Vol. 110: 33–54, 2014
doi: 10.3354/dao02738

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published July 24

Contribution to DAO Special 7 'Microcell parasites of molluscs'

FREE ACCESS

Phylogenetics of *Bonamia* parasites based on small subunit and internal transcribed spacer region ribosomal DNA sequence data

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ABSTRACT: The genus *Bonamia* (Haplosporidia) includes economically significant oyster parasites. Described species were thought to have fairly circumscribed host and geographic ranges: *B. ostreae* infecting *Ostrea edulis* in Europe and North America, *B. exitiosa* infecting *O. chilensis* in New Zealand, and *B. roughleyi* infecting *Saccostrea glomerata* in Australia. The discovery of *B. exitiosa*-like parasites in new locations and the observation of a novel species, *B. perspora*, in non-commercial *O. stentina* altered this perception and prompted our wider evaluation of the global diversity of *Bonamia* parasites. Samples of 13 oyster species from 21 locations were screened for *Bonamia* spp. by PCR, and small subunit and internal transcribed spacer regions of *Bonamia* sp. ribosomal DNA were sequenced from PCR-positive individuals. Infections were confirmed histologically. Phylogenetic analyses using parsimony and Bayesian methods revealed one species, *B. exitiosa*, to be widely distributed, infecting 7 oyster species from Australia, New Zealand, Argentina, eastern and western USA, and Tunisia. More limited host and geographic distributions of *B. ostreae* and *B. perspora* were confirmed, but nothing genetically identifiable as *B. roughleyi* was found in Australia or elsewhere. Newly discovered diversity included a *Bonamia* sp. in *Dendostrea sandvicensis* from Hawaii, USA, that is basal to the other *Bonamia* species and a *Bonamia* sp. in *O. edulis* from Tomales Bay, California, USA, that is closely related to both *B. exitiosa* and the previously observed *Bonamia* sp. from *O. chilensis* in Chile.

KEY WORDS: *Bonamia* · Haplosporidia · Phylogeny

INTRODUCTION

Bonamia species are protozoan parasites of oysters in the phylum Haplosporidia whose characteristic cell form is a 2–3 µm uninucleate 'microcell' (Pichot et al. 1980, Carnegie & Cochennec-Laureau 2004). There are 3 described species, from 3 different oyster hosts: *Bonamia ostreae* (Pichot et al. 1980) in *Ostrea edulis* L.; *B. exitiosa* (Hine et al. 2001) in *O. chilensis* Philippi, 1845; and *B. perspora* (Carnegie

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et al. 2006) in *O. stentina* Payraudeau, 1826 (formerly *Ostreola equestris* Say, 1834). A fourth parasite, *Mikrocytos roughleyi* (Farley et al. 1988) from *Saccostrea glomerata* Gould 1850, was reassigned to the genus *Bonamia* based on molecular analyses (Cochennec-Laureau et al. 2003), but it is not clear whether the *Bonamia* sp. detected in *S. glomerata* is distinct from *B. exitiosa* (Hill et al. 2010). A recent analysis argued that the parasite genetically identified as *B. roughleyi* was actually a mis-identified *B. exitiosa* (Carnegie et al. 2014).

Questions regarding the diversity and geographic distribution of *Bonamia* species began to emerge in conjunction with new discoveries of the geographic and host ranges of these parasites. Prior to 2004, *Bonamia* parasites were viewed as having fairly circumscribed host and geographic distributions — *B. ostreae* in *O. edulis* in the temperate Northern Hemisphere, *B. exitiosa* in *O. chilensis* in southern New Zealand, and the little studied *B. roughleyi* restricted to New South Wales, Australia. However, this perspective began to change when Burreson et al. (2004) found a *B. exitiosa*-like species infecting experimentally deployed *Crassostrea ariakensis* Fujita 1913 in Bogue Sound, North Carolina, USA. This parasite was subsequently observed infecting native oyster *O. stentina* (=*Ostreola equestris*; Shilts et al. 2007) in North Carolina, a species that was additionally found to host the novel parasite described as *B. perspora* (Carnegie et al. 2006, Hill et al. 2010). Additional observations of *Bonamia* parasites were made in *O. chilensis* from Chile (Campalans et al. 2000), *O. puelchana* from Argentina (Kroeck & Montes 2005), *O. angasi* from Australia (Corbeil et al. 2006), *O. edulis* from Spain (Abollo et al. 2008) and Italy (Narcisi et al. 2010), and *O. stentina* from the Mediterranean (Hill et al. 2010), but whether or not these were distinct from a described *Bonamia* species, particularly *B. exitiosa*, was not certain.

A phylogeographic study of *Bonamia* parasites is important given the ecological and economic destruction these parasites can cause in oyster hosts. *B. ostreae*, for example, contributed greatly to the collapse of *O. edulis* populations and fisheries in Europe (Grizel et al. 1988), and *B. exitiosa* has devastated *O. chilensis* populations in New Zealand (Hine et al. 2001). The objective of this study was to assess the global distribution of *Bonamia* parasites by collecting potential oyster hosts from around the world to better understand the geographic and host ranges and the genetic diversity of these parasites. Separate phylogenies were constructed based on the small subunit ribosomal RNA gene (SSU rDNA) and internal transcribed spacer (ITS) region rDNA (defined as ITS-1 rDNA, the 5.8S rRNA gene, and ITS-2 rDNA and hereafter referred to as ITS rDNA) to better understand the host and geographic boundaries and the evolutionary relationships of *Bonamia* species that were found in several new hosts and locations. Additionally, given the morphological similarities of all *Bonamia* parasites, we used *in situ* hybridization of archival materials to re-examine microcell observations from the pre-molecular era and revisit previous conclusions regarding the geographic distribution of *Bonamia* parasites.

MATERIALS AND METHODS

Sample collection and processing

Samples of 11 oyster species were obtained from 21 locations around the world (Table 1). Most oysters were collected from natural habitats. However, samples of *Crassostrea ariakensis* from Florida, USA, were hatchery-produced by the Virginia Institute of Marine Science and experimentally deployed; *Ostrea denselamellosa* from the Okayama Prefecture, Japan, were cultured as part of a restoration program; and *O. angasi* from Australia, *Saccostrea glomerata* from Australia, and one sample of *O. chilensis* from Chile were obtained from commercial aquaculture. With the exception of the *C. ariakensis* samples, the hatchery sources of the cultured oysters are not known. Oysters were shucked, and small pieces of gill and mantle tissue (~3–5 mm^3) were either preserved individually in 95% ethanol or placed directly in lysis solution (QIAamp DNA Kit, QIAGEN) for molecular analyses. The only exceptions were *O. edulis* and 2004 *O. lurida* tissue samples collected from California, USA. Tissues from *O. lurida* individuals were stored at −80°C in pools of 3 or 4 oysters per tube, and then preserved in 100% ethanol for shipping. The California *O. edulis* samples were preserved in 95% ethanol, but again pooled: 14 pools of 4 individuals, and 1 pool of 2 individuals in 1 sample. All instruments used for dissection were sterilized with 95% ethanol and flamed between each sample. Remaining tissues, including gill, mantle, and visceral mass, were fixed in Davidson's fixative (Shaw & Battle 1957) for standard histopathology. For *Isognomon* sp. from Florida, *O. edulis* from the Netherlands, *O. chilensis* from Chile, and the 2006 *Dendostrea sandvicensis* samples, tissues were collected for molecular analyses only.

Table 1. Collection information and *Bonamia* sp. PCR prevalence data. PCR primers Bon-319F + Bon-524R were used for amplifications unless otherwise noted. n/a: not applicable

Oyster species	Location	Collection date (mo/d/yr)	Sample size	*Bonamia* spp. PCR prevalence (%)
Crassostrea ariakensis	Fort Pierce, Florida, USA	4/16/2007	17	6/17 (35.3%)
Crassostrea ariakensis	Fort Pierce, Florida, USA	6/14/2007	19	8/19 (42.1%)
Isognomon sp.	Long Key, Florida, USA[a]	7/10/2009	30	0/30 (0%)
Ostrea stentina	Old House Creek, South Carolina, USA	4/12/2006	55	2/55 (3.6%)[c]
Ostrea stentina	Wilmington, North Carolina, USA	6/22/2005	200	8/200 (4.0%)[d]
Ostrea stentina	Morehead City Port, North Carolina, USA	8/1/2005	150	3/150 (2.0%)[e]
Ostrea puelchana	San Antonio Bay, Argentina	3/22/2005	57	6/57 (10.5%)[c]
Ostrea stentina	San Antonio Bay, Argentina	04/2007	3	3/3 (n/a[f])
Ostrea lurida	Lemmens Inlet, British Columbia, Canada	4/01/2005	25	0/25 (0%)
Ostrea lurida	Drakes Estero, California, USA	7/7/2004	15[b]	0/15[b] (0%)
Ostrea lurida	Elkhorn Slough, California, USA	5/27/2004	15[b]	13/15[b] (21.7–86.7%)
Ostrea lurida	Elkhorn Slough, California, USA	09/17/2009	60	53/60 (88.3%)
Ostrea edulis	Tomales Bay, California, USA	10/7/2005	32[b]	21/32[b] (20.7–82.0%)
Dendostrea sandvicensis	Kaneohe Bay, Hawaii, USA[a]	06/2006	120	79/120 (65.8%)
Dendostrea sandvicensis	Kaneohe Bay, Hawaii, USA	10/18-20/2007	60	42/60 (70.0%)
Ostrea denselamellosa	Seto Inland Sea, Okayama Prefecture, Japan	7/13/2009	40	0/40 (0%)
Ostrea angasi	Pambula River, New South Wales, Australia	11/2006	42	1/42 (2.4%)
Saccostrea glomerata	Georges River, New South Wales, Australia	08/2007	200	1/200 (0.5%)
Ostrea stentina	Tamaki Estuary, Glendowie, New Zealand	8/14/2007	44	4/44 (9.1%)
Ostrea stentina	Karaka Bay, Tamaki Estuary, Auckland, New Zealand	3/13/2009	90	5/90 (5.6%)
Ostrea chilensis	Foveaux Strait, New Zealand	01 & 03/2005	5	4/5 (n/a[f])
Ostrea chilensis	Foveaux Strait, New Zealand	06/13/2004	40	2/40 (5.0%)
Saccostrea glomerata	Whangarei Harbour, Northland, New Zealand	3/2/2008	50	0/50 (0%)
Saccostrea glomerata	Whangarei Harbour, Northland, New Zealand	5/1/2009	60	0/60 (0%)
Ostrea chilensis	Chiloe Island, Chile[a]	12/2003	1	1/1 (n/a[f])
Ostrea chilensis	Chiloe Island, Chile[a]	10/2003	32	6/32 (18.8%)
Ostrea stentina	Hammamet, Tunisia	06/2007	85	8/85 (10.6%)
Ostrea edulis	Lake Grevelingen, The Netherlands[a]	05/2008	1	1/1 (n/a[f])

[a]Samples collected for molecular analyses (PCR, sequencing) only. [b]More than one individual in a single DNA extraction, and therefore PCR reaction. [c]C_F + C_R primers used instead of Bon-319F + Bon-524R. [d]C_F + C_R primers used instead of Bon-319F + Bon-524R. Five of these were *B. perspora* only, one was *B. exitiosa* only, and 2 were co-infections. [e]C_F + C_R primers used instead of Bon-319F + Bon-524R. One of three was *B. perspora*. [f]Sample sent to the Virginia Institute of Marine Science was confirmed *Bonamia* sp. positive as determined by histology

DNA extraction and diagnostic PCR

Genomic DNA from each oyster sample was extracted using a QIAamp DNA Kit. DNA was eluted in 100–225 µl of elution buffer and stored at 4°C. For the pooled *O. edulis* samples from California, each pool was divided into 2 individuals per extraction (except one pool that had larger pieces of tissue, for which the 4 individuals were divided into 4 separate extractions). This was done in order to obtain a better estimate of prevalence.

After each extraction, DNA was quantified using a GeneQuant *pro* spectrophotometer (Amersham Biosciences). Oysters were screened for *Bonamia* spp. DNA using either generic PCR primers Bon-319F and Bon-524R (Hill et al. 2010), which target a 206 bp portion of *Bonamia* spp. SSU rDNA, or generic primers C_F and C_R (Carnegie et al. 2000), which amplify a 760 bp portion of *Bonamia* spp. SSU rDNA. (The approximate primer binding sites for this and all other primer pairs used in this study are presented in Fig. 1.) For the Bon-319F + Bon-524R PCR, a 25 µl total volume reaction contained 1× PCR buffer (Applied Biosystems), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µg µl^{-1} bovine serum albumin (BSA), 0.25 µM primer mix, 0.024 units µl^{-1} Ampli*Taq* DNA polymerase (Applied Biosystems), and 200–250 ng (= 0.5–1.6 µl) template DNA. A 4 min initial denaturation at 94°C was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, and then by a final extension at 72°C for 7 min. Products were electrophoresed on 2.5% agarose gels (100 V, 30 min), subsequently stained with ethidium bromide, and visualized under a UV light.

The 25 µl C_F + C_R reaction contained the same reagents and concentrations, but thermal cycling

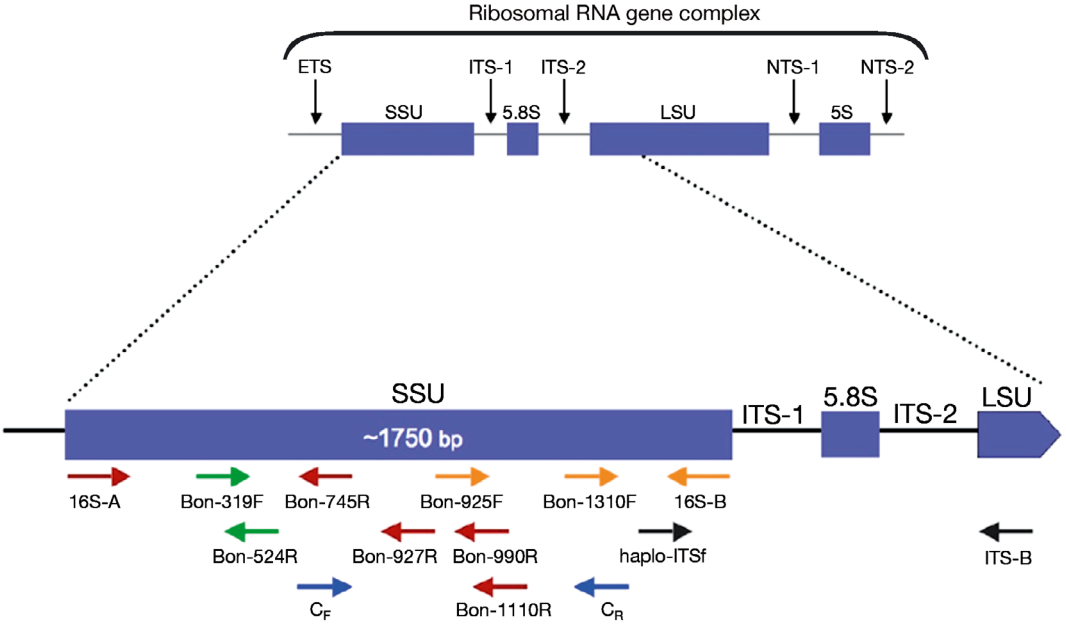

Fig. 1. Schematic representation of ribosomal RNA gene complex-targeted PCR assays used in this study. Forward primers (F) were paired with reverse primers (R), with colors indicating which primers were used in combination. ITS: internal transcribed spacer; SSU: small subunit; LSU: large subunit

was performed as described in Carnegie et al. (2000): a 4 min initial denaturation at 94°C was followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min, and then by a final extension at 72°C for 10 min. These products were electrophoresed on 2% agarose gels (100 V, 30 min), stained with ethidium bromide, and visualized under UV light.

SSU rDNA sequencing

PCR products from infected oysters were used to generate *Bonamia* spp. SSU rDNA sequence. One to 3 PCR-positive oysters, 2 to 3 primer sets, and 3 to 9 clones per primer set were used to obtain complete SSU rDNA sequences. In order to generate sequence for the entire SSU rDNA region (~1750 bp), multiple PCR amplifications had to be performed. Several primer pairs were tested, and the pair that yielded a single amplicon of appropriate size was used for subsequent cloning reactions. In order to amplify the 5′ end of *Bonamia* spp. SSU rDNA region, a reverse *Bonamia*-generic primer (Bon-745R, Bon-927R, Bon-990R, Bon-1110R, or Bon-1050R) was paired with primer 16S-A (Medlin et al. 1988), a universal primer that amplifies eukaryotic SSU rDNA (Table 2). The 3′ end of *Bonamia* spp. SSU was generated using a forward *Bonamia*-generic primer (Bon-925F or Bon-1310F) paired with primer 16S-B (Medlin et al. 1988) (Table 2). In most cases, a third PCR had to be performed in order to complete the SSU rDNA region. Either C_F + C_R or Bon-319F + Bon-990R primer pairs were used to generate sequencing data for the gap (Table 2). For the *Bonamia* sp. from *D. sandvicensis* in Hawaii, a new primer, HIBon-620F, was designed using MacVector 8.0 (Oxford Molecular) in order to obtain the middle portion of the SSU rDNA sequence, and was paired with Bon-1110R (White 2008) (Table 2). For each PCR, a 25 µl total reaction volume contained the same reagents at the same concentrations as the Bon-319F + Bon-524R PCR described above. A 4 min initial denaturation was followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 54–58°C for 45 s, extension at 72°C for 1 min (for products <~800 bp) or 1.5 min (for products >~800 bp), and then by a final extension at 72°C for 6 min (except for the C_F + C_R PCR, which was performed as described above). The selected amplification products from triplicate PCR reactions were pooled and purified using a QIAquick PCR purification kit (QIAGEN).

The reactions to obtain the *Bonamia* sp. SSU rDNA found in *O. edulis* from California differed from the above descriptions in that a 25 µl total reaction volume contained 1× PCR buffer (Invitrogen), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM forward primer,

Table 2. *Bonamia* spp. sequences of PCR primers and *in situ* hybridization probes. Binding site ranges defined using *B. exitiosa* in *Ostrea chilensis* from New Zealand (GenBank accession no. JF495410) as the target sequence unless noted. ITS: internal transcribed spacer; SSU: small subunit; ISH: *in situ* hybridization; FISH: fluorescent *in situ* hybridization; n/a: not applicable

Name	Sequence (5′–3′)	*Bonamia* sp. SSU binding site	Use	Reference
C_F	CGG GGG CAT AAT TCA GGA AC	663–682[a]	*Bonamia* spp. PCR; SSU seq.	Carnegie et al. (2000)
C_R	CCA TCT GCT GGA GAC ACA G	1401–1383	*Bonamia* spp. PCR; SSU seq.	Carnegie et al. (2000)
Bon-319F	TTT GAC GGG TAA CGG GGA ATG CG	301–323	*Bonamia* spp. PCR; SSU seq.	Hill et al. (2010)
Bon-524R	CTT GCC CTC CGC TGG AAT TC	505–486	*Bonamia* spp. PCR	Hill et al. (2010)
Bon-745R	CTA ATG CAT TCA GGC GCG AG	754–735	*Bonamia* spp. PCR; SSU seq.	Carnegie et al. (2006)
Bon-925F	ATT CCG GTG AGA CTA ACT TAT G	885–906	*Bonamia* spp. PCR; SSU seq.	White (2008)
Bon-927R	CAT AAG TTA GTC TCA CCG GAA TT	906–884	*Bonamia* spp. PCR; SSU seq.	This study
Bon-990R	CTT AGT CGA CAT CGT TTA TGG TTG GG	1011–986	*Bonamia* spp. PCR; SSU seq.	White (2008)
Bon-1110R	CCT TTA AGT TTC ACT CTT GCG AG	1102–1080	*Bonamia* spp. PCR; SSU seq.	White (2008)
Bon-1310F	GAG ACC CCA CCC ATC TAA C	1288–1306	*Bonamia* spp. PCR; SSU seq.	Carnegie et al. (2006)
HIBon-620F	CGG CAC GCA CGT AAG TGG AG	620–639[b]	Hawaiian *Bonamia* PCR; SSU seq.	This study
16S-A	AAC CTG GTT GAT CCT GCC AGT	5′ end SSU	Universal SSU PCR; seq.	Medlin et al. (1988)
16S-B	GAT CCT TCC GCA GGT TCA CCT AC	3′ end SSU	Universal SSU PCR; seq.	Medlin et al. (1988)
haplo-ITSf	GGG ATA GAT GAT TGC AAT TRT TC	1529–1551	Haplosporidia[c] PCR; ITS seq.	Hill et al. (2010)
ITS-B	TAT GCT TAA ATT CAG CGG GT	5′ end LSU	Universal ITS PCR; ITS seq.	Goggin (1994)
16Sar	CGC CTG TTT ATC AAA AAC AT	n/a	Universal mt16S PCR; host mtDNA seq	Kessing et al. (1989)
16Sbr	CCG GTC TGA ACT CAG ATC ACG T	n/a	Universal mt16S PCR; host mtDNA seq	Kessing et al. (1989)
CaBon166	CGA GCA GGG TTT GTC ACG TAT	166–146	*B. exitiosa* dig-ISH	Hill et al. (2010)
CaBon461	TTC CGA ATA GGC AAC CGA AG	461–442	*B. exitiosa* dig-ISH	Hill et al. (2010)
CaBon1704	CAA AGC TTC TAA GAA CGC GCC	1704–1684	*B. exitiosa* dig-ISH	Hill et al. (2010)
Bost171	CCG CCG AGG CAG GGT TTG T	187–169[a]	*B. ostreae* dig-ISH	Present study
BON1490	GTC AAG CCG GGT CAA ACT CGT TG	1473–1495	*Bonamia* dig-ISH	Present study
HIBon-167	CTA ATA TGC ACA GCC GCC AG	186–167[b]	Hawaiian *Bonamia* FISH	Present study
HIBon-634	CGA TTA TGG CCT CTC TCC AC	653–634[b]	Hawaiian *Bonamia*-FISH	Present study
Oe-309	TCA TGC TCC CTC TCC GG	n/a	Universal SSU probe	Cáceres-Martínez et al. (2012)

[a]Binding site ranges defined using *Bonamia ostreae* in *Ostrea edulis* from Maine (GenBank accession no. AF262995) as the target sequence. [b]Binding site ranges defined using *Bonamia* sp. in *Dendostrea sandvicensis* from Hawaii, USA (GenBank accession no. JF831803) as the target sequence. [c]Primer was designed to anneal to most haplosporidian genera

0.25 µM reverse primer, 0.05 units µl^{-1} Platinum *Taq* DNA polymerase (Invitrogen), and 200–250 ng template DNA. The thermal cycling program was as stated above. Duplicate reactions were individually purified; amplification products were not pooled as for other samples.

Purified PCR products were cloned into the plasmid vector pCR4-TOPO using the TOPO TA Cloning kit (Invitrogen), and then transformed into One Shot TOP10 competent *Escherichia coli* cells (Invitrogen). Colonies were screened either using phenol/chloroform/isoamyl-alcohol (PCI) extractions or by PCR using M13 vector primers. Clones with inserts of desired size were cultured and plasmids were extracted using the QIAprep Spin Miniprep Kit protocol (QIAGEN), and sequenced on either a LI-COR 4200L or a 16-capillary 3130*xl* Genetic Analyzer (Applied Biosystems).

ITS region rDNA sequencing

Bonamia sp. ITS rDNA region sequences were characterized for oysters that initially tested positive for a *Bonamia* sp. using SSU rDNA-based assays above. One to 7 *Bonamia*-positive oysters per species were characterized by ITS rDNA sequencing. The primers HaploITSf (Hill et al. 2010) and ITS-B (= reverse primer D; Goggin 1994) were used (Table 2). This primer pair amplifies ~220 bp of the 3′ end of the SSU rRNA gene, the complete ITS-1, 5.8S gene, and ITS-2 region rDNA, and a short fragment of the large subunit (LSU) rDNA of most haplosporidians, yielding a product of approximately 750 bp from *Bonamia* spp. Initially, PCR reactions were performed in triplicate and pooled as described above for cloning and SSU rDNA sequencing. These reactions were carried out using Applied Biosystems reagents at the same con-

centrations as described above for the Bon-319F + Bon-524R PCR. After June 2008, however, duplicate reactions were performed on each individual or pool of individuals, with each reaction individually cloned, sequenced, and compared. The objective of this change in protocol was to allow the detection of more rare variant sequences. A 25 µl total reaction contained 1× PCR Buffer (Invitrogen), 2–2.5 mM $MgCl_2$, 0.2 mM dNTPs, each primer at 0.25 µM, 0.05 U $µl^{-1}$ Platinum *Taq* DNA polymerase (Invitrogen), and 200–250 ng (=0.5–1.6 µl) template DNA. Regardless of reagents used, a 7 min initial denaturation was followed by 35 cycles of denaturation at 95°C for 1 min, annealing between 55 and 61°C for 1 min, and extension at 72°C for 1.5 min, and then by a final extension at 72°C for 7 min. These products were purified, cloned, transformed, screened, and sequenced as in the above description of SSU rDNA sequencing. One to 29 clones per individual host were sequenced.

Oyster mt16S rDNA sequencing

All oysters were presumptively identified based on morphological characteristics and sample locations. However, universal primers 16Sar and 16Sbr (Kessing et al. 1989) (Table 2) were used to amplify a portion of the mitochondrial 16S (mt16S) rRNA gene of *Bonamia*-positive host species to confirm oyster identity of *O. stentina* (=*O. aupouria*; Shilts et al. 2007) from New Zealand, *O. lurida* and *O. edulis* from California, *O. stentina* from Argentina, *O. stentina* from Tunisia, and *D. sandvicensis* from Hawaii. A 25 µl total reaction contained 1× PCR Buffer (Applied Biosystems), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µg $µl^{-1}$ BSA, 0.20 µM primer mix, 0.024 units $µl^{-1}$ Ampli*Taq* DNA polymerase (Applied Biosystems), and 200–250 ng (= 0.5–2.5 µl) template DNA. Reaction conditions were modified from Kirkendale et al. (2004): initial denaturation at 95°C for 4 min was followed by 38 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 2 min, and by a final extension at 72°C for 7 min. Products were electrophoresed and visualized as above. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) and quantified using either a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech) or a NanoDrop 2000 (Thermo Fisher Scientific) as per the manufacturers' instructions. Three to 10 nanograms of the purified PCR product was then added to the reagents from a BigDye® Terminator v3.1 cycle sequencing kit (using manufacturer's instructions, modified for 5 µl reactions) and the 16Sar and 16Sbr primers (Kessing et al. 1989). Reactions were then cleaned, precipitated, and sequenced as above. Resulting sequences were compared with sequences in the National Center for Biotechnology Information (NCBI) GenBank database by using the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1997).

Sequence alignments and molecular phylogenetics

Newly generated SSU rDNA *Bonamia* spp. sequences from *C. ariakensis* from Florida, *O. stentina* from Argentina, *O. edulis* and *O. lurida* from California, *D. sandvicensis* from Hawaii, *S. glomerata* from Australia, *O. stentina* and *O. chilensis* from New Zealand, and *O. edulis* from The Netherlands were aligned with published SSU rDNA sequences. These included *B. ostreae* (GenBank accession numbers AF262995 and AF192759), *B. exitiosa* (AF337563), *B. roughleyi* (AF508801), and *B. perspora* (DQ356000); the *Bonamia* spp. from *O. stentina* from Tunisia (GQ385242), *C. ariakensis* from North Carolina (AY542903), *O. edulis* from Spain (EU016528) and Italy (EU598800, EU598801), *O. angasi* from Australia (DQ312295, JF495408), *O. chilensis* from Chile (AY860060, GQ366703), and *O. puelchana* from Argentina (JF495409); and outgroup species *Minchinia tapetis* (AY449710), *M. teredinis* (U20319), *M. chitonis* (AY449711), and *M. mercenariae* (FJ518816). *Minchinia* spp. were chosen as an outgroup because *Minchinia* is sister to *Bonamia* in the haplosporidian phylogeny (Reece et al. 2004).

Bonamia spp. ITS rDNA sequences amplified from *C. ariakensis* from Florida, *O. angasi* and *S. glomerata* from Australia, *O. stentina* and *O. chilensis* from New Zealand, *O. chilensis* from Chile, *O. lurida* and *O. edulis* from California, *O. edulis* from the Netherlands, *O. stentina* and *O. puelchana* from Argentina, *O. stentina* from North and South Carolina and Tunisia, and *D. sandvicensis* from Hawaii were aligned with GenBank-deposited *Bonamia* spp. ITS rDNA sequences from *C. ariakensis* from North Carolina, *O. puelchana* from Argentina, *O. angasi* from Australia, and *O. chilensis* from New Zealand and Chile, with *B. ostreae* sequences from *O. edulis* from Maine, and with *B. perspora* sequences from *O. stentina* from North Carolina. GenBank accession numbers can be found in Table 3. All sequences were bidirectional except some of the GenBank-deposited *Bonamia* sp. sequences from *O. angasi* (EU780686–EU780692). Both the SSU and the ITS rDNA alignments were carried out using the automatic setting in MAFFT v. 6 (Katoh & Toh 2008).

Table 3. GenBank accession numbers of the *Bonamia* spp. internal transcribed spacer rDNA sequences used in the alignments for subsequent phylogenetic and distance analyses

Bonamia species	Sampling location	Host species	GenBank accession nos.
B. exitiosa	Florida, USA	*Crassostrea ariakensis*	JF712867–JF712869
B. exitiosa	North Carolina, USA	*C. ariakensis*	EU709024[a], EU709025[a], EU709027–EU709031[a], EU709033–EU709039[a], EU709041–EU709045[a], EU709051–EU709054[a]
B. exitiosa	North Carolina, South Carolina, USA	*Ostrea stentina*	JF831575–JF831580, JF831585–JF831590, JF831592–JF831594, JF831596–JF831599, JF831601, JF831602
B. exitiosa	Argentina	*O. stentina*	JF831556, JF831557, JF831559–JF831563, JF831565–JF831574
B. exitiosa	Argentina	*O. puelchana*	EU709055[a], EU709057–EU709061[a], EU709063[a], EU709064[a], EU709068[a], JF831603, JF831604[a], JF831605–JF831610, JF831611[a], JF831612–JF831615, JF831617–JF831624, JF831626[a], JF831627[a], JF831628- JF831638
B. exitiosa	New Zealand	*O. chilensis*	EU709069[a], EU709071[a], EU709073–EU709075[a], JF831639, JF831640, JF831641[a], JF831642–JF831655, JF831656[a], JF831657
B. exitiosa	New Zealand	*O. stentina*	JF831658–JF831669, JF831671–JF831677
B. exitiosa	Australia	*O. angasi*	EU723225[a], EU780686–EU780692[a], JF831678–JF831680
B. exitiosa	Australia	*Saccostrea glomerata*	JF831681–JF831683
B. exitiosa	Tunisia	*O. stentina*	GU356032–GU356035, JF831685–JF831689, JF831691–JF831713, JF831715–JF831718
B. exitiosa	California, USA	*O. lurida*	JF831719–JF831738, JF831740, JF831741, JF831743–JF831761, JF831763–JF831767, JF831769–JF831774, JF831776, JF831778–JF831788, JF831790, JF831792–JF831800
B. ostreae	California, USA	*O. edulis*	JF831830–JF831840, JF831842–JF831848
B. ostreae	Maine, USA	*O. edulis*	EU709105–EU709111[a]
B. ostreae	New Zealand	*O. edulis*	JF831857–JF831862
B. perspora	North Carolina, USA	*O. stentina*	EU709112[a], EU709115–EU709121[a], EU709123–EU709129[a], EU709131[a], EU709132[a]
Bonamia sp.	Hawaii, USA	*Dendostrea sandvicensis*	JF831863–JF831879
Bonamia sp.	California, USA	*O. edulis*	JF831808–JF831818, JF831820, JF831821, JF831823–JF831825, JF831827–JF831829
Bonamia sp.	Chile	*O. chilensis*	EU709079–EU709084[a], EU709086[a], EU709087[a], EU709090[a], EU709091[a], EU709093[a], EU709095[a], EU709096[a], EU709098–EU709102[a], EU709104[a], JF831849–JF831856

[a]From White (2008)

For the ITS rDNA distance analyses, the SSU rDNA portion of the amplicon produced by the HaploITSf + ITS-B primer set was removed from the alignment. Incomplete sequences were also removed from the above ITS rDNA alignment so that all sequences spanned identical regions. The data set was then realigned using MAFFT v. 6, again on the automatic setting (Katoh & Toh 2008).

Parsimony analysis of SSU rDNA sequence data was conducted using PAUP* 4.0b10 (Swofford 2002). One thousand bootstrap replicates with 100 random additions were performed. Gaps were treated as missing. Parsimony analysis of ITS region sequence data was performed using TNT v. 1.1 (Tree analysis using New Technology; Goloboff et al. 2008). A new technology search was completed with 100 bootstrap replicates and 10 random additions. A 50% majority rule unrooted consensus tree was generated using PAUP* 4.0b10.

Bayesian inference analyses of SSU and ITS rDNA sequence data were conducted using MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003). MrModeltest v. 2.3 was used to determine the best model for each data set. Are We There Yet? (AWTY; Wilgenbusch et al. 2004) was used to determine whether stationarity had been reached for the SSU rDNA dataset. Fifty percent majority rule consensus trees were generated using PAUP*4.0 b10 (Swofford 2002).

Distance analyses

Distance analyses were performed using MEGA v. 4 (Tamura et al. 2007). For the *Bonamia* spp. SSU rDNA data set, uncorrected p-distances were calculated between the different *Bonamia* clades that were indicated by the parsimony

and Bayesian analyses. Gaps and missing data were only eliminated in pairwise sequence comparisons (pairwise deletion option). Standard error estimates were determined by a bootstrap procedure (1000 replicates). For the ITS rDNA data set, uncorrected p-distances were calculated between and within each *Bonamia* clade, again as indicated by the parsimony and Bayesian analyses of the ITS rDNA sequences. Again, the pairwise deletion option was used and standard error estimates were determined by a bootstrap procedure (1000 replicates).

In situ hybridization of contemporary samples

Standard, chromogenic *in situ* hybridization (ISH) assays specific for *B. ostreae* and *B. exitiosa* were performed with digoxigenin-labeled anti-sense probes on tissue sections of *C. ariakensis* from Florida, *O. stentina* from New Zealand, and *O. lurida* and *O. edulis* from California that were PCR-positive for a *Bonamia* sp. The *B. exitiosa* specific ISH used a cocktail of 3 probes, CaBon461 + CaBon166 + CaBon1704, at 2 ng μl^{-1} each (Hill et al. 2010) (Table 2) and the *B. ostreae*-specific assay used a single probe, Bost171, at 3 ng μl^{-1} (Table 2). For each sample, experiments included the following treatments: a no-probe control (25 µl hybridization buffer only), a positive control (*B. exitiosa*-infected *C. virginica* or *C. ariakensis* from North Carolina, USA, for the *B. exitiosa*-specific assays; and *B. ostreae*-infected *O. edulis* from Maine, USA, for the *B. ostreae*-specific assays), and a standard experimental treatment using CaBon probes (Hill et al. 2010) and/or Bost171 (Table 2).

Tissue sections for ISH were processed as described previously (Stokes & Burreson 1995) with a few modifications as follows. Protease treatment was changed to 50 µg ml^{-1} Proteinase K in PBS for 18 min at 37°C. After hybridization, the washes in 0.5× SSC (2 × 10 min) were at 42°C for the Bost171 probe and 45°C for the CaBon probe cocktail. Slides were counterstained with Bismarck Brown Y after color development as in Stokes & Burreson (2001) and were examined on an Olympus BX51 light microscope.

A fluorescent *in situ* hybridization (FISH) assay was used to visualize the *Bonamia* sp. found in *D. sandvicensis*. Anti-sense probes HIBon-167 and HIBon-634 (Table 2) tagged with Alexa Fluor 488 labels (Invitrogen) were designed in MacVector 8.0 to specifically target this parasite's SSU rRNA. The assay was optimized and tested for specificity against available *Bonamia* species (*B. exitiosa*, *B. perspora*, and *B. ostreae*). Slides were treated with HIBon-167 + HIBon-634 (each at 10 ng μl^{-1}), a no-probe control (hybridization buffer only), and a positive control (Oe-309 at 10 ng μl^{-1}) for ensuring general probe accessibility to oyster tissue (Table 2). The FISH assay was performed as in Carnegie et al. (2006), except a descending ethanol series was used for rehydration instead of a descending isopropanol series. As an added component to the FISH experiment, fluorescently labeled *B. exitiosa*-specific CaBon probes were hybridized to *B. exitiosa*-infected *C. ariakensis* and to the *Bonamia* sp.-infected *D. sandvicensis* individual, with experimental conditions as above. Slides were evaluated on an Olympus Provis epifluorescence microscope equipped with a red–green dual bandpass filter.

In situ hybridization of archival material

Paraffin-embedded oyster tissue was re-cut from archival blocks in which microcells were originally identified by Farley et al. (1988) using histology. These samples were identified by those authors as *Mikrocytos mackini* in *C. gigas* from Kaneohe Bay, Hawaii from 1972 (code S-124A-45); as *B. ostreae* in experimental *O. edulis* that were transferred from Milford, Connecticut, USA, to Oxford, Maryland, USA, and 'fed tissues of moribund *O. edulis* from Pigeon Point, CA' (p. 158) from 1968 (code FMT-B-1-3); and as *B. ostreae* in *O. edulis* produced in Milford and maintained in Chincoteague Bay, Virginia, USA, from 1961 (code FK-3-1). Mercury removal from sections originally fixed in Zenker's fixative was performed as in Mitchell (1966), and chromogenic ISH was performed as above using generic and *B. exitiosa*-specific probes, BON1490 and the CaBon cocktail, respectively (Table 2).

RESULTS

Bonamia-generic PCR

Putative *Bonamia* spp. were detected in 9 out of 11 oyster species examined, at numerous new locations (Table 1, Fig. 2). No *Bonamia* spp. were detected from *Isognomon* sp. from Florida and *Ostrea denselamellosa* from Japan. Two oyster species were *Bonamia*-positive at some locations but not others. No *Bonamia* spp. were detected in *Saccostrea glomerata* from Whangarei Harbour, New Zealand, or in *O. lurida* from Drakes Estero, California, or British

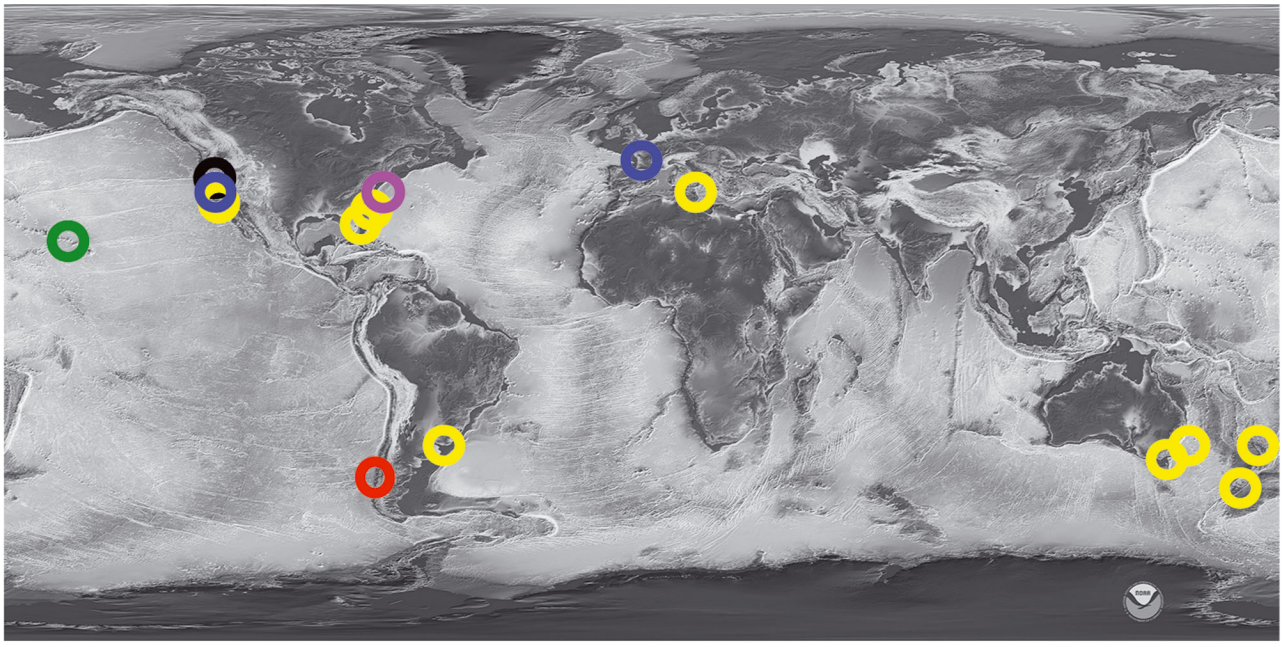

Fig. 2. Locations at which *Bonamia* parasites were analyzed in this study. Included are *B. ostreae* in Europe and North America (blue); *B. exitiosa* from North and South America, Australia, New Zealand, and the Mediterranean (yellow); *B. perspora* from North America (purple); and undescribed species from Hawaii (green) and California, USA (black), and Chile (red). See Table 1 for specific locations. Image: NOAA

Columbia, Canada. However, these oyster species were positive for a *Bonamia* sp. at other locations: *S. glomerata* in New South Wales, Australia, and in *O. lurida* from Elkhorn Slough, California. PCR prevalence of *Bonamia* spp. parasitism in positive samples ranged from 0.5 to 88.3% overall.

SSU rDNA sequencing

Complete SSU rDNA sequences were generated from putative *Bonamia* sp. parasites infecting *Crassostrea ariakensis* from Florida (GenBank accession number JF831807), *O. stentina* from Argentina (JF831801), *O. edulis* (JF831804 and JN040832) and *O. lurida* (JF831805) from California, *Dendostrea sandvicensis* from Hawaii (JF831803), *S. glomerata* from Australia (JF831802), *O. chilensis* (JF495410) and *O. stentina* (JF831806) from New Zealand, and *O. edulis* from the Netherlands (JN040831).

Putative *Bonamia* spp. SSU rDNA consensus sequences ranged from 1749 to 1766 bp in length, with *B. ostreae* and *B. exitiosa* sequences ~1750 bp, *B. perspora* 1762 bp, and the *Bonamia* sp. from *D. sandvicensis* 1766 bp in length. The SSU rDNA sequences of putative *Bonamia* sp. found in *C. ariakensis* from Florida, *O. stentina* from New Zealand, *S. glomerata* from Australia, *O. lurida* from California, and *O. stentina* from Argentina were 99–100% identical to the published SSU rDNA sequences of *B. exitiosa* from Australia (DQ312295) and New Zealand (AF337563), *B. roughleyi* (AF337563), *Bonamia* sp. in *O. stentina* from Tunisia (GQ385242), and *Bonamia* sp. in *C. ariakensis* from North Carolina (AY542903).

Two putative *Bonamia* spp. were detected in *O. edulis* from California. One sequence (JF831804) was 99% similar to *B. exitiosa*, while the other (JN040832) was 99% similar to published SSU rDNA sequences from *B. ostreae* (AF262995, AF192759). The *Bonamia* sp. detected in *O. edulis* from the Netherlands (JN040831) was also 99% similar to *B. ostreae*.

The putative *Bonamia* sp. SSU rDNA sequence found in *D. sandvicensis* from Hawaii was unique and only 90–91% similar to published sequences from *B. exitiosa*, *B. ostreae*, and *B. perspora* (DQ356000).

ITS region rDNA sequencing

Putative *Bonamia* sp. ITS rDNA sequences were amplified from all hosts from which presumed *Bonamia* sp. SSU rDNA sequences were characterized. GenBank accession numbers that were used in

subsequent ITS rDNA phylogenetic and distance analyses are listed in Table 3. Most *Bonamia* sp. ITS rDNA sequences were 98–100% similar to those from *B. exitiosa* in *O. chilensis* from New Zealand. Two sets of putative *Bonamia* spp. ITS rDNA sequences, those from *O. chilensis* from Chile and from *O. edulis* from California, were only 83–85% similar to the sequences from *B. exitiosa*; sequences from these same *Bonamia* spp. had been 99–100% similar to those from *B. exitiosa* in the SSU rDNA region. There was only 83–86% ITS rDNA sequence similarity between the groups from Chile and California *O. edulis*.

Both sets of putative *B. ostreae* ITS rDNA sequences found in *O. edulis* from California and the Netherlands showed similarity to *B. ostreae* from Maine (EU709108 and EU709110): 90–99% and 95–98%, respectively. A BLAST search of putative *Bonamia* sp. ITS rDNA sequences from *D. sandvicensis* from Hawaii resulted in significant alignments from only *Bonamia* spp., *Haplosporidium costale*, and *H. nelsoni* sequences. However, these were aligning only in the SSU rDNA portion of the amplicon; there were no identical or close matches to the ITS rDNA.

Molecular phylogenetics

For the Bayesian analyses, MrModeltest v. 2.3 chose GTR + I + Γ for the SSU and ITS rDNA data sets. For the SSU rDNA data, 10 000 trees were generated (10 000 000 generations, sample frequency = 1000), and the first 25% were removed as burn-in. For the ITS rDNA data, 40 000 000 generations were performed (sample frequency = 1000), resulting in 40 000 trees with the first 34 999 trees removed in order to compute the consensus. A 50% majority rule Bayesian consensus tree was generated based on the remaining 5001 trees.

Parsimony and Bayesian analyses of the SSU rDNA sequences yielded trees with similar topologies (Figs. 3 & 4). Both analyses suggested with strong support that the *Bonamia* sp. found in *D. sandvicensis* is basal to other *Bonamia* lineages. Both analyses portrayed *B. perspora* and *B. ostreae* as sister species, albeit with only weak support, and both placed the remaining *Bonamia* spp. sequences on a monophyletic clade with *B. exitiosa* and *B. roughleyi*. These included the *Bonamia* spp. from *O. edulis* and *O. lurida* from California, *O. chilensis* from Chile, *O. puelchana* and *O. stentina* from Argentina, *O. stentina* from Tunisia, *O. edulis* from Spain, *O. stentina* from New Zealand, *O. angasi* and *S. glomerata* from Australia, *C. ariakensis* from North Carolina and Florida, *O. chilensis* from New Zealand, and 2 partial *Bonamia* sp. SSU rDNA sequences from *O. edulis* from Italy.

Parsimony and Bayesian analyses of the ITS rDNA data revealed 6 well-supported clades (Figs. 5 & 6). All of the *Bonamia* sp. sequences found in *D. sandvicensis* from Hawaii formed one monophyletic clade, the *B. ostreae* sequences from Maine, California, France, and the Netherlands formed a second, and the *B. perspora* sequences formed a third. The remaining 3 clades belonged to a single monophyletic lineage that included all the ITS region sequences associated with parasites and SSU rDNA sequences that grouped with *B. exitiosa* in the SSU rDNA-based analyses. One of these clades, shaded in orange in Figs. 5 & 6, comprised ITS rDNA sequences from *B. exitiosa* from *O. chilensis* as well as *Bonamia* spp. from *O. stentina* from New Zealand, *C. ariakensis* from North Carolina and Florida, *O. stentina* from North Carolina and South Carolina, *O. stentina* and *O. puelchana* from Argentina, *O. lurida* from California, *O. angasi* and *S. glomerata* from Australia, and *O. stentina* from Tunisia. A second clade (shaded in gray) comprised the sequences amplified from *O. edulis* from California, and a third (shaded in red) comprised the *Bonamia* sp. sequences amplified from *O. chilensis* from Chile. Sister relationships were suggested between the *Bonamia* sp. from *O. edulis* from California and the *Bonamia* sp. from *O. chilensis* from Chile in the parsimony analysis (Fig. 5), and between the *Bonamia* sp. from California *O. edulis* and the *B. exitiosa*-containing clade in the Bayesian analysis (Fig. 6), but in neither case with strong support.

Distance analyses

Uncorrected p-distances between *Bonamia* spp. clades as indicated by the SSU rDNA phylogenetic analyses are shown in Table 4. The final data set contained an analysis of 1670 positions. Uncorrected p-distances among *B. ostreae*, *B. perspora*, and the clade containing *B. exitiosa* ranged from from 0.025 to 0.031, but were 0.084 to 0.086 in the comparisons including the Hawaiian *Bonamia* sp.

Mean uncorrected p-distances within each *Bonamia* clade as indicated by the ITS rDNA phylogenetic analyses ranged from 0.004 to 0.020 (Table 5). The greatest uncorrected p-distances (0.020) were among *B. ostreae* sequences and among the Hawaiian *Bonamia* sp. sequences. Values within the other clades ranged from 0.004 to 0.007. Mean uncor-

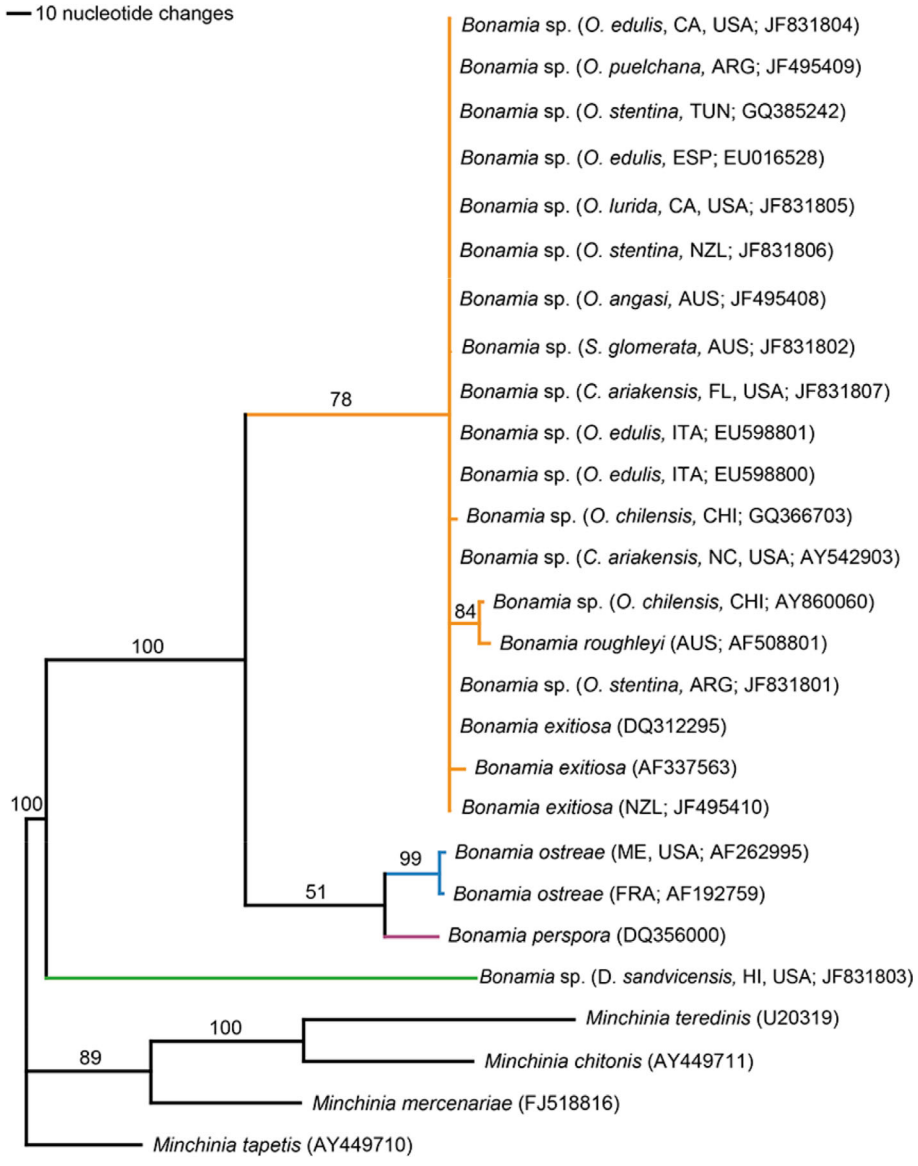

Fig. 3. *Bonamia* spp. small subunit (SSU) rDNA consensus phylogram. Parsimony analysis (1000 bootstrap replicates with 100 random additions) based on 285 informative characters was performed in PAUP* 4.0b10 (Swofford 2002) following alignment of *Bonamia* spp. SSU rDNA sequences using MAFFT v. 6 (Katoh & Toh 2008). Gaps were treated as missing. *Minchinia* species were chosen as the outgroup. Colors indicate distinct *Bonamia* lineages. CA: California; ARG: Argentina; TUN: Tunisia; ESP: Spain; NZL: New Zealand; AUS: Australia; FL: Florida; ITA: Italy; CHI: Chile; NC: North Carolina; ME: Maine; FRA: France; HI: Hawaii. See Table 1 for full species names

rected p-distances between each *Bonamia* clade as indicated by the ITS rDNA analyses ranged from 0.094 to 0.324 (Table 6). The lowest p-distances corresponded to comparisons among the *B. exitiosa* clades, i.e. those related to the parasites of *O. chilensis* from Chile and *O. edulis* from California (0.094–0.169). The p-distances for all other interclade comparisons ranged from 0.259 to 0.324. A total of 717 positions were analyzed in the ITS rDNA final data set containing 242 sequences from *B. exitiosa* and the other *Bonamia* sp. parasites whose sequences were drawn to its clade; 27 sequences from the *Bonamia* sp. from Chilean *O. chilensis*; 17 sequences from the *Bonamia* sp. from Hawaiian *D. sandvicensis*; 19 sequences from the *Bonamia* sp. from California *O. edulis*; 17 sequences from *B. perspora*; and 31 sequences from *B. ostreae*.

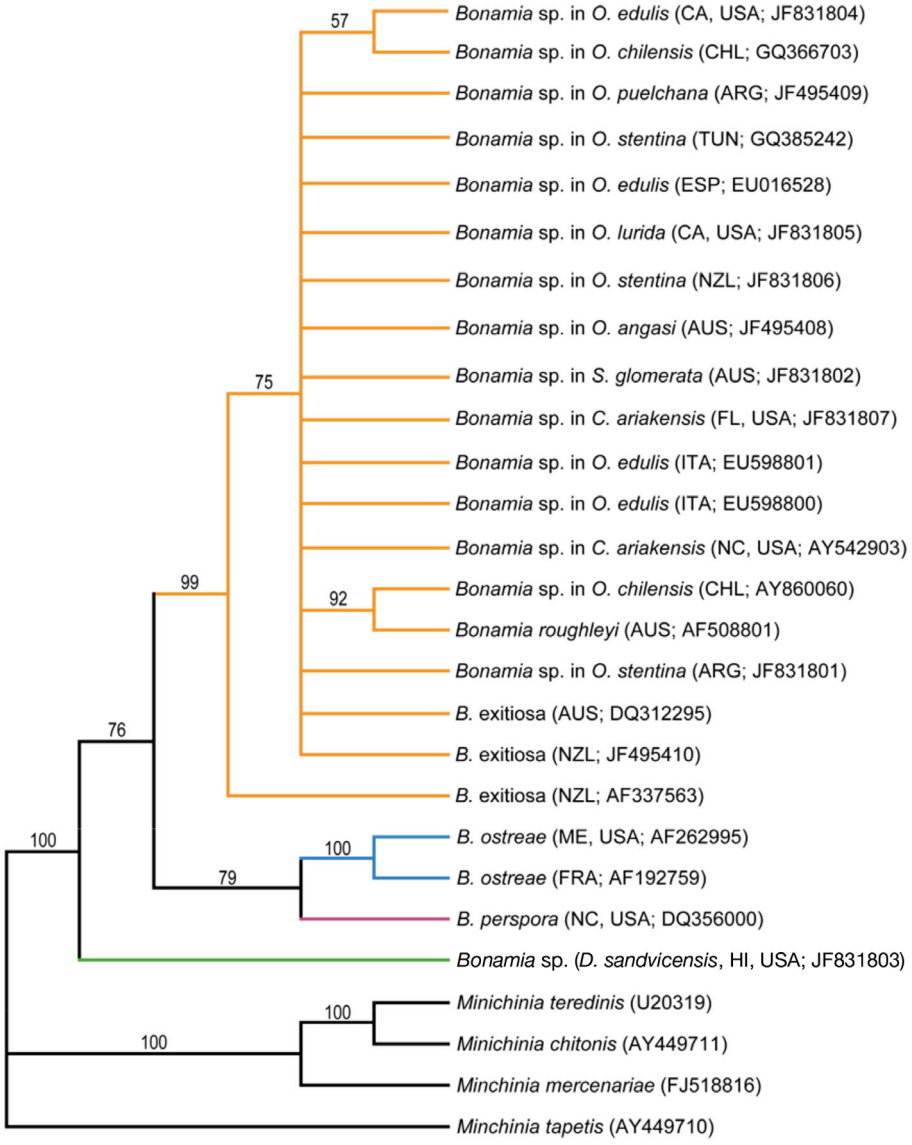

Fig. 4. *Bonamia* spp. small subunit (SSU) rDNA Bayesian 50% majority rule consensus tree. Bayesian inference analysis was conducted in MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) using model GTR + I + Γ, as determined by MrModeltest v. 2.3, following alignment using MAFFT v. 6 (Katoh & Toh 2008) of *Bonamia* spp. SSU rDNA sequences. Ten thousand trees were produced (10 000 000 generations, sample frequency = 1000), with 25% removed as burn-in. The consensus tree was generated using PAUP *4.0b10 (Swofford 2002) using *Minchinia* species as the outgroup. Colors indicate distinct *Bonamia* lineages. See Fig. 3 legend for location abbreviations; see Table 1 for full species names

Table 4. Mean uncorrected p-distance between group means for the small subunit rDNA data set. (below diagonal), and standard error estimates obtained by 1000 bootstrap replicates (above diagonal)

Species group	*B. exitiosa*	Hawaiian *Bonamia* sp.	*B. ostreae*	*B. perspora*
Bonamia exitiosa	–	0.007	0.004	0.004
Hawaiian *Bonamia* sp.	0.086	–	0.007	0.007
Bonamia ostreae	0.031	0.086	–	0.004
Bonamia perspora	0.025	0.084	0.029	–

Oyster mt16S rDNA sequencing

Mitochondrial 16S rDNA sequencing of infected host species confirmed the morphological identifications of oysters. GenBank BLAST searches revealed that the *O. edulis* mt16S rDNA sequence from California was identical to deposited *O. edulis* 16S rDNA sequences (DQ093488, DQ280032).

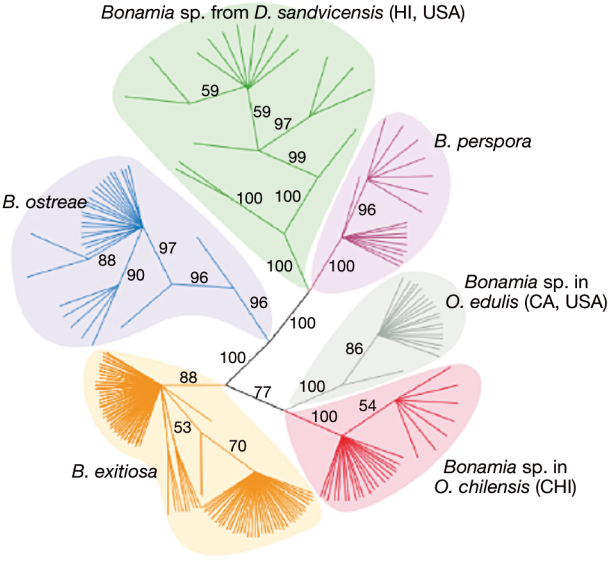

Fig. 5. *Bonamia* spp. internal transcribed spacer (ITS) rDNA unrooted parsimony consensus tree (50% majority rule). Parsimony analysis (100 bootstrap replicates with 10 random additions) conducted using a new technology search in TNT v. 1.1 (Goloboff et al. 2008) following alignment in MAFFT v. 6 (Katoh & Toh 2008) of *Bonamia* spp. ITS rDNA sequences. Bootstrap values less than 50 are not shown. The consensus tree was generated using PAUP* 4.0b10 (Swofford 2002). Colors indicate distinct *Bonamia* lineages. See Fig. 3 legend for location abbreviations; see Table 1 for full species names

Table 5. Mean uncorrected p-distance within *Bonamia* clades for the internal transcribed spacer region rDNA data set. See Table 1 for full species names

Bonamia clade	p-distance	No. sequences analyzed
Bonamia exitiosa	0.005	242
Chilean *Bonamia* sp. in *O. chilensis*	0.007	27
Hawaii *Bonamia* sp. in *D. sandvicensis*	0.020	17
California *Bonamia* sp. from *O. edulis*	0.004	19
Bonamia perspora	0.005	17
Bonamia ostreae	0.020	31

The mt16S rDNA sequence of *O. stentina* was 99% similar to *O. equestris* (=*O. stentina*; Shilts et al. 2007; AY376603) and *O. aupouria* (=*O. stentina*; Shilts et al. 2007; AF052064). The *O. lurida* mt16S rDNA sequence from California was identical to that of *O. lurida* from British Columbia, Canada (FJ768589). The *O. sandvicensis* mt16S region rDNA was 99% similar to that of the fingerprint oyster, *Dendostrea crenulifera* (EU815984, EU815985), and 94–95% similar to *Alectryonella plicatula* Gmelin 1790 (AF052072).

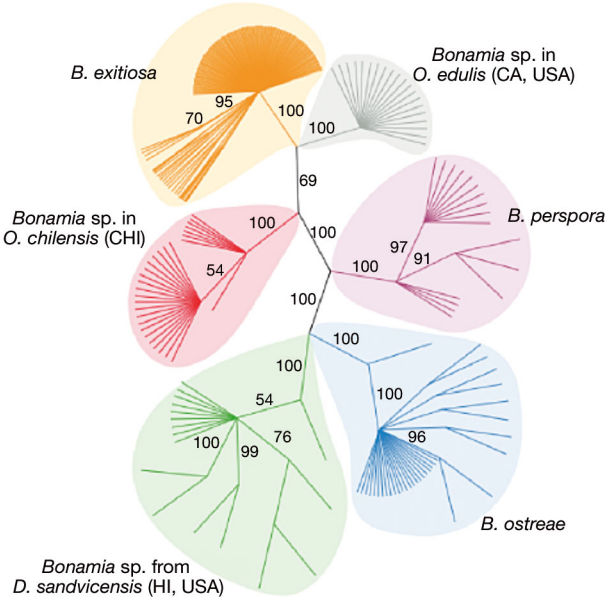

Fig. 6. *Bonamia* spp. internal transcribed spacer (ITS) rDNA unrooted Bayesian consensus tree (50% majority rule). Bayesian inference analysis was conducted in MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) using model GTR + I + Γ, as determined by MrModeltest v. 2.3, following alignment using MAFFT v. 6 (Katoh & Toh 2008) of *Bonamia* spp. ITS rDNA sequences. A total of 40 000 trees were produced (40 000 000 generations, sample freq. = 1000), with the first 34 999 trees removed in order to compute a consensus tree. Posterior probabilities less than 50 are not shown. A consensus of the remaining 5001 trees was generated using PAUP* 4.0b10 (Swofford 2002). Colors indicate distinct *Bonamia* lineages. See Fig. 3 legend for location abbreviations; see Table 1 for full species names

There were no *D. sandvicensis* sequences in GenBank. Molecular confirmation of the identity of *Bonamia* spp. PCR-positive *O. stentina* from Argentina was performed by Dr. Ami Wilbur at the University of North Carolina Wilmington (pers. comm.), and confirmation of the identity of *O. stentina* from Tunisia was described in Hill et al. (2010).

Histopathology and *in situ* hybridization

Microcells were observed in at least one individual from each oyster host that was PCR-positive for *Bonamia* spp. except *O. stentina* from New Zealand. New observations in *O. lurida* from California, *O. edulis* from California, and *D. sandvicensis* from Hawaii are illustrated in Fig. 7. The histological presentation of the parasite in each host was typical of infection by 'microcell haplosporidian' (Carnegie & Cochennec Laureau 2004) *Bonamia* spp.: parasite

Table 6. Mean uncorrected p-distances among *Bonamia* clades for the internal transcribed spacer region rDNA data set. (below diagonal), and standard error estimates obtained by 1000 bootstrap replicates (above diagonal)

Species group	*Bonamia exitiosa*	*Bonamia* sp. (HI, USA)	*Bonamia* sp. (Chile)	*Bonamia* sp. (CA, USA)	*Bonamia perspora*	*Bonamia ostreae*
Bonamia exitiosa	–	0.022	0.012	0.013	0.021	0.021
Bonamia sp. (HI, USA)	0.298	–	0.022	0.023	0.020	0.021
Bonamia sp. (Chile)	0.094	0.284	–	0.016	0.022	0.021
Bonamia sp. (CA, USA)	0.104	0.324	0.169	–	0.022	0.020
Bonamia perspora	0.275	0.259	0.295	0.292	–	0.021
Bonamia ostreae	0.263	0.313	0.292	0.286	0.281	–

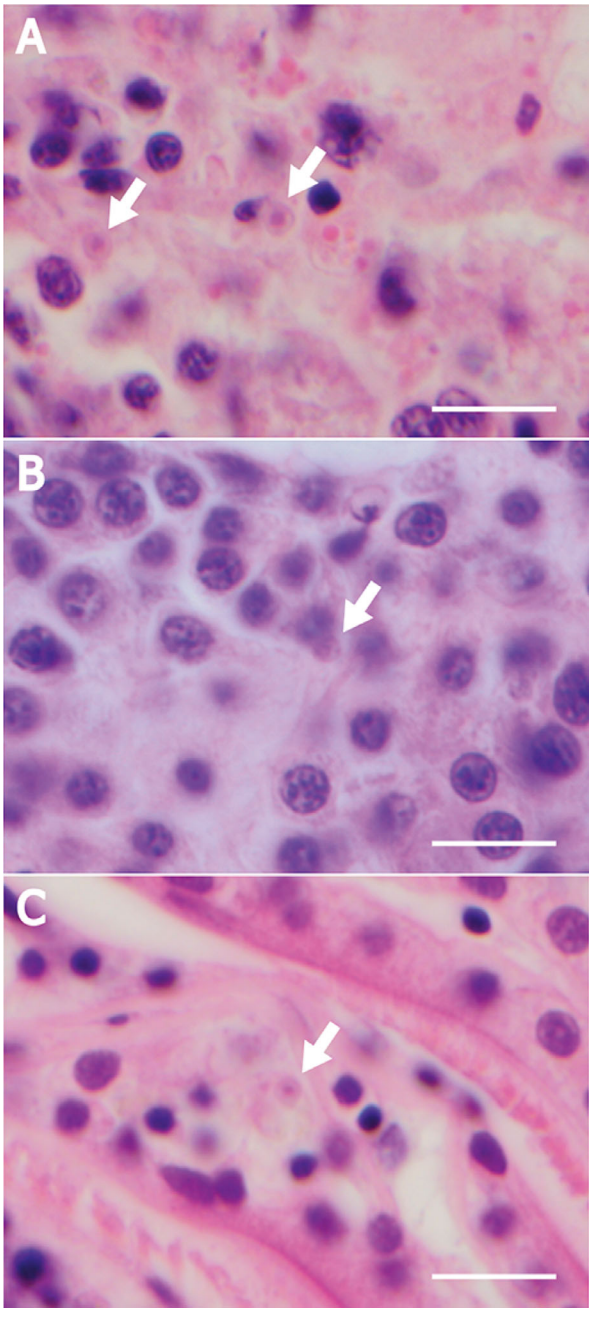

Fig. 7. Light microscopy of *Bonamia* spp. in hematoxylin & eosin-stained oyster tissue sections. (A) *Bonamia exitiosa* microcells (arrows) in an *Ostrea lurida* section from California, USA. (B) *Bonamia exitiosa*-like sp. microcell (arrow) in an *O. edulis* section from California. (C) *Bonamia* sp. microcell (arrow) in the gills of a *Dendostrea sandvicensis* section from Hawaii, USA. Scale bars = 10 µm

cells were generally intrahemocytic, sometimes extracellular where hemocytes had lysed, with no presentation of more conventionally haplosporidian forms such as spores or large multinucleate plasmodia. Infections were light to moderate in intensity except in *C. ariakensis*, which can be heavily infected (Burreson et al. 2004), and light to moderate hemocytosis was typically observed as a host response. Disruption of tissue was only modest.

In situ hybridization using digoxigenin-labeled probes was performed on 4 *C. ariakensis* from Florida, 4 *O. lurida* from California (3 individuals from the 2004 sample and 1 individual from the 2009 sample), 4 *O. edulis* from California, and 3 *O. stentina* from New Zealand. Hybridization within the tissues of the *B. exitiosa*-specific probes, which target SSU rRNA, was observed in *C. ariakensis* and in *O. lurida* and *O. edulis* (Fig. 8A,B), but not conclusively in the New Zealand *O. stentina*. Most infections as detected using this method were light. No case showed hybridization to the *B. ostreae*-specific probe, and positive and negative controls performed appropriately (Fig. 8C,D).

ISH using digoxigenin-labeled probes was also performed on 3 archival samples to verify microcell observations by histopathology and ultrastructural methods described in Farley et al. (1988). Parasites originally identified as *Mikrocytos mackini* in *Crassostrea gigas* from Kaneohe Bay, Hawaii, from 1972 hybridized to both the generic *Bonamia* spp. probe and the *B. exitiosa*-specific probes, as did the '*B. ostreae*' infecting *O. edulis* in oysters maintained in Chincoteague Bay (an indication that the generic identification of the latter parasite was correct, but

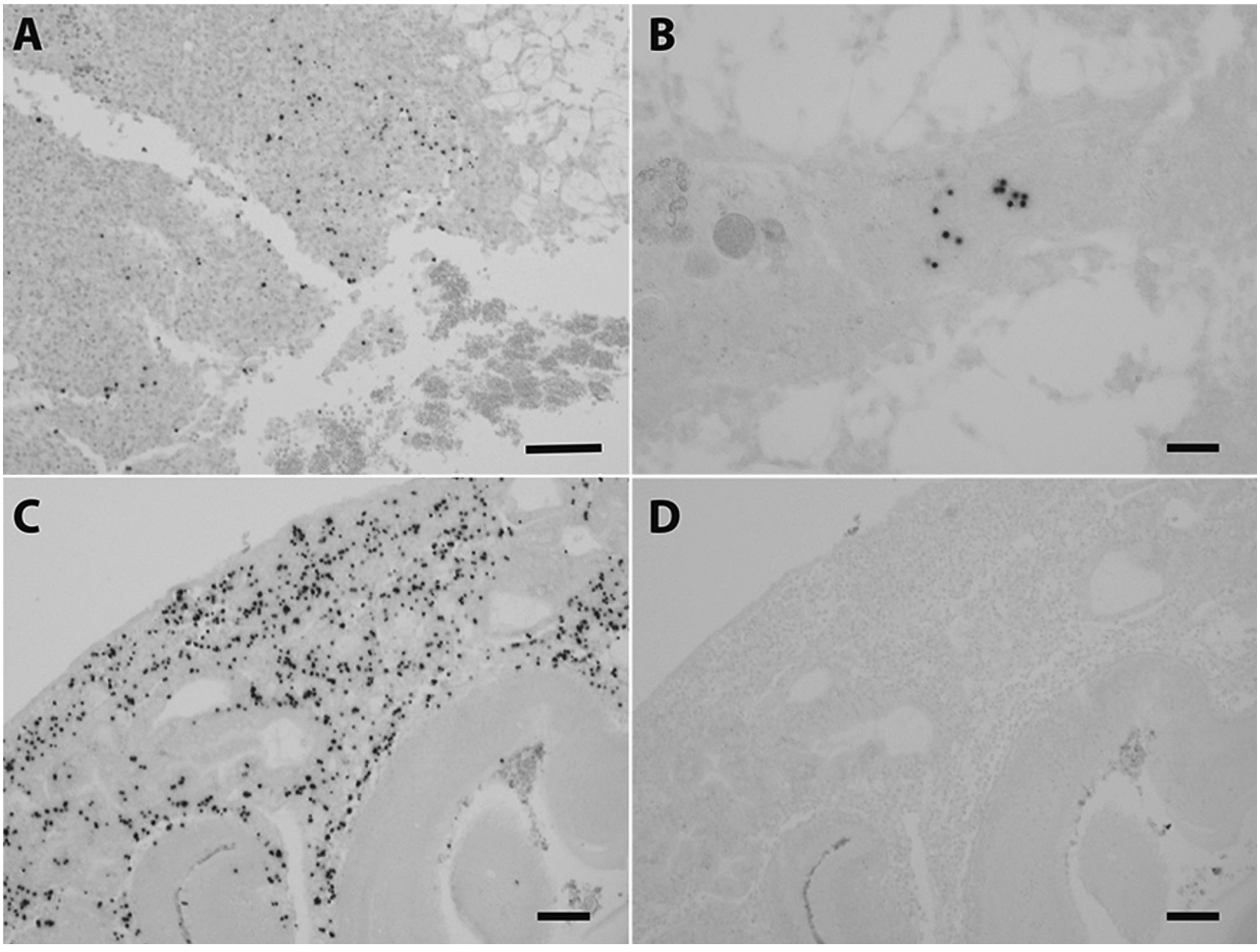

Fig. 8. *In situ* hybridization (ISH) images for molecular confirmation of histopathological diagnoses. Digoxigenin-labeled probe-based assays were specific for members of the *Bonamia exitiosa* small subunit rDNA clade in all cases. (A) Hybridization to *B. exitiosa* in *Ostrea lurida* from California, USA. Scale bar = 50 µm. (B) Hybridization to *Bonamia* sp. in *O. edulis* from California, USA. Scale bar = 20 µm. (C) Positive control hybridization to *B. exitiosa* infecting *Crassostrea virginica* from North Carolina, USA. Scale bar = 50 µm. (D) No-probe control. Scale bar = 50 µm

the specific identification was not). No hybridization to any *Bonamia* spp. probes occurred to parasites in the sample of experimental *O. edulis* that were 'fed tissues of moribund *O. edulis* from Pigeon Point, CA' (p. 158, Farley et al. 1988) from 1968, which were originally identified as *B. ostreae*. Again, all controls performed as expected.

FISH was performed on *D. sandvicensis* from Hawaii using probes HIBon-167 and HIBon-634 (Table 2) specific to this particular *Bonamia* species. Hybridization was observed in an oyster (the only oyster evaluated) that was diagnosed as a moderate, systemic infection by histology (Fig. 9A). The no-probe control showed no hybridization, and hybridization with these probes did not occur in samples infected with other known *Bonamia* species (e.g. *B. exitiosa*, Fig. 9B). Fluorescently labeled *B. exitiosa*-specific probes did not hybridize to the *D. sandvicensis* section (Fig. 9C), though they did to *B. exitiosa*-infected *C. virginica* (Fig. 9D).

DISCUSSION

From this study, it is apparent that *Bonamia* species have a wider geographic and host distribution and are more diverse than first appreciated. *Bonamia* spp. were detected by PCR, histology, and ISH in most oyster hosts and locations examined (Table 1). *Bonamia exitiosa* appears to be particularly widespread, infecting a variety of oyster hosts around the world, while *B. ostreae* and *B. perspora* seem to be

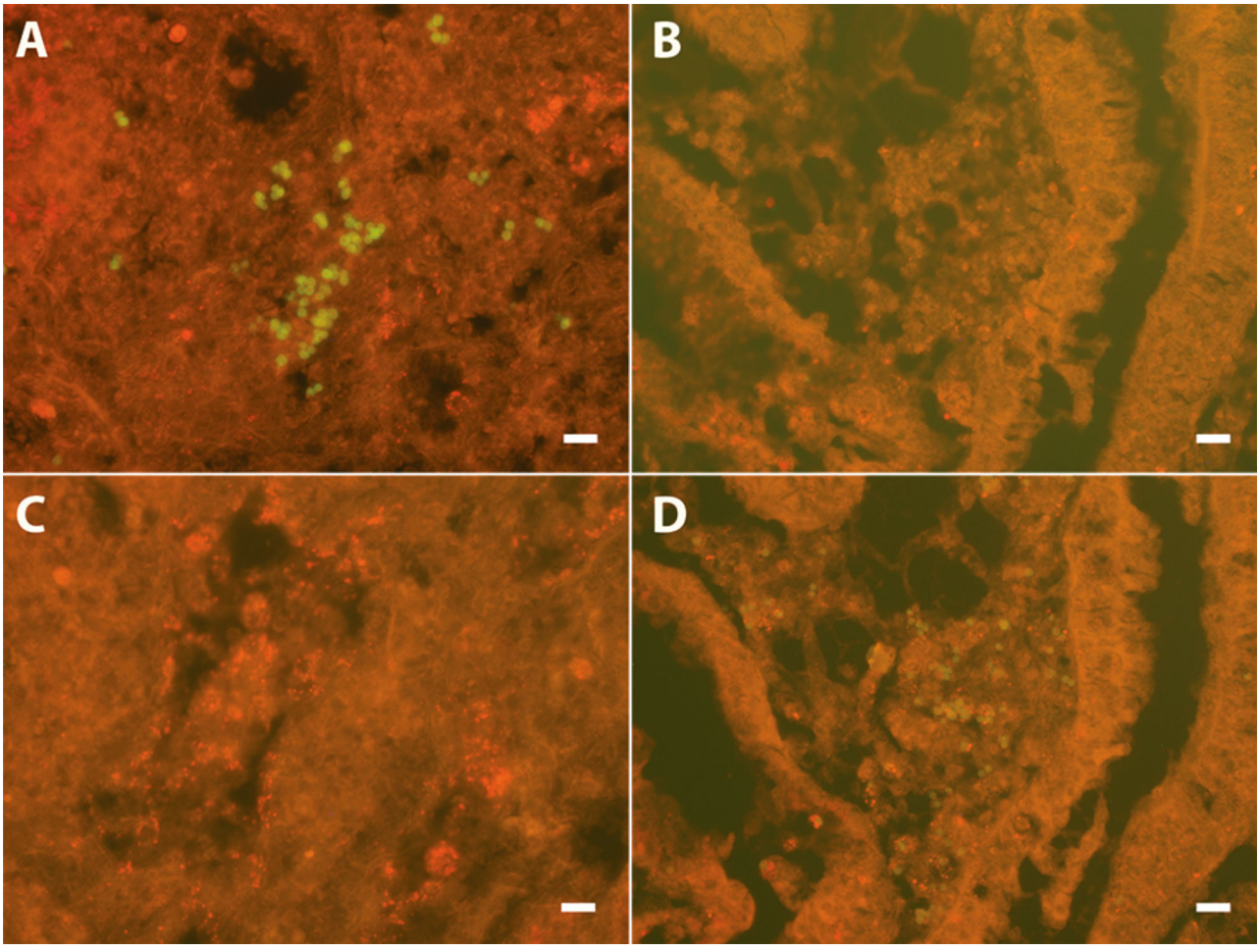

Fig. 9. Fluorescent *in situ* hybridization to the *Bonamia* sp. in *Dendostrea sandvicensis* from Hawaii, USA. (A) Hawaiian *Bonamia* sp.-specific probes hybridized to a *Bonamia* sp.-infected *D. sandvicensis* section. (B) Hawaiian *Bonamia* sp.-specific probes hybridized to a *B. exitiosa*-infected *Crassostrea virginica* section. (C) *B. exitiosa*-specific probes hybridized to a *Bonamia* sp.-infected *D. sandvicensis* section. (D) *Bonamia exitiosa*-specific probes hybridized to a section of *B. exitiosa*-infected *Crassostrea virginica* from North Carolina, USA. Scale bars = 20 µm

host specialists maintaining well-defined geographic ranges. Cryptic diversity was uncovered in Hawaiian *Dendostrea sandvicensis*, Chilean *Ostrea chilensis*, and Californian *O. edulis* hosts.

Bonamia exitiosa and its relatives

Phylogenetic analyses of SSU and ITS rDNA revealed the affinity of most newly characterized *Bonamia* sp. sequences from various hosts to *B. exitiosa*. In all phylogenetic analyses (Figs. 3–6), *Bonamia* sequences found in *Crassostrea ariakensis* from Florida and North Carolina, *O. stentina* from North Carolina, South Carolina, Argentina, Tunisia, and New Zealand, *O. puelchana* from Argentina, *O. lurida* from California, *O. angasi* and *Saccostrea glomerata* from Australia, and *O. chilensis* from New Zealand appeared in the same clade as *B. exitiosa*. *Bonamia* spp. sequences from *O. edulis* from California and *O. chilensis* from Chile appeared in the *B. exitiosa* clade in SSU rDNA phylogenetic analyses (Figs. 3 & 4), but diverged from this clade in the ITS rDNA analyses (Figs. 5 & 6). Histological presentation of parasites in these clades was similar in all cases, underscoring the limited utility of microscopy for uncovering *Bonamia* diversity. The more high-resolution ITS rDNA-based analyses were capable of resolving distinct lineages, and thus are useful for conservatively determining the species boundaries of *B. exitiosa*. We conclude that all the *Bonamia* sp. sequences appearing in the same clade as *B. exitiosa* in both the SSU and ITS rDNA phylogenetic analyses should be identified as *B. exitiosa*. Inclusion within *B. exitiosa* of the para-

sites from *O. chilensis* in Chile and *O. edulis* in California may not be justified, and these lineages may represent novel species. Further analyses using more expansive genetic analyses and experimental challenge systems may provide more perspective here. There was some more modest ITS rDNA sequence divergence evident within the *B. exitiosa* clade (Figs. 5 & 6), but this had no strongly supported pattern with regard to geography or host that could be discerned from these analyses.

ISH detection not only of a member of the genus *Bonamia* but of a parasite closely related to *B. exitiosa* in archival histological materials of *C. gigas* from Hawaii and *O. edulis* from Chincoteague Bay, Virginia, was unexpected. Without ITS rDNA region sequence data, it is impossible to know whether these parasites belonged to the *B. exitiosa* lineage proper, to the 2 related lineages comprising sequences from the *Bonamia* sp. infecting *O. chilensis* in Chile and *O. edulis* in California, or to another related group; ISH using the CaBon probe cocktail of Hill et al. (2010) could not distinguish among these possibilities. Given the very wide geographic and host range of *B. exitiosa*, it is conceivable that our observations revealed the presence of this particular parasite. Determining whether these locations presently harbor *B. exitiosa* or a related species in one host or another (*C. gigas* or *D. sandvicensis* in Hawaii, *C. virginica* in Virginia) should be a priority for aquatic animal health managers.

Histopathology and ISH provided visual confirmation of infections for all *B. exitiosa* and *Bonamia* spp. observations (Figs. 7–9), except for the parasite amplified from *O. stentina* from New Zealand. For the New Zealand samples, there was considerable tissue loss during the slide washing process, so it is possible that *Bonamia* cells were lost along with the tissue. However, *B. exitiosa* has been visualized in *O. stentina* from North Carolina, South Carolina, Argentina, and Tunisia, so this oyster is a known host. It is plausible, therefore, that *B. exitiosa* also infects not only *O. chilensis* but *O. stentina* as well in New Zealand.

Bonamia ostreae and *B. perspora*

Based on the locations surveyed and previous studies, *B. ostreae* and *B. perspora* demonstrate narrower host and geographic ranges than *B. exitiosa*. *Bonamia ostreae* has only been detected in *O. edulis*. Putative *Bonamia* sp. SSU and ITS rDNA sequences found in *O. edulis* from the Netherlands and some sequences found in *O. edulis* from California are within the same clade as *B. ostreae* from Maine and France (Figs. 3–6). This study did not examine southern hemispheric *O. edulis*, which have been reported to occur in Australia (Morton et al. 2003) and in South Africa (FAO 2007–2011, Haupt et al. 2010). Therefore, the question remains as to whether *B. ostreae* is present within its type host in the Southern Hemisphere. A host specialist strategy and a potential inability to survive variable environmental conditions (i.e. it prefers cool, temperate climates) may provide barriers to dispersal for this particular species.

The data collected to date suggest that *B. perspora*, like *B. ostreae*, is also a host specialist, but with an even more restricted geographic distribution. *B. perspora* has only been detected in *O. stentina* from North Carolina (Carnegie et al. 2006), and *B. perspora* was never detected in *O. stentina* evaluated elsewhere (Argentina, Tunisia, and New Zealand). One explanation could be that *B. perspora* has only been found at very low prevalence (<5.6% prevalence; Carnegie et al. 2006), so perhaps larger sample sizes will be needed in order to detect this parasite. Alternatively, *B. perspora* could have been established in North Carolina *O. stentina* after a geographic separation of conspecific hosts. More sampling would need to be done in order to test either hypothesis.

While a sister relationship between *B. perspora* and *B. ostreae* was suggested by both the parsimony and Bayesian analyses of SSU rDNA data (Figs. 3 & 4), it was not well supported. Analyses of additional loci may shed further light on this relationship. The fact that the ITS rDNA-based analyses failed to show this sister relationship should not be taken as lack of support. The divergence of ITS rDNA sequences among *Bonamia* species may result in alignments that are reliable intra-specifically but unreliable across species, making resolution of inter-specific relationships impossible using this locus.

Bonamia sp. in *Dendostrea sandvicensis* from Hawaii

While analyses based on SSU rDNA sequences do not provide sufficient resolution to distinguish all *Bonamia* species, the divergence of this parasite's sequence at this locus as well as the phylogenetic analyses based on this region suggest that the species found in *D. sandvicensis* from Hawaii is a novel species.

We expected that the putative *Bonamia* sp. SSU rDNA sequence detected in *D. sandvicensis* would be

another observation of *B. exitiosa*, since this parasite was detected by ISH in archival *C. gigas* tissue sections taken in 1972 from Hawaii. However, when contemporary sequences were compared with those from other described *Bonamia* spp., they were only 90–91% similar, whereas the identity among currently described *Bonamia* species is 94–98%. The Hawaiian *Bonamia* sp. was 85% similar to SSU rDNA of *Minchinia tapetis* (AY449710) and *M. mercenariae* (FJ518816), species within the sister genus to *Bonamia* in the haplosporidian phylogeny (Reece et al. 2004). The variability in SSU rDNA among *Bonamia* species is low (similarity ≥91% among *Bonamia* spp. overall, and ≥97% when the Hawaiian *Bonamia* sp. is excluded) when compared with other closely related taxa, such as *Minchinia* species, where similarity ranges from 86 to 91% between SSU rDNA sequences of differing species within the genus (from BLAST results). Based on SSU rDNA sequencing data alone, then, it is unlikely that the *Bonamia* sp. from Hawaii is *B. exitiosa* or any other described species. The presentation of the parasite conforms to that of typical microcell haplosporidian *Bonamia* species: a 2–3 µm, uninucleate cell predominantly associated with hemocytes (Fig. 7C). It is unlike the presentation of *Minchinia* spp. and the atypical *B. perspora*, in which uninucleate cells would be primarily extracellular and accompanied by multinuclear and sporogonic forms (Carnegie et al. 2006, Ford et al. 2009). Therefore, while this *Bonamia* sp. is relatively divergent from the other *Bonamia* species, its histological presentation does support its inclusion in the genus *Bonamia*.

Both SSU rDNA sequence phylogenetic analyses provided strong support for the inclusion of the Hawaiian *Bonamia* sp. in the *Bonamia* clade (Figs. 3 & 4). The ITS rDNA analyses provided further support for this being a distinct species as the *Bonamia* sp. sequences obtained from *D. sandvicensis* form a distinct monophyletic clade in unrooted trees (Figs. 5 & 6). The basal position of the parasite in the SSU rDNA-based analyses in particular raises interesting questions. First, did the *Bonamia* genus arise in cool temperate euhaline environments, in which its members have generally been known to exist? Or, given the basal position of a tropical *Bonamia* sp. infecting a tropical oyster host, is this a genus whose origins are among the fauna of tropical seas? Related to this is the phylogenetic position of the host itself. *Bonamia* spp. typically infect members of the families Ostreinae (which includes the genus *Ostrea*) and Crassostreinae (which includes *Crassostrea* and *Saccostrea*; Harry 1985), but *D. sandvicensis*, which the basal Hawaiian *Bonamia* sp. infects, is a member not of the Ostreinae but of the family Lophinae, which includes generally tropical genera (Harry 1985). Members of the Lophinae are thought to be evolutionarily older than the Ostreinae, based on paleontological analyses (Stenzel 1971) and molecular evidence including partial 28S rDNA (Littlewood 1994) and 16S mtDNA sequences (Jozefowicz & Ó Foighil 1998). Therefore, if the placement of the Hawaiian *Bonamia* sp. at the base of the entire *Bonamia* clade is correct, origins of the genus *Bonamia* not only in the tropics but also among tropical lophine oyster hosts might be hypothesized.

A second and more important question arises from the basal position of the Hawaiian *Bonamia* sp. What does the phylogeny say about the evolution of *Bonamia* sp. life strategies? Of all the known *Bonamia* spp., the only one that clearly has retained the ancestral haplosporidian traits of extracellular infection by various vegetative and sporogonic cell forms and, presumably, a complex life cycle is *B. perspora*. The other *Bonamia* spp., including the Hawaiian *Bonamia* sp. and the *B. exitiosa*-related lineages infecting *O. chilensis* in Chile and *O. edulis* in California, are typical microcell haplosporidians (Carnegie & Cochennec-Laureau 2004), presenting small uninucleate ameboid cells almost exclusively, intracellularly infecting oyster hemocytes, and displaying (presumably) direct transmission among hosts. Yet while its histological presentation and expression of cell forms suggests that *B. perspora* should be basal to the other *Bonamia* spp., which would lead to the parsimonious hypothesis of a single adoption of the microcell haplosporidian life cycle (intracellularity, abandonment of sporulation, direct transmission; see Fig. 10A) in the Haplosporidia, the SSU rDNA-based phylogenies suggest that it is not. Three possible explanations are thus raised. First, the SSU rDNA-based gene trees may be inaccurate representations of the species relationships. Second, switching between life strategies actually happened several times in the evolution of the genus. The microcell haplosporidian life strategy may have been adopted on 3 separate occasions, in lineages giving rise to *B. ostreae*, to the Hawaiian *Bonamia* sp., and to the *B. exitiosa*/Chilean *Bonamia* sp./Californian *Bonamia* sp. lineage (Fig. 10B). Or, less plausibly, it may have been adopted once, but with the 'conventional' haplosporidian strategy arising once again on the lineage giving rise to *B. perspora* (Fig. 10C). A third possible explanation is that all *Bonamia* spp. can pursue either strategy, depending on host and environmental circumstances, and that our present perspective is impaired by our limited observations. Comparative genomic analyses

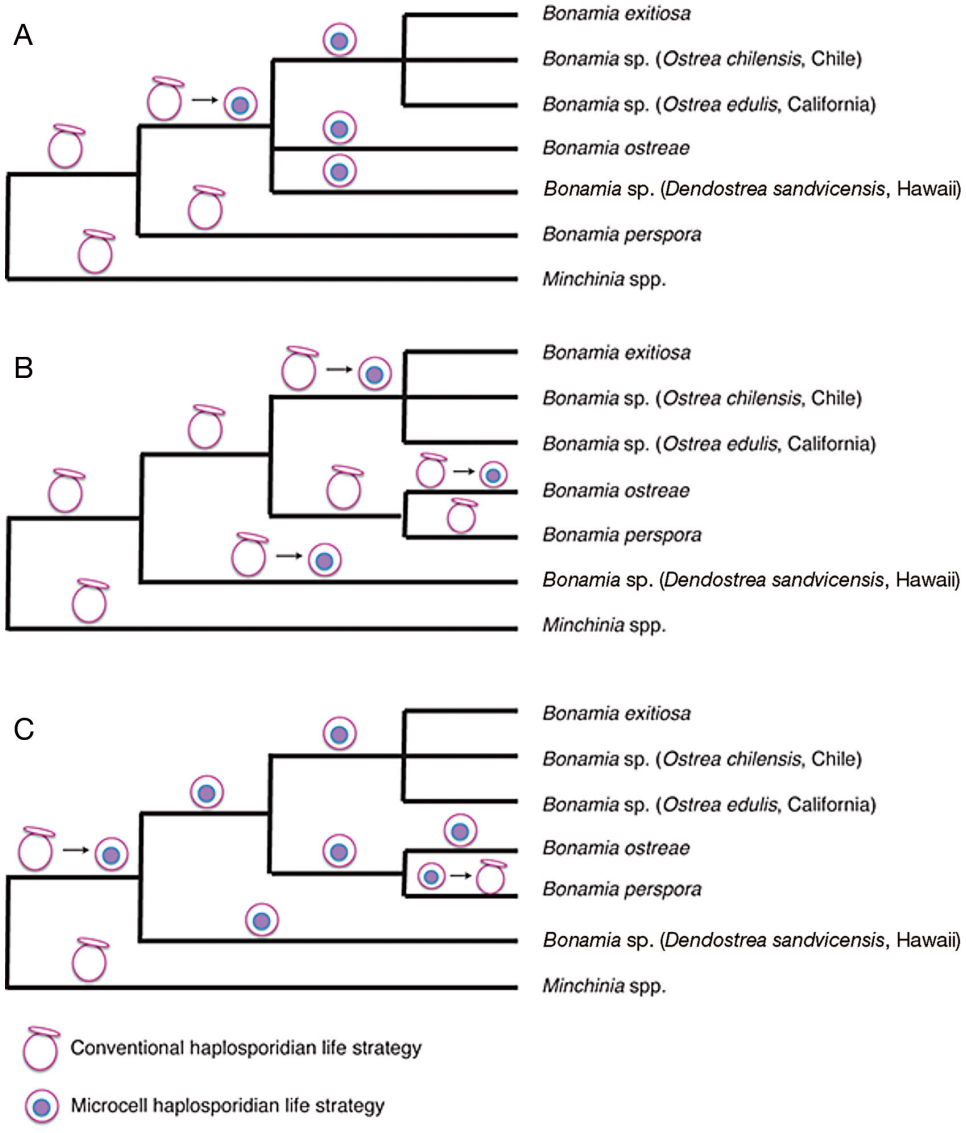

Fig. 10. Models of life history evolution in the genus *Bonamia*. (A) Most parsimonious tree based on morphology, with a single adoption of the microcell haplosporidian strategy of direct transmission of uninucleate, generally intrahemocytic microcells. (B) First alternative model overlaid on the small subunit (SSU) rDNA phylogenies determined herein, implausibly hypothesizing 3 separate adoptions of the microcell haplosporidian strategy. (C) Second alternative overlaid on the SSU rDNA phylogenies, hypothesizing a single adoption of the microcell haplosporidian strategy and then a subsequent restoration of a convention haplosporidian strategy. A fourth model (not illustrated) would hypothesize that *Bonamia* species can alternate between the 2 strategies, depending on host and environmental conditions

may ultimately reveal whether the architecture underlying sporulation and the conventional haplosporidian life strategy has been lost or is still maintained in species such as *B. ostreae* and *B. exitiosa*.

Samples with no detection of *Bonamia* species

Non-detection by PCR of a *Bonamia* sp. in some samples may have been due to low sample sizes in some cases (Table 1), if not an actual absence from some locations. There has never been a report of a *Bonamia* sp. infecting *O. denselamellosa* in Japan, although this species has not received much parasitological attention. While *Isognomon* sp. from Florida were also negative, it is not known whether members of the bivalve family Pteriidae are susceptible to *Bonamia* sp. parasitism at all. We must also consider the possibility that a *Bonamia* sp. might have been present in some samples but genetically

divergent at primer binding sites, leading to false negative results.

The most noteworthy non-observation was of *B. roughleyi* in Australian or New Zealand samples of *S. glomerata*. Sequences bearing the *B. roughleyi*-diagnostic signatures identified by Cochennec-Laureau et al. (2003) were not found in any sample at any location, and cryptic ITS rDNA region lineages such as were observed in Chilean *O. chilensis* and Californian *O. edulis*, which might indicate the presence of *B. roughleyi*, were not observed in *Bonamia*-positive oysters from the region. Our present work supports the conclusions of Hill et al. (2010) and Carnegie et al. (2014) that there is no genetic basis for identifying any *Bonamia* sp. besides *B. exitiosa* in Australia or New Zealand.

Inter- and intra-clade distances, and patterns of diversity

Two observations emerge from the phylogenetic analyses that are reinforced by the distance analyses. First, the *Bonamia* sp. from Hawaii is the most divergent *Bonamia* species known based on SSU rDNA sequence data, with p-distances in every comparison more than twice as great as those of any of the other *Bonamia* species (Table 4). While the ITS rDNA-based p-distances do not show the same level of inter-lineage divergence (Table 6), this may again relate to the unreliability of the ITS rDNA-based analyses for reconstructing interspecific relationships. The ITS rDNA data are stronger with regard to intra-specific comparisons, and here another notable observation emerges: while the ITS rDNA-based analyses showed divergence to be greatest among the sequences from the Hawaiian *Bonamia* sp. ($p = 0.20$, Table 5), the intra-specific divergence among *B. ostreae* sequences was just as high. Intra-specific divergence among sequences of *B. perspora*, *B. exitiosa*, and the *B. exitiosa*-related lineages was lower by a factor of 3–5, and divergence was unrelated to the intensity of sampling of the lineages. While one may speculate that the lack of divergence in *B. exitiosa* may represent a recent radiation, particularly given the very recent detection of this parasite (and its relatives) over such a broad range, we must recognize that the new observations of *B. exitiosa* are due partly to a new interest in applying molecular tools to uncover *Bonamia* diversity. It does not necessarily indicate a rapid radiation and dispersal, i.e. a *B. exitiosa* panzootic. The similar lack of divergence in *B. perspora*, a very different parasite in its display of the ancestral haplosporidian cell forms and presumably life cycle, would also have to be explained. Further exploration of the patterns of diversity within and among *Bonamia* lineages must await application of a wider suite of molecular tools.

CONCLUSIONS

The diversity of *Bonamia* species is wider than previously appreciated, including 3 valid described species (*B. ostreae*, *B. exitiosa*, and *B. perspora*) plus 3 additional lineages, infecting *O. chilensis* in Chile, *O. edulis* in California, and *D. sandvicensis* in Hawaii, that warrant descriptions as new species. The undescribed parasites from *O. chilensis* and *O. edulis* are distinguished from *B. exitiosa* only at the ITS rDNA locus, which we recommend should be used for determination of the species boundaries of *B. exitiosa* as well as other *Bonamia* species.

The discovery of a *Bonamia* sp. infecting Hawaiian *D. sandvicensis* underscores the potential for important new observations to emerge when surveys of diversity include little-studied, non-commercial marine fauna. It also raises new questions concerning the evolution of haplosporidian life strategies. Most intriguingly, we might ask whether all *Bonamia* species, and not just *B. perspora*, may be capable of 'conventional' haplosporidian replication and transmission under appropriate conditions.

Acknowledgements. We thank the many colleagues who assisted with collection of materials. Allen Fraser (New Zealand Ministry of Fisheries), Wilma Blom and Gordon Nicholson (Auckland War Memorial Museum, New Zealand), and Rodney Roberts (Cawthron Institute) performed oyster collections in New Zealand. Susan Laramore and John Scarpa (Harbor Branch Oceanographic Institute) provided the *Crassostrea ariakensis* from Florida. Jessica Moss Small and Hamish Small (Virginia Institute of Marine Science) provided the *Isognomon* sp. from Florida. Loren Coen (South Carolina Department of Natural Resources) provided *Ostrea stentina* from South Carolina. Naoki Itoh (Tohoku University, Miyagi, Japan) provided *O. denselamellosa*. Nejla Bejaoui (Institut National Agronomique de Tunisie, Tunis, Tunisia) provided Tunisian *O. stentina*. Marc Engelsma (Central Veterinary Institute, Lelystad, the Netherlands) provided samples of Dutch *O. edulis*. Gary Meyer provided *O. lurida* from British Columbia. Rita Crockett and Susan Denny (Virginia Institute of Marine Science) performed all histology. TNT v. 1.1 software was available through the sponsorship of the Willi Hennig Society. This is VIMS Contribution Number 3367.

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Editorial responsibility: Marc Engelsma, Lelystad, The Netherlands

Submitted: July 29, 2013; Accepted: February 27, 2014
Proofs received from author(s): May 22, 2014