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Molecular characterisation of an Australian isolate of *Bonamia exitiosa*

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ABSTRACT: An Australian (New South Wales) isolate of *Bonamia* was characterised at the molecular level by sequencing the 18S-ITS-1 region of the small subunit rRNA operon obtained from flat oysters *Ostrea angasi* shown to be infected by histological examination. Sequence data alignment with homologous genes from 2 other isolates of *Bonamia* (New Zealand and France) revealed high levels of nucleotide identity with both isolates. However, the Australian *Bonamia* is shown to be more closely related to the New Zealand isolate, suggesting the existence of an oceanic subgroup of *Bonamia*.

KEY WORDS: *Bonamia* species · *Ostrea angasi* · Flat oyster · Small subunit rRNA operon

INTRODUCTION

Translocation of live molluscs is generally recognised as a major underlying cause of the spread of molluscan pathogens and diseases (Berthe et al. 1999). Bonamiosis, an infectious disease caused by haplosporidian parasites of the genus *Bonamia*, is one such example; in the northern hemisphere, the disease caused by *B. ostreae* is believed to have been introduced into Europe via European flat oysters *Ostrea edulis* imported from California, USA (Cigarría & Elston 1997, Grizel 1997). These studies were based on available documentation of trade and transfer of oyster spat between California and Europe. Subsequent molecular characterisation and sequencing of the small subunit (ssu) rRNA operon of *Bonamia* species confirmed the broad northern hemisphere distribution of *B. ostreae* (Carnegie et al. 2000, Cochennec et al. 2000). Conversely, since its redesignation as *B. exitiosa* (Hine et al. 2001, Berthe & Hine 2003), the exact distribution and geographical range of this antipodean *Bonamia* is unclear (Hine 1996, Campalans et al. 2000, Burreson et al. 2004, Kroeck & Montes 2005), but this information is of central importance to better address management of disease caused by this serious pathogen (OIE 2003). Sequence comparison of the 18S gene and Internal Transcribed Spacer 1 (ITS-1) region of the ssu rRNA operon has helped clarify the taxonomic position of these intra-haemocytic parasites and has led to the development of diagnostic methods capable of detecting and distinguishing different isolates — resources essential for the development of health surveillance, disease monitoring and oyster stock translocation programmes (Carnegie & Cochennec 2004).

A parasite belonging to the genus *Bonamia* was first reported in Australian flat oysters *Ostrea angasi* from Port Phillip Bay (Victoria), Georges Bay (Tasmania) and Albany (Western Australia) in the early 1990s (Hine & Jones 1994), with no further certitude regarding species affiliation. Specification of *Bonamia* sp. is important in order to include or exclude *B. exitiosa*, as well as exclude *Mikrocytos roughleyi*, which was redescribed recently as a *Bonamia* bona fide (Cochen-

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nec-Laureau et al. 2003). A recent epidemiological study of bonamiosis carried out in southern New South Wales (NSW), Australia (Heasman et al. 2004), has allowed us to identify and characterise a *Bonamia* isolate at the molecular level and to establish its relatedness to other described *Bonamia* species.

MATERIALS AND METHODS

Australian isolate of *Bonamia* sp. Ethanol (70%)-fixed, *Bonamia*-infected flat oysters *Ostrea angasi*, collected from the Merimbula estuary in NSW (during 2003), were provided by Dr. Symon Dworjanyn (NSW Fisheries, Port Stephens) (Heasman et al. 2004). A total of 200 oysters, ranging from 80 to 120 mm in diameter, were sampled from the site, and histological sections were examined by light microscopy. Of several samples confirmed positive, 3 were used for sequencing analysis.

DNA extraction. Oyster tissues (mantle and gills) were rinsed in phosphate-buffered saline (PBS; pH 7.4), and then DNA was extracted using a QiaAmp DNA minikit (Qiagen) according to the manufacturer's instructions. DNA was eluted and resuspended in a final volume of 100 µl sterile deionised water.

Table 1. Primer sequences used for amplifying and sequencing *Bonamia* rDNA 18S-ITS-1 gene sequences. The positions of the primer sequences correspond to the Australian *Bonamia* sp. 18S-ITS-1 rRNA sequence deposited in GenBank (Accession Number DQ312295)

Name	Sequence 5′-3′	Primers position
18S For-A	TGCATGTCCAAGTATAAACACG	53–74
18S Rev-A	AACGCGATCCGACAAAATA	250–233
18S For-B	TCGCGGGAGTGCATATTAG	186–204
18S Rev-B	GGATGTGGTAGCCGTTTCTC	389–370
18S For-C	TGAGAAACGGCTACCACATC	369–388
18S Rev-C	GGGGGCAGATATCCAACTAC	617–598
18S For-D	CGACTAAGCATTGGGCTACC	1026–1045
18S Rev-D	GTGGGGTCTCGTCCGTTAT	1319–1301
18S For-E	CGAGACCCCACCCATCTAAC	1309–1328
18S Rev-E	CAAAGGGCAGGGACGTAATC	1640–1621
18S For-F	GCTGTTAAAACGCTCGTAGTTG	583–604
18S Rev-F	ATCATTACTCCAGCTCAAAACCA	856–830
18S For-G	CGGGCCAGAGGTAAAATTC	884–902
18S Rev-G	CCCCCTGAGTCCGAAAAC	1094–1077
18S For-J	GTGCATGGCCGTTCTTAGTC	1253–1272
18S Rev-J	TTGCCCCAATCTTCCATCT	1436–1418
18S For-M	GAATGCGGGTTCGATTC	339–355
18S Rev-M	TTGGGTAATTTGCGCACC	420–403
18S For-N	CTTCGGCGCCGCCAC	797–811
18S Rev-N	GCTTTCGCATAAGTTAG	935–919
ITS For-7	ATTACGTCCCTGCCCTTTGT	1622–1641
ITS Rev-7	ACGAGGTTCGCGGTTTTTAT	1859–1840
ITS For-8	CGTAACAAGGTTTCCGTAGGT	1757–1777
ITS Rev-8	TGCTTTTTGCGTTTGTGTAGT	1911–1891

PCR amplification of the rRNA 18S gene and ITS-1 region. Taq DNA polymerase (Promega) was used for PCR assays. In all PCR assays, the primers (Table 1) were used at a final concentration of 1 µM. Aliquots (2 µl) of DNA samples were added to 23 µl of reaction mixture for amplification. Thermocycling conditions for the 18S gene PCR were: 95°C for 2 min (1 cycle), followed by 94°C for 30 s, 55°C for 45 s and 72°C for 45 s (40 cycles), followed by 72°C for 5 min (1 cycle). PCR cycling conditions for the ITS region were: 95°C for 2 min (1 cycle), 94°C for 30 s, 50°C for 45 s and 72°C for 45 s (40 cycles), followed by 72°C for 5 min (1 cycle). The resulting amplicons were resolved by electrophoresis in 2% agarose gels in 1 × TAE (0.004 M tris-acetate, 0.001 M EDTA) buffer. Gels were stained with ethidium bromide (0.5 µg ml^{-1}). Amplicons were then extracted using a gel extraction kit (Qiagen) before sequencing.

Sequence determination and comparison. The 18S rRNA gene and the ITS region (GenBank Accession Number DQ312295) of the Australian *Bonamia* isolate were determined by direct sequencing of the PCR product using an ABI PRISM Ready Reaction Big dye Termination Cycle Sequencing Kit (Perkin-Elmer) and an ABI PRISM Model 377XL DNA sequencer (Sequencing Analysis 3.4.1 software Version 2.6) (Perkin-Elmer). All sequencing was performed on 3 different samples. Twelve pairs of primers were used to amplify overlapping fragments covering the 18S-ITS-1 gene sequences (Table 1). Confirmation of the 5′-end of the 18S gene was performed by cloning the PCR product, amplified by the primers Suni (CAA CCTGGT TGA TCC TGC CC, Besnard-Cochennec 2001) and Boas (Cochennec et al. 2000), into the TA cloning vector (Invitrogen). Sequence determination of the cloned fragments was performed using the plasmid primers Topo F (GAC CAT GAT TAC GCC AAG C) and Topo R (CCC AGT CAC GAC GTT G) and the Big Dye V3 sequencing kit (Applied Biosystem).

Bonamia sequences were aligned using the CLUSTAL W algorithm (Thompson et al. 1994, Biomanager, Australian National Genomic Information Service).

Nucleotide sequences from *Bonamia ostreae* and *B. exitiosa* (Accession Number AF262995 and AF337563, respectively) were obtained from the GenBank database. The *B. exitiosa* ITS-1 sequence was obtained from Besnard-Cochennec (2001), and the Chilean *Bonamia* sp. ITS-1 sequence (Accession Number AY539840) was obtained from the GenBank database.

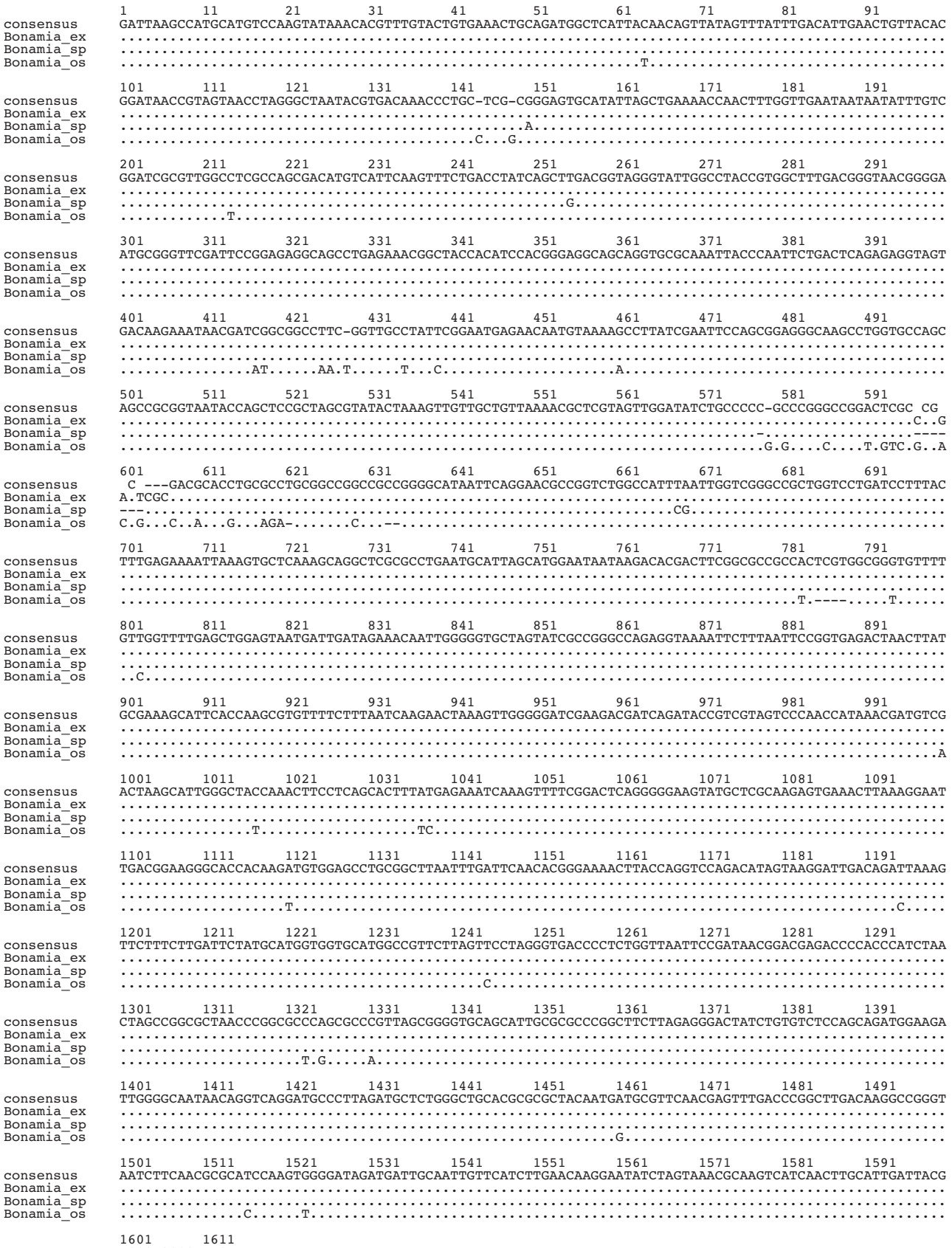

Fig. 1. *Bonamia* spp. Alignment of 18S rRNA partial gene sequences from Australia (*Bonamia* sp., Bonamia_sp), New Zealand (*B. exitiosa*, Bonamia_ex) and France (*B. ostreae*, Bonamia_os). Nucleotide mismatches are indicated below the consensus sequence; dashes represent nucleotide deletions with respect to consensus

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               1         11        21        31        41        51
consensus         TT  TTATT T TGCA  AATAA AACAT  ATAAA    AACCGCGAACCT   T
Bon_NSW        TAT..GT.....A.-....A-.....A.....TT.....----.............CGT.C
Bon_NZ         TAT..GT.....A.-....T-.....A.....TT.....----.............CGT.C
Bon_Chile      --C..TA.....G.A....AC..C..C.....GC.A...TATA..........A.TTC.G
Bon_France     --------------CAC..CATT.T.TTG..CG-.....ATTC...........CACA.T

               61        71        81        91        101       111
consensus      TTTT ATTAAT TTGCTGACTACCGATTTAC -ACA---CAAACGCA A--AAG AT T
Bon_NSW        ....A......T...................T..............A......C..A.-
Bon_NZ         ....A......T...................T..............A......C..A.-
Bon_Chile      ....T.AA...A...................AC...AAG........T...T.TAT.T.T
Bon_France     ..A.T...GCAAACT...G....A....G..A...........T...GCGC...G..T.T

               121       131       141       151       161
consensus      A ATA G   TTTGCA-AGACA   TGC-CGACATCAATCTTTGCA
Bon_NSW        .C...T.ACT............---.....................
Bon_NZ         .C...T.ACT............---.....................
Bon_Chile      .T...C.CGA............ACA.....................
Bon_France     GTGC.A.GAA.....GC.A.GTTCT...G.TG..AA.C..CCGA..
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Fig. 2. *Bonamia* spp. Alignment of ITS-1 rRNA sequences from the Australian *Bonamia* sp.(Bon_NSW), the New Zealand *B. exitiosa* (Bon_NZ), the Chilean *Bonamia* sp. (Bon_Chile) and the French *B. ostreae* (Bon_France). Nucleotide identities are presented on the consensus lines; dashes represent nucleotide deletions

RESULTS AND DISCUSSION

The aim of this study was to explore the relationship, at a molecular level, of an Australian isolate of *Bonamia* sp. with previously described species of the genus, *B. ostreae* and *B. exitiosa*. In order to evaluate the relatedness of this Australian isolate to isolates from New Zealand and France, sequencing of the 18S gene and ITS-1 region of the rRNA operon of *Bonamia* sp. was performed. The sequences obtained from the 3 NSW *Bonamia* isolates were identical. A nucleotide sequence comparison between the isolates from Australia, France and New Zealand was conducted. Results indicated that a higher level of nucleotide identity exists between the *Bonamia* sp. from Australia and *B. exitiosa* (New Zealand) than between *Bonamia* sp. and *B. ostreae* from France (Fig. 1). A 1598 base pair (bp) sequence of the 18S gene of the Australian isolate presents 4 base pair mismatches and 11 base pair deletions when compared with the *B. exitiosa* sequence. On the other hand, differences between *Bonamia* sp. and *B. ostreae* sequences are more numerous, with 41 base pair mismatches, 5 base pair insertions and 10 base pair deletions.

Alignment of the ITS-1 rRNA sequences from *Bonamia* sp. and *B. exitiosa* presents further evidence of the genetic similarity between the Australian and New Zealand *Bonamia*. The isolates present only 1 base pair mismatch (Fig. 2). In addition, the ITS-1 sequence of a recently characterised *Bonamia* isolate from Chile (southern hemisphere) shows a higher level of similarity with the oceanic isolates than *B. ostreae* from France (northern hemisphere) (Fig. 2). It has been shown that the Chilean flat oyster *Ostrea chilensis* originates from New Zealand (Ó Foighil et al. 1999); it is therefore logical that their respective *Bonamia* isolates are closely related. Sequence analysis of the Australian isolate of *Bonamia* sp. demonstrates closer affinity with *B. exitiosa* compared to *B. ostreae*. In the light of this information, it is suggested that this *Bonamia* isolate from Australia be considered as a member of *B. exitiosa*. It has been hypothesised (Hine & Jones 1994, Hine 1996) that, in this region, the parasite may have evolved in New Zealand and spread to Australia when commercial-size oysters were shipped live from New Zealand and laid in Victorian and Tasmanian waters prior to consumption (Dartnall 1969). With regards to surveillance of bonamiosis, the genetic similarity of the New Zealand and Australian isolates will allow the development of differential molecular diagnostic tests at various levels of specificity. A PCR-restriction fragment length polymorphism (PCR-RFLP) assay has been proposed to differentiate *B. ostreae*, *B. exitiosa* and *B. roughleyi* (Cochennec-Laureau et al. 2003). With ITS-1 sequences, it should be possible to design specific primers that would allow amplification of a given species in a single-step assay. However, more isolates need to be examined at the molecular level to provide further information on the diversity of *Bonamia* species before clear delineation of species and possible subspecies is justified. This is particularly true since recent reports of *Bonamia* sp. outside New

Zealand and Australia have significantly broadened the recognised geographical distribution of this parasite (Campalans et al. 2000, Burreson et al. 2004, Kroeck & Montes 2005). With regards to those recent isolates from Chile, the USA and Argentina, it is believed that the sequence data provided in this paper will help to clarify taxonomic relationships within the genus *Bonamia* and, more particularly, the species *B. exitiosa*. If *B. exitiosa* appears to be widely distributed within this geographical area, then, tools providing sound strain or subspecies identification will assist decision making concerning translocation of live molluscs. Together with robust epidemiological studies and biological evidence of biodiversity, the implementation of such tools could help avert the detrimental affects of translocation of live molluscs.

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