin genes present in *B. exitiosa* genome.

High variability was found regarding the number of introns in actin genes of Haplosporidia (Reece *et al.* 2004). *Bonamia exitiosa*, together with *H. nelsoni* and *U. crescens*, could be included in the group of species with an unique actin gene and not

Table 4. Summary of clones analysed and the actin sequences per oyster sample with the corresponding accession number

Location	<i>Ostrea</i> sp.	Samples	Clones	Sequences	Nomenclature	Accession Number
Tunisia (TN)	<i>O. stentina</i>	2	2	1	Be_ <i>O. stentina</i> _TN	KM073090
Turkey (TR)	<i>O. edulis</i>	2	4	1	Be_ <i>O. edulis</i> _TR	KM073091
United Kingdom (UK)	<i>O. edulis</i>	1	3	1	Be_ <i>O. edulis</i> _UK	KM073092
France Atlantic coast (FR-Atl)	<i>O. edulis</i>	3	8	1	Be_ <i>O. edulis</i> _FR-Atl	KM073093
Italy (IT)	<i>O. edulis</i>	1	3	1	Be_ <i>O. edulis</i> _IT	KM073094
Chile (CL)	<i>O. chilensis</i>	1	1	1	Bsp_ <i>O. chilensis</i> _CL	KM073095
New Zealand (NZ)	<i>O. chilensis</i>	2	5	2	Be_ <i>O. chilensis</i> _NZ_clone1	KM073096
					Be_ <i>O. chilensis</i> _NZ_clone2	KM073097
France Mediterranean coast (FR-Med)	<i>O. edulis</i>	2	6	2	Be_ <i>O. edulis</i> _FR-Med_clone1	KM073098
					Be_ <i>O. edulis</i> _FR-Med_clone2	KM073099
Australia (AU)	<i>O. angasi</i>	2	3	3	Be_ <i>O. angasi</i> _AU_clone1	KM073100
					Be_ <i>O. angasi</i> _AU_clone2	KM073101
					Be_ <i>O. angasi</i> _AU_clone3	KM073102
Spain (SP)	<i>O. edulis</i>	1	5	4	Be_ <i>O. edulis</i> _SP_clone1	KM073103
					Be_ <i>O. edulis</i> _SP_clone2	KM073104
					Be_ <i>O. edulis</i> _SP_clone3	KM073105
					Be_ <i>O. edulis</i> _SP_clone4	KM073106

introns. Intronless sequences were described to be orthologous (Reece *et al.* 2004). It was hypothesized that the increase in the number of introns could explain the emergence of multiple genes in haplosporidians (López-Flores *et al.* 2007).

Comparison of amino acid sequence showed that *B. exitiosa* actin was more similar to *B. ostreae* actin gene-1. Both sequences shared 21% of the amino acid residues and also the 3' end of the fragment amplified by the degenerate primers. The evolutionary divergence between sequences estimated by pairwise distance revealed that the number of base substitutions per site among sequences was lower between *B. exitiosa* actin and *B. ostreae* actin gene-1 than between *B. exitiosa* actin and *B. ostreae* actin gene-2, confirming results obtained by multiple alignment analysis. The actin sequence of the host, *O. edulis*, was also included in the analysis and confirmed that the sequence that we obtained did not correspond to the host. The divergence between the deduced amino acid sequences of *B. exitiosa* actin and *B. ostreae* actin gene-1 was lower than the divergence observed between *B. ostreae* actin genes (López-Flores *et al.* 2007). Divergence between species and within species can be similar at protein level, as was previously described in Dinoflagellates (Kim *et al.* 2011). The percentage of amino acid divergence between actin isoforms in protozoans was described to be very variable reaching 16–18% in foraminiferans and 40% in the ciliate *Paramecium tetraurelia* (Wesseling *et al.* 1988; Keeling 2001; López-Flores *et al.* 2007; Sehring *et al.* 2007).

*Bonamia* genus was described to belong to haplosporidian group among Cercozoa (Carnegie *et al.* 2000; Cochenec-Laureau *et al.* 2000; Cavalier-Smith 2002; Cavalier-Smith and Chao, 2003; Prado-Alvarez *et al.* 2013). The phylogenetic relationship among haplosporidians has been previously ascertained using ribosomal DNA and actin genes (Carnegie *et al.* 2000, 2006; Reece *et al.* 2004; López-Flores *et al.* 2007; Carrasco *et al.* 2012). Regarding *Bonamia* representatives, phylogenetic analysis based on ribosomal DNA placed *B. exitiosa* and *B. roughleyi* (*B. roughleyi nomen dubitum*, Carnegie *et al.* 2014) in a sister group to *B. ostreae* and *B. perspora* (Cochenec-Laureau *et al.* 2003; Carnegie *et al.* 2006; Abollo *et al.* 2008). The topology of our phylogenetic tree based on actin sequences including the novel *B. exitiosa* actin was in concordance with these previous analyses. *Bonamia exitiosa* grouped within the clade of *Bonamia* genus closer to *B. ostreae* actin gene-1 and in a sister branch to *B. ostreae* actin gene-2 suggesting that *B. exitiosa* have evolved after the differentiation of *B. ostreae* actin paralogs.

The actin sequence obtained in purified *B. exitiosa* parasites perfectly aligned with the currently considered *B. exitiosa* infected samples from New Zealand,



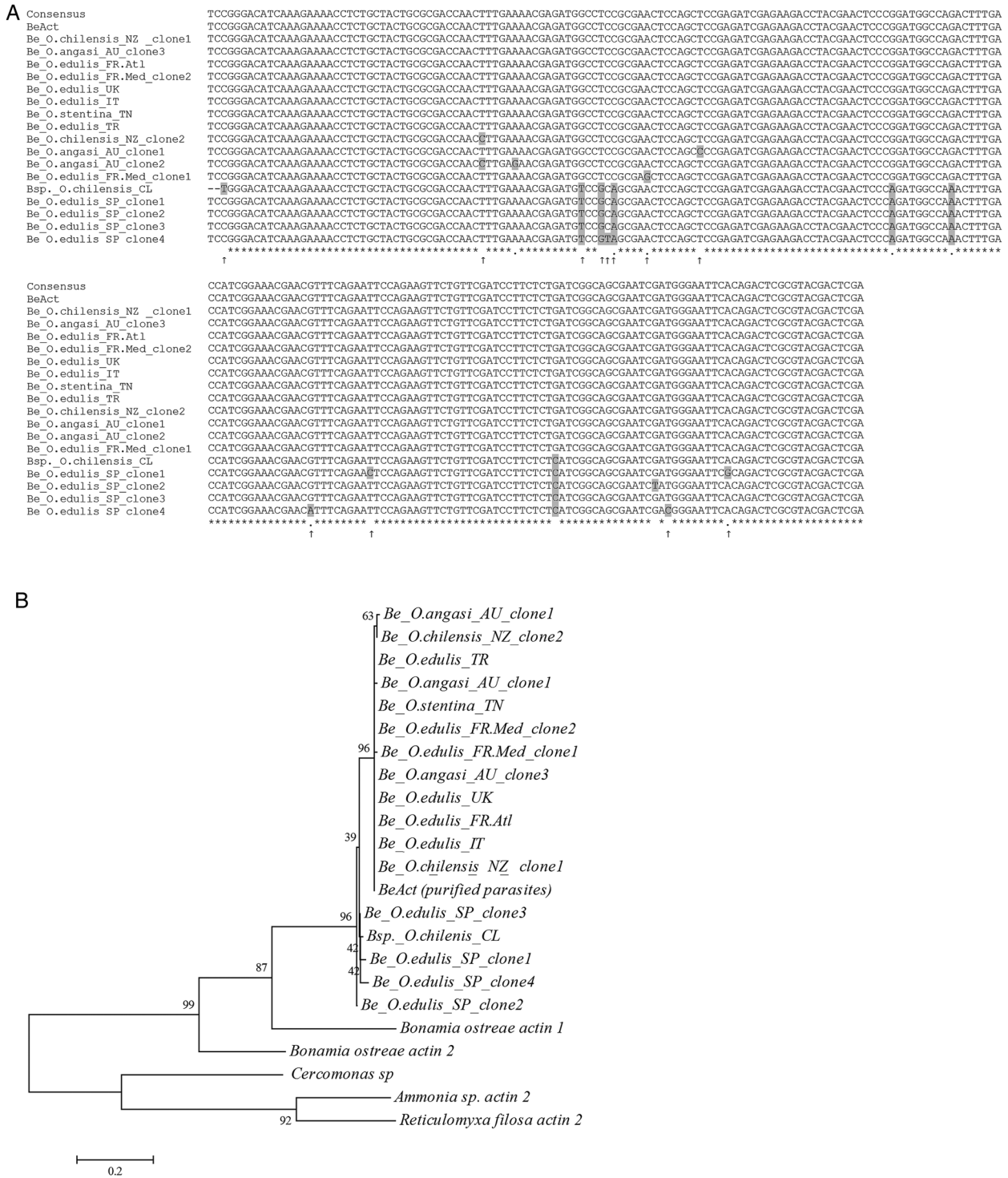


Fig. 4. (A) Multiple alignment of actin nucleotide sequence obtained in *Bonamia* sp. infected oysters including *Bonamia exitosa* actin sequence obtained from purified parasites. Distinctive residues compared with the consensus sequence are marked in grey and non-synonymous changes with arrows. (B) Phylogenetic tree between nucleotide actin sequences obtained in *Bonamia* sp. infected oysters and *B. ostreae* actin genes and other haplosporidian representatives inferred by Neighbour-Joining. Bootstrap of 1000 repetitions.

Australia, the UK, Italy, France-Mediterranean coast and Tunisia (Hine *et al.* 2001; Corbeil *et al.* 2006; Arzul *et al.* 2011). Sequences obtained in *Bonamia* sp. infected oysters from France-Atlantic coast and Turkey were also included in the same

group, concluding that these uncategorized parasites might be indeed considered *B. exitosa*.

The phylogenetic study based on actin sequences supported the inclusion of all the samples in a sister clade to *B. ostreae* sequences with a strong

Table 5. Pairwise distance between *Bonamia exitiosa* actin (BeAct), *Bonamia ostreae* actin gene-1 (BoAct1) and *B. ostreae* actin gene-2 (BoAct2) and actin sequences obtained in *Bonamia* sp. infected oysters from different geographical origin (Maximum Composite Likelihood model)

	Geographical origin	BeAct	BoAct1	BoAct2
Be_ <i>O.chilensis</i> _NZ_clone1	New Zealand	0.000	0.258	0.281
Be_ <i>O.angasi</i> _AU_clone3	Australia	0.000	0.258	0.281
Be_ <i>O.edulis</i> _FR.Atl	France-Atlantic Coast	0.000	0.258	0.281
Be_ <i>O.edulis</i> _FR.Med_clone2	France-Mediterranean Coast	0.000	0.258	0.281
Be_ <i>O.edulis</i> _UK	United Kingdom	0.000	0.258	0.281
Be_ <i>O.edulis</i> _IT	Italy	0.000	0.258	0.281
Be_ <i>O.edulis</i> _TR	Turkey	0.000	0.258	0.281
Be_ <i>O.stentina</i> _TN	Tunisia	0.000	0.258	0.281
Be_ <i>O.chilensis</i> _NZ_clone2	New Zealand	0.005	0.263	0.286
Be_ <i>O.angasi</i> _AU_clone1	Australia	0.005	0.263	0.286
Be_ <i>O.edulis</i> _FR.Med_clone1	France-Mediterranean Coast	0.005	0.258	0.286
Be_ <i>O.angasi</i> _AU_clone2	Australia	0.009	0.267	0.290
Be_ <i>O.edulis</i> _SP_clone3	Spain	0.029	0.244	0.286
Be_ <i>O.edulis</i> _SP_clone2	Spain	0.034	0.244	0.281
Bsp_ <i>O.chilensis</i> _CL	Chile	0.034	0.249	0.290
Be_ <i>O.edulis</i> _SP_clone1	Spain	0.039	0.253	0.295
Be_ <i>O.edulis</i> _SP_clone4	Spain	0.044	0.249	0.295

bootstrap support. Molecular analysis comparisons based on nucleotide alignment and pairwise distances among actin sequences revealed that samples from *O. chilensis* from Chile and *O. edulis* from Spain were the most divergent compared with *B. exitiosa* actin sequence. These samples shared 6 of the 17 mismatches found in the multiple alignment and resolved in separated branches within the strongly supported cluster where actin sequence from *B. exitiosa* purified parasites was included. Previous phylogenetic studies based on SSU rDNA placed the Chilean and the Spanish samples in the same group as *B. exitiosa* (Hill *et al.* 2010; Carrasco *et al.* 2012). However, the analysis of ITS1-5, 8-ITS2 sequences placed the Chilean clone in a separate branch inside the group of *Bonamia* sp. representatives (Hill *et al.* 2010). Our phylogenetic analyses based on the actin gene supported this topology, suggesting a closer relationship between Chilean and Spanish samples. Based on our analysis, we do not have enough support to conclude that Chilean samples were infected with *B. exitiosa*. Therefore, the affiliation of this sample remains uncertain and further investigation would be necessary to clarify its position among *Bonamia* representatives.

The NGS methods have recently been applied to the protozoan *M. mackini* obtaining novel information that clarified the phylogenetic position of this organism (Burki *et al.* 2013). However, the impossibility to obtain proper genomic material could limit the use and potential of the NGS in some organisms such as unculturable protozoans. In this sense, the WGA method allows the obtaining of reliable NGS results from limited starting material, reducing also the percentage of undesirable products and enhancing the efficiency of the technique. Some technologies combining both methods have

been applied to Eukaryotes and Prokaryotes (Pamp *et al.* 2012; Young *et al.* 2012; Korfhage *et al.* 2013). Data presented in this work demonstrated the successful use of the WGA technique in the unculturable protozoa *B. exitiosa* which might facilitate the application of new promising methodologies in these organisms.

Using Whole-Genome amplified genomic DNA we characterized for first time the actin gene on *B. exitiosa*, increasing the genomic data available in this specie. These new data allow us examining the phylogenetic affiliation of *Bonamia* sp. representatives and clarified their uncertain classification. To our knowledge this is the first time that WGA methodology is applied in a haplosporidian. This technique might also allow the developing of necessary and specific diagnostic tools to discriminate between *B. ostreae* and *B. exitiosa* parasites.

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