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Phylogeographic perspective on the distribution and dispersal of a marine pathogen, the oyster parasite *Bonamia exitiosa*

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ABSTRACT: The significance of infectious disease has intensified as our marine ecosystems are increasingly altered, with molluscan taxa being among the affected. One of the important pathogens to emerge in recent years, the oyster parasite *Bonamia exitiosa,* has a broad geographic distribution and has been found to infect a number of oyster species. In order to better understand how *B. exitiosa* achieved this wide distribution, a gene genealogy was constructed using internal transcribed spacer region ribosomal DNA sequencing data from across the host species range. The analysis revealed population structure in the form of 4 well-defined groups of sequences: 3 corresponding to geographic regions (temperate Atlantic and Pacific waters of the Southern Hemisphere, California, and the western Atlantic along the coast of the Americas) and the fourth geographically cosmopolitan. Inclusion of *B. exitiosa* sequences from New Zealand, Australia, and Argentina in the Southern Hemisphere group may reflect natural dispersal of the parasite via rafting with oyster hosts, whereas the California group may reflect limited anthropogenic movement of a host species, *Ostrea lurida*. The extensive geographic distribution of *B. exitiosa* parasites belonging to the cosmopolitan and Atlantic Coast groups may relate to both natural and anthropogenic dispersal of a single host, *O. stentina*, which is distributed from the eastern Americas to the Mediterranean and African coast to New Zealand — that is, in most regions where *B. exitiosa* has been found to occur.

KEY WORDS: Marine disease · *Bonamia* · Haplosporidia · Phylogeography · *Ostrea stentina*

INTRODUCTION

Infectious disease has grown in significance in our increasingly impacted and altered marine ecosystems (Harvell et al. 1999), with mollusks being among the affected taxa (Ward and Lafferty 2004). Protozoan parasites in the genus *Bonamia* (Haplosporidia; Sprague 1979) are one of the major threats to oyster populations, yet we know little about how and when they came to achieve their current distributions. *B. exitiosa* (Hine et al. 2001, Berthe & Hine 2003) in particular has caused large-scale mortalities in New Zealand *Ostrea chilensis* since it was discovered in Foveaux Straight, New Zealand, in the 1980s (Dinamani et al. 1987), with retrospective evidence that it was associated with disease events as early as 1964 (Hine et al. 2001). Since its description, *B. exitiosa* has been observed in several commercial and non-commercial oyster hosts in various locations around the world (Hill et al. 2014). In addition to *O. chilensis* from New Zealand, it has been noted to infect *O. angasi* and *Saccostrea glomerata* in Australia; *O. puelchana* and *O. stentina* (= *O. equestris*, Shilts et al. 2007) from Argentina; wild *O. stentina* (=

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O. equestris, Shilts et al. 2007) and experimental *Crassostrea ariakensis* along the southeastern US coast; *O. lurida* in California, USA; *O. edulis* in England, Spain, and Italy; and *O. stentina* in Tunisia (Kroeck & Montes 2005, Corbeil et al. 2006, Abollo et al. 2008, Hill et al. 2010, 2014, Narcisi et al. 2010, Longshaw et al. 2013). *B. exitiosa* also potentially infects *O. stentina* (= *O. aupouria*, Shilts et al. 2007) from New Zealand (based on PCR results only; Hill et al. 2014). It is not yet clear how the disease it causes (known as bonamiasis) impacts some populations, especially with respect to non-commercial hosts. However, because *B. exitiosa* has been the cause of severe mortality in some oyster species in the wild (Doonan et al. 1994, Cranfield et al. 2005) and in aquaculture systems (Burreson et al. 2004), understanding how it came to achieve its wide distribution is important. If the more recent observations of *B. exitiosa* are the result of contemporary introductions rather than long-established presences that have gone unnoticed, it is imperative that preventative measures be taken to obviate similar economic and ecological losses due to accidental introductions elsewhere.

Phylogeographic studies explore the principles and processes involved in the geographical distributions of genealogical lineages, especially those within and among closely related species (Avise 2000). With the rise in global connectivity, these studies are becoming increasingly useful for tracking the concomitant dispersal of organisms around the world. Some *B. exitiosa* dispersal hypotheses have already been proposed, but these have been based mainly on the occurrence of epizootics and/or conjecture about the parasite's supposed presence/absence. For example, *B. exitiosa* purportedly reached Australia from its presumed origins in New Zealand through shipment of live, commercial-sized oysters, which were held in Victorian and Tasmanian waters in the early 1990s (Hine & Jones 1994, Hine 1996). Additionally, Abollo et al. (2008) detected *B. exitiosa* in *O. edulis* in Galicia, NW Spain, and they hypothesized that the parasite could have been inadvertently introduced through the legal or illegal importation of oysters from *B. exitiosa*-endemic areas. The authors also suggested the possibility of an introduction via the ballast water and outer hulls of ships, which was a hypothesis proposed by Bishop et al. (2006) regarding the presence of *B. exitiosa* (then identified only as *Bonamia* sp.) in North Carolina *C. ariakensis* and *O. stentina*. We sought to test the validity of existing hypotheses and to develop additional hypotheses regarding the dispersal of *B. exitiosa* using network analysis to examine internal transcribed spacer region ribosomal DNA (ITS rDNA) sequences of *B. exitiosa* found in New Zealand, Australia, Argentina, Tunisia, and along the east and west coasts of the USA.

MATERIALS AND METHODS

Sample collection and DNA extraction

Samples of 7 oyster species were obtained from 10 locations (Table 1). Oysters were shucked, and small pieces of gill and mantle tissue (~3–5 mm^3) were preserved individually in 95% ethanol or placed directly in lysis solution (QIAamp DNA Kit, Qiagen), except for the 2004 California *Ostrea lurida* samples, where 3 to 4 individuals were placed in the same tube and stored at −80°C until being transferred to 100% ethanol for shipping. Genomic DNA from each oyster sample was extracted using a Qiagen QIAamp DNA Kit. DNA was eluted in 100–225 µl of elution buffer and stored at 4°C.

PCR, cloning, and sequencing

PCR, cloning, and sequencing of ITS region rDNA was performed as described by Hill et al. (2014). Briefly, primers HaploITSf (Hill et al. 2010) and ITS-B (= reverse primer D; Goggin 1994) were used to amplify a ~750 base pair (bp) product, which includes ~220 bp of the 3′ end of the small subunit ribosomal RNA (SSU rRNA) gene, the complete ITS-1, 5.8S gene, and ITS-2 region rDNA, and a short fragment (~20 bp) of large subunit (LSU) rDNA. 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. 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, followed by a final extension at 72°C for 7 min.

Purified PCR products were cloned into plasmid vector pCR4-TOPO using the TOPO TA Cloning Kit (Invitrogen), and then transformed into One Shot TOP10 competent *Escherichia coli* cells (Invitrogen). Bacterial colonies containing plasmid inserts of the appropriate size were cultured and then extracted using the QIAprep Spin Miniprep Kit protocol (Qiagen). Primers HaploITSf (Hill et al. 2010) and ITS-B

Table 1. Collection information detailing host species names, locations, and dates from which *Bonamia exitiosa* internal transcribed spacer region rDNA sequences were recovered, and cloning data including GenBank accession numbers of sequences used in TCS and genetic differentiation analyses. Sample size and prevalence information can be found in Hill et al. (2014). The number of individuals sequenced indicates the number of individual oysters from which *B. exitiosa* sequences were recovered

Sampling location	Host	Collection date(s)	No. of ind. sequenced	Avg no. of *B. exitiosa* clones per ind.	Avg no. of unique *B. exitiosa* clones per ind.	Total no. of *B. exitiosa* clones	Total no. of unique *B. exitiosa* clones	GenBank accession numbers
Argentina								
San Antonio Bay	*Ostrea puelchana*	Mar 2005	5	14.0	9.2	70	46	JF831556–JF831574
	Ostrea stentina	Apr 2007	3	8.0	6.3	24	19	EU709055–EU709061; EU709063; EU709064; EU709067; JF831603–JF831638
Australia								
Pambula River, New South Wales	*Ostrea angasi*	Nov 2006	1	4.0	4.0	4	3	JF831678–JF831680
Georges River, New South Wales	*Saccostrea glomerata*	Aug 2007	1	11.0	4.0	11	4	JF831681–JF831684
California, USA								
Elkhorn Slough	*Ostrea lurida*	May 2004; Sep 2009	5	20.8	16.4	104	82	JF831719–JF831800
New Zealand								
Tamaki Estuary, Auckland	*Ostrea stentina*	Aug 2007; Mar 2009	4	8.0	5.0	32	20	EU709069; EU709071; EU709073–EU709075; JF831639–JF831657
Foveaux Strait	*Ostrea chilensis*	Jun 2004; Jan, Mar 2005	3	9.3	8.0	28	24	JF831658–JF831677
Southeastern USA								
Fort Pierce, Florida	*Crassostrea ariakensis*	Apr, Jun 2007	2	4.0	2.5	8	5	JF712867–JF712871
Wilmington and Morehead City, North Carolina	*Crassostrea ariakensis*	Sep, Nov 2005	4	7.3	5.3	29	21	EU709024–EU709037; EU709039–EU709045
	Ostrea stentina	Jun, Aug 2005	3	9.7	6.3	29	19	JF831575–JF831584; JF831586–JF831592; JF831600; JF831602
Old House Creek, South Carolina	*Ostrea stentina*	Apr 2006	2	8.0	4.5	16	9	JF831585; JF831593–JF831599; JF831601
Tunisia								
Hammamet	*Ostrea stentina*	Jun 2007	4	13.8	9.8	55	38	GU356032–GU356035; JF831685–JF831718

(= reverse primer D; Goggin 1994) were used for bidirectional sequencing, which was performed on either a LI-COR 4200L or a 16-capillary 3130*xl* Genetic Analyzer (Applied Biosystems). Complementary sequences were compared to one another and to their chromatograms using MacVector 8.0 (Oxford Molecular) or CodonCode Aligner.

Sequence alignment and network analysis

Bonamia exitiosa sequences were aligned using MAFFT v. 6 (Katoh & Toh 2008). When identical *B. exitiosa* sequences were recovered from an individual oyster host, a single representative sequence was used in the alignment since it is not possible to discriminate between the case of multiple *B. exitiosa* parasites with identical sequences and the same *B. exitiosa* clone that is recovered multiple times from the same individual. The GenBank accession numbers of the sequences used are listed in Table 1. Once the alignment was produced, the ends of longer sequences were removed so that, with gaps, the sequences were of equal length.

To examine the genealogical relationships among sequences, the *B. exitiosa* sequence alignment was analyzed using TCS (Clement et al. 2000). Gaps were treated as a fifth state for the network analysis, and the program calculated maximum connection steps at 95%.

Genetic differentiation

Maximum likelihood fits of 24 different nucleotide substitution models were conducted in MEGA6 (Tamura et al. 2013) and the lowest Bayesian information criterion score was considered to best describe the observed substitution pattern among sequences. Sequences were collapsed into unique haplotypes using FaBox v. 1.41 (Villesen 2007). Population pairwise Φ_{ST} values were calculated between geographic collections with and without consideration of *B. exitiosa* sequences recovered from different host species as different groups. To assess the magnitude of differences between groups recovered by the TCS analysis, pairwise Φ_{ST} values between groups were calculated. Significance was assessed using 10 000 permutations of the data. All Φ_{ST} values between sequences were based on a Kimura 2-parameter (K2P) distance method using the Arlequin v. 3.1.5.3 software package (Excoffier & Lischer 2010). Nucleotide diversity per site (π), gene diversity (h), and the average number of pairwise nucleotide differences within and between sequences from different locations and groups (k) were also calculated using the Arlequin software assuming a K2P model. Here, gene diversity (h) is defined as the probability that 2 randomly chosen sequences are different in a population (Nei 1987). Genetic differentiation estimates and tests of population subdivision including both haplotype- and nucleotide-based statistics were calculated using DNAsp (Librado & Rozas 2009).

RESULTS

We found 290 *Bonamia exitiosa* ITS region rDNA sequences from a total of 410 cloned and sequenced PCR fragments (720 bp) recovered from 7 oyster host species after excluding identical sequences recovered from a single oyster. Information regarding the number of individual oysters per geographic region from which *B. exitiosa* sequences were obtained and other information regarding clone number and averages per sampling location and host are presented in Table 1. *B. exitiosa* sequences were obtained from 1 to 11 oysters per location. The average number of clones sequenced per individual varied from 4.0 to 20.8, and the average number of unique sequences per individual ranged from 2.5 to 16.4 (Table 1). The total number of clones sequenced per region ranged from 14 (Australia) to 104 (California), with the total number of unique sequences ranging from 7 (Australia) to 82 (California).

Of the 290 *B. exitiosa* sequences analyzed, 234 unique sequences were present, and 4 well-defined groups emerged and were designated as 'Cosmopolitan,' 'Southern Hemisphere,' 'western Atlantic,' and 'California' based on their geographic distribution (Figs. 1 & 2). The most common sequence belonged to the Cosmopolitan group and was found at every sampling location where *B. exitiosa* was detected by PCR except in California, and in every oyster host species except *Ostrea lurida* from California, *Saccostrea glomerata* from Australia, and *O. chilensis* from New Zealand. This sequence was found in a total of 23 individuals: 4 *O. stentina* from Tunisia, 4 *O. puelchana* and 3 *O. stentina* from Argentina, 1 *O. stentina* from New Zealand, 1 *O. angasi* from Australia, and 5 *O. stentina* and 5 *Crassostrea ariakensis* from North Carolina, South Carolina, and/or Florida, making up 36.7% of haplotypes in the Cosmopolitan group. A second sequence (4.4% of haplotypes) within the Cosmopolitan group was found in 4 individuals: 1 *O. stentina* from Tunisia, 2 *O. puelchana* from

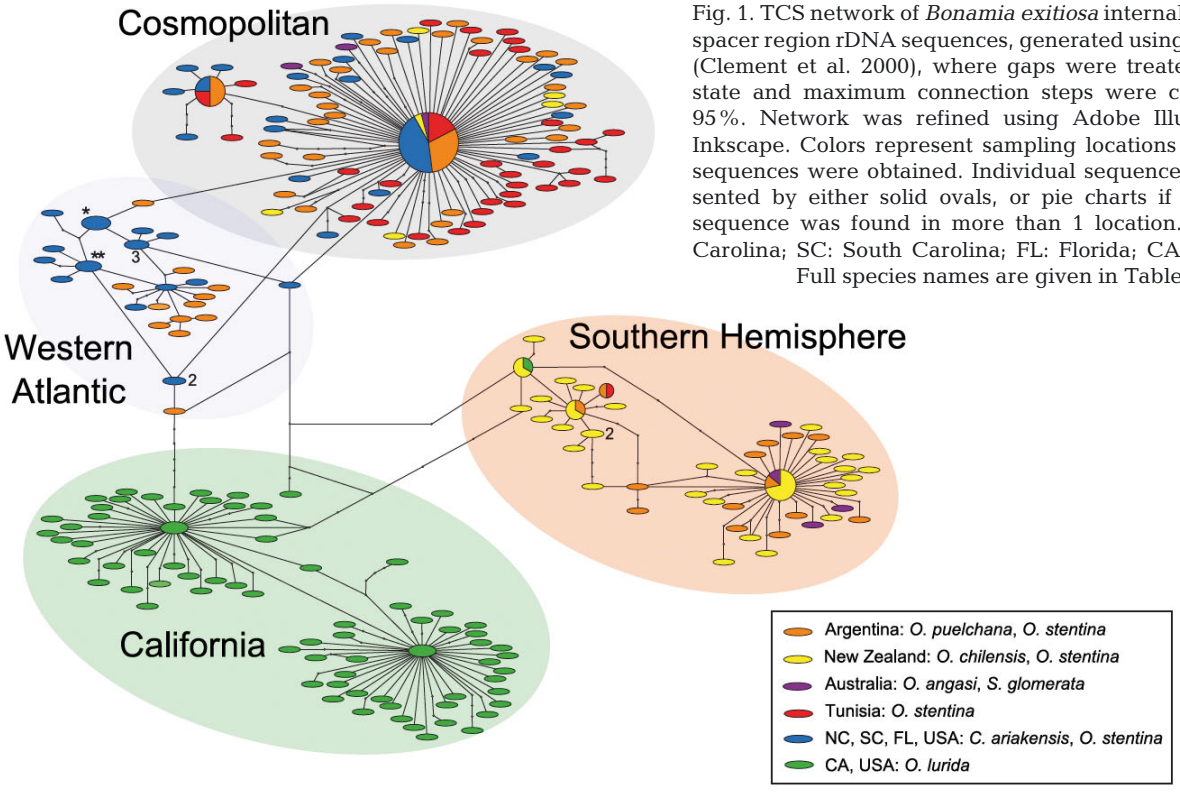

Fig. 1. TCS network of *Bonamia exitiosa* internal transcribed spacer region rDNA sequences, generated using TCS v. 1.21 (Clement et al. 2000), where gaps were treated as a fifth state and maximum connection steps were calculated at 95%. Network was refined using Adobe Illustrator and Inkscape. Colors represent sampling locations from which sequences were obtained. Individual sequences are represented by either solid ovals, or pie charts if the specific sequence was found in more than 1 location. NC: North Carolina; SC: South Carolina; FL: Florida; CA: California. Full species names are given in Table 1

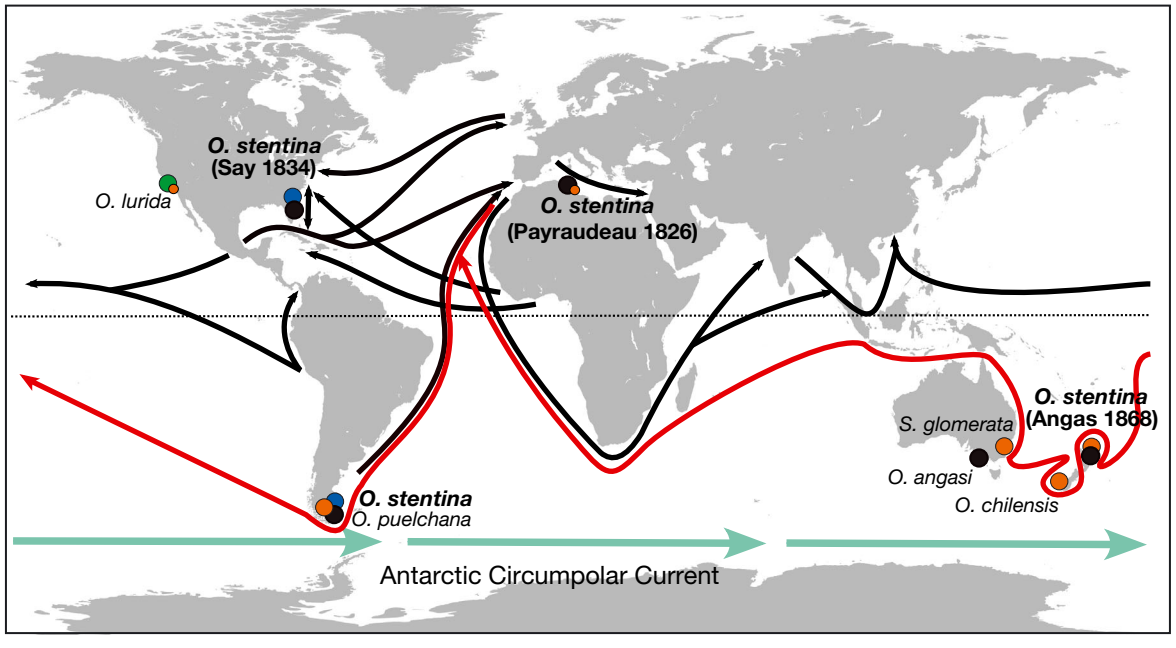

Fig. 2. Current geographic distribution of *Bonamia exitiosa* lineages and host *Ostrea stentina*, with depictions of proposed dispersal hypotheses: major global trade routes from the 1400s to the 1800s (black arrows; Rodrigue 2013) and an example of 1 voyage (red arrows; Turnbull 2004) out of hundreds made during the Age of Exploration. Of the 2 sizes of orange circles, the smaller depicts finding a Southern Hemisphere *B. exitiosa* outside of its geographic range

Table 2. Diversity indices of *Bonamia exitiosa* by sampling location and by TCS analysis results. n: number of sequences analyzed; *h*: gene diversity; *k*: average number of pairwise nucleotide differences within and between sequences from different locations and groups; π: nucleotide diversity per site

Geographic region	Host(s)	n	*h*	*k*	π
Sampling location					
Australia	*Saccostrea glomerata*	4	1.000 ± 0.177	2.509 ± 1.690	0.004 ± 0.003
Australia	*Ostrea angasi*	3	1.000 ± 0.272	4.026 ± 2.741	0.006 ± 0.005
Tunisia	*O. stentina*	38	0.996 ± 0.008	3.155 ± 1.670	0.004 ± 0.003
Argentina	*O. puelchana*	46	0.993 ± 0.008	3.140 ± 1.657	0.004 ± 0.003
Argentina	*O. stentina*	19	0.983 ± 0.026	2.277 ± 1.307	0.003 ± 0.002
New Zealand	*O. chilensis*	24	0.989 ± 0.015	1.632 ± 0.998	0.002 ± 0.002
New Zealand	*O. stentina*	20	0.984 ± 0.024	2.529 ± 1.420	0.004 ± 0.002
North Carolina	*C. ariakensis*	21	0.976 ± 0.023	1.764 ± 1.064	0.002 ± 0.002
North and South Carolina	*O. stentina*	28	0.950 ± 0.025	1.645 ± 0.999	0.002 ± 0.002
Florida	*Crassostrea ariakensis*	5	0.900 ± 0.161	1.605 ± 1.131	0.002 ± 0.002
California	*O. lurida*	82	0.994 ± 0.004	2.660 ± 1.433	0.004 ± 0.002
TCS grouping					
Southern Hemisphere	*O. stentina, O. chilensis, O. puelchana, S. glomerata*	57	0.983 ± 0.009	1.923 ± 1.110	0.003 ± 0.002
California	*O. lurida*	81	0.994 ± 0.004	2.643 ± 1.426	0.004 ± 0.002
Cosmopolitan	*O. stentina, O. puelchana, C. ariakensis, O. angasi*	116	0.964 ± 0.014	2.637 ± 1.418	0.004 ± 0.002
Western Atlantic	*O. stentina, C. ariakensis*	36	0.960 ± 0.020	1.600 ± 0.972	0.002 ± 0.002
TCS Cosmopolitan group					
Tunisia	*O. stentina*	37	0.996 ± 0.008	3.231 ± 1.705	0.005 ± 0.003
Argentina	*O. stentina, O. puelchana*	39	0.970 ± 0.020	2.623 ± 1.433	0.004 ± 0.002
Western Atlantic (North & South Carolina, Florida, USA)	*O. stentina, C. ariakensis*	30	0.897 ± 0.053	1.807 ± 1.072	0.003 ± 0.002
Australia/New Zealand	*O. stentina, O. angasi*	9	0.972 ± 0.064	2.904 ± 1.680	0.004 ± 0.003

Argentina, and 1 *C. ariakensis* from North Carolina. All but one *B. exitiosa* sequence from Tunisian *O. stentina* (97.4%) belonged to the Cosmopolitan group. Sequences within the Cosmopolitan group (n = 116) had a nucleotide sequence diversity of 0.004 ± 0.002, a gene diversity of 0.964 ± 0.014, and sequences differed by an average of 2.637 ± 1.418 nucleotides (Table 2).

The second cluster of sequences, the Southern Hemisphere group, was found almost exclusively in oysters sampled in Argentina, New Zealand, and Australia (96.3% of Southern Hemisphere sequences were from these 3 sampling locations, Figs. 1 & 2). Sequences within the Southern Hemisphere group (n = 57) had a nucleotide sequence diversity of 0.003 ± 0.002, a gene diversity of 0.983 ± 0.010, and sequences differed by an average of 1.923 ± 1.110 nucleotides (Table 2). The most common sequence in this group, representing 15.2% of Southern Hemisphere haplotypes, was found in 7 individuals: 1 *O. puelchana* from Argentina, 2 *O. chilensis* and 3 *O. stentina* from New Zealand, and 1 *S. glomerata* from Australia. A second sequence in the Southern Hemisphere group (6.5% of Southern Hemisphere haplotypes) was found in 3 individuals: 2 *O. chilensis* from New Zealand and 1 *O. stentina* from Argentina. Southern Hemisphere sequences were also recovered from 2 oysters sampled from outside of this geographic region. One sequence from an *O. stentina* from Tunisia was found to belong to the Southern Hemisphere group and was identical to a sequence found in an *O. stentina* from Argentina. A second Southern Hemisphere sequence was found in 1 *O. lurida* from California, and this sequence was identical to 2 sequences recovered from New Zealand, 1 found in *O. chilensis* and 1 found in *O. stentina*.

A third cluster of sequences, the western Atlantic group, was predominantly found in samples taken from North Carolina, South Carolina, and Florida; 66.7% of the western Atlantic group sequences were from these locations. However, several sequences from oysters sampled in Argentina also belonged to this group. Overall, sequences within the western Atlantic group (n = 36) had a nucleotide sequence diversity of 0.002 ± 0.002, a gene diversity of 0.960 ± 0.020, and sequences differed by an average of 1.600 ± 0.972 (Table 2). The most common western Atlantic group sequence was found only in North and South Carolina (Fig. 1). This sequence, which represented 24% of haplotypes in this group, was found in 6 indi-

viduals: 4 *O. stentina* (3 from North Carolina and 1 from South Carolina) and 2 *C. ariakensis* from North Carolina. A second sequence was shared between 2 *O. stentina* and 2 *C. ariakensis* from North Carolina (16% of haplotypes in this group). A third sequence was found in 2 *O. stentina* (1 from North Carolina and 1 from South Carolina) and 1 *C. ariakensis* from North Carolina (8%), and a fourth sequence was found in 2 *O. stentina*: 1 from North Carolina and 1 from South Carolina (8%). All other sequences were unique. Twelve sequences recovered from oysters sampled in Argentina were found to belong to the western Atlantic group, 9 from *O. puelchana* and 3 from *O. stentina*, comprising 18.5% of the sequences from Argentina.

The 2 most common sequences in the California group were each found 5 times (each representing 6.8% of haplotypes in this group) and differed from each other by 1 bp (Fig. 1). These sequences were only found in *O. lurida* from California. The other sequences belonging to this group were unique. Sequences within the California group (n = 81) had a nucleotide sequence diversity of 0.004 ± 0.002, a gene diversity of 0.994 ± 0.004, and sequences differed by an average of 2.643 ± 1.426 nucleotides (Table 2).

Overall, gene diversity was 0.99, and there were an average of 7.94 nucleotide differences among the groups. The number of uncorrected average pairwise distances between groups ranged from 1.888 between the Southern Hemisphere and western Atlantic groups and 4.220 between Southern Hemisphere and California groups. Population pairwise Φ_{ST} values ranged from 0.043 between the Atlantic Coast and California groups to 0.450 between the Southern Hemisphere and California groups. Estimates of genetic differentiation including both gene- and nucleotide-based estimates indicated that there were significant differences among all groups ($p < 0.001$; Table 3A)

To examine the relationships among geographic regions, sequences were grouped by collection location (without regard to TCS clustering). If *B. exitiosa* was found in multiple host species in a single geographic region, host species were analyzed

Table 3. (A) Population pairwise Φ_{ST} values among all groups of *Bonamia exitiosa* based on a Kimura 2-parameter (K2P) distance method. (B) Population pairwise Φ_{ST} values among sampling locations and hosts based on a K2P distance method. Significance was assessed using 10000 permutations. Location abbreviations — ARG: Argentina; NZL: New Zealand; AUS: Australia; USA_NSC: North and South Carolina; USA_NC: North Carolina; TUN: Tunisia; USA_CA: California; USA_FL: Florida. Host abbreviations — Opu: *Ostrea puelchana*; Ost: *O. stentina*; Och: *O. chilensis*; Sgl: *Saccostrea glomerata*; Oan: *O. angasi*; Car: *Crassostrea ariakensis*; Olu: *O. lurida*. Values in **bold** are significant ($p < 0.05$); $^{*}p \leq 0.01$, $^{**}p \leq 0.001$

(A)	Cosmopolitan	Southern Hemisphere		Western Atlantic						
Southern Hemisphere	**0.27173****									
Western Atlantic	**0.14651****	**0.05543****								
California	**0.26563****	**0.44975****		**0.04313****						
(B)	ARG_Opu	ARG_Ost	NZL_Ost	NZL_Och	AUS_Sgl	AUS_Oan	USA_NSC_Ost	USA_NC_Car	TUN_Ost	USA_CA_Olu
ARG_Ost	−0.01506									
NZL_Ost	**0.02979**	0.02701								
NZL_Och	**0.12262****	**0.15566****	**0.03588***							
AUS_Sgl	**0.09404**	**0.13563**	0.03456	0.06272						
AUS_Oan	0.04674	0.09822	**0.15168**	**0.35523****	0.18964					
USA_NSC_Ost	−0.00913	0.00023	**0.07711***	**0.22634****	**0.25433***	0.15966				
USA_NC_Car	−0.00122	0.00796	**0.10581****	**0.26994****	**0.27902****	0.13322	−0.00431			
TUN_Ost	**0.04372****	**0.04334****	**0.13993****	**0.27314****	**0.22642****	0.04601	**0.02879****	0.0091		
USA_CA_Olu	**0.20343****	**0.22317****	**0.34826****	**0.43669****	**0.41631****	**0.23594****	**0.15263****	**0.1875****	**0.26362****	
USA_FL_Car	−0.00061	0.0363	**0.15039***	**0.3752****	**0.33792***	0.07226	0.04148	0.00407	−0.04474	**0.24576****

Table 4. Pairwise differences between populations of *Bonamia exitiosa* based on a Kimura 2-parameter distance method are shown above the diagonal and the corrected average pairwise difference between populations ($\pi_{between\ xy} - (\pi_{within\ x} + \pi_{within\ y}) / 2$) are below the diagonal. Location and host abbreviations as in Table 3

	ARG_Opu	ARG_Ost	NZL_Ost	NZL_Och	AUS_Sgl	AUS_Oan	USA_ NSC_Ost	USA_NC_ Car	TUN_Ost	USA_ CA_Olu	USA_FL_ Car
ARG_Opu		2.702	2.961	2.793	3.254	3.634	2.402	2.489	3.321	3.639	2.534
ARG_Ost	–0.030		2.493	2.324	2.762	3.193	1.974	2.053	2.884	3.233	2.094
NZL_Ost	0.100	0.068		2.174	2.643	3.572	2.272	2.422	3.361	4.023	2.586
NZL_Och	0.386	0.353	0.073		2.117	3.509	2.140	2.346	3.404	4.061	2.626
AUS_Sgl	0.397	0.340	0.092	0.020		3.975	2.605	2.811	3.858	4.516	3.091
AUS_Oan	0.012	0.007	0.257	0.646	0.663		2.846	2.892	3.647	3.973	2.850
USA_NSC_Ost	–0.013	–0.005	0.163	0.485	0.499	–0.024		1.714	2.507	2.610	1.719
USA_NC_Car	0.015	0.015	0.254	0.632	0.647	–0.037	–0.008		2.524	2.812	1.723
TUN_Ost	0.145	0.143	0.491	0.988	0.992	0.016	0.083	0.040		3.933	2.414
USA_CA_Olu	0.724	0.753	1.414	1.906	1.911	0.603	0.447	0.590	1.008		3.095
USA_FL_Car	0.138	0.134	0.496	0.989	1.005	0.000	0.075	0.020	0.009	0.951	

separately. The highest pairwise Φ_{ST} values among geographic regions were between *B. exitiosa* sequences recovered from *O. lurida* from California and *O. chilensis* from New Zealand (0.437, $p < 0.001$, Table 3B); these sequences differed by an uncorrected average of 4.1 bp among sequences from the 2 locations (Table 4). Within a collection location, significant differences were seen between *B. exitiosa* from *O. stentina* and *O. chilensis* in New Zealand ($\Phi_{ST} = 0.036$, $p = 0.005$), and these sequences differed by an average of 2.2 bp. Significant differences were also observed between parasite sequences collected from *O. angasi* and *S. glomerata* collected in Australia ($\Phi_{ST} = 0.190$, $p = 0.027$). Sequences taken from these 2 hosts differed by an average of 4.0 bp; however, the overall number of sequences examined was small (n = 7). There were no significant differences between *B. exitiosa* sequences sampled from *O. puelchana* and *O. stentina* collected in Argentina or between *O. stentina* and *C. ariakensis* collected in North and South Carolina nor between either Carolina oyster and *C. ariakensis* collected in Florida.

Since the Cosmopolitan group was distributed among all sampling locations, with the exception of California, patterns of diversity among locations within this group were examined separately. Cosmopolitan *B. exitiosa* sequences from Tunisia were the most diverse, having the highest gene diversity (0.996 ± 0.008), nucleotide diversity (0.005 ± 0.003), and number of pairwise differences among sequences (3.231 ± 1.705). The lowest values of all measures within this group occurred in sequences recovered from North and South Carolina and Florida (Table 2).

DISCUSSION

The analyses in this study revealed that *Bonamia exitiosa* has a significant level of population structure based on ITS region rDNA sequences, demonstrating that while some sequences are distributed broadly, others appear to be confined to particular geographic areas. The network displays a strong geographic signal in the distribution of *B. exitiosa* sequences, and these differences are statistically significant. Samples comprise 4 reasonably well-defined groups: (1) the Cosmopolitan group, which represents *B. exitiosa* sequences from almost all sampling locations except California; (2) the western Atlantic group, which represents sequences from North and South Carolina, Florida, and Argentina; (3) the Southern Hemisphere group, which is composed mostly of sequences from Argentina, New Zealand, and Australia, with the exception of a sequence from California and another from Tunisia; and (4) the California group, which only includes sequences found in California. These phylogeographic groupings likely indicate that natural historical factors at least partly shaped the current distribution of *B. exitiosa*. However, contemporary anthropogenic factors (e.g. intentional and unintentional introduction or transplantation of oysters for aquaculture or fisheries restoration, or via ship hulls or ballast) also seem to be influencing its distribution as seen with the wide distribution of Cosmopolitan group sequences and the occasional detection of Southern Hemisphere *B. exitiosa* sequences in northern locations (Tunisia and California).

The predominant cell form of *B. exitiosa* in host tissue is a naked, uninucleate microcell less than 5 µm in size, and it is unknown what form the parasite takes

when released from the host and into the environment. The duration of *B. exitiosa* survival outside of the host is unknown, but Arzul et al. (2009) found that purified *B. ostreae* cells from host tissue had a clear preference for specific environmental conditions such as temperature (<25°C) and salinity (euhaline), and further demonstrated that the percentage of *B. ostreae* cells producing esterase activity (a measure of cell viability) decreased significantly at most salinities after 48 h in suspension (Arzul et al. 2009). *B. perspora* Carnegie et al. 2006 is currently the only *Bonamia* species for which spores have been observed. This finding could suggest that other species within the genus also produce these more resistant life-stages (Carnegie et al. 2006) and support the hypothesis of dispersal via ballast water and/or along oceanic currents. However, having not observed these stages in *B. exitiosa*, it seems unlikely that the parasite could disperse great distances through varied environments on its own, suggesting that natural and/or anthropogenic co-dispersal of the parasite and host(s) appears most likely. Based on the geographic patterns resulting from the TCS network analysis, as well as the diversity of the sequences at each location, we discuss potential distribution hypotheses of *B. exitiosa*.

Southern Hemisphere *Bonamia exitiosa*

Some *B. exitiosa* sequences seem to be restricted to the Southern Hemisphere, with grouping of sequences from Argentina, northern and southern New Zealand, and Australia, with 2 exceptions: 1 sequence from Tunisia that is identical to a sequence found in Argentina in the same oyster host, *O. stentina*, and another sequence from California that is identical to a sequence found in both northern and southern New Zealand in 3 different oyster hosts (*O. lurida*, *O. stentina*, and *O. chilensis*, respectively). These sequences are clearly nested within the Southern Hemisphere group, potentially indicating a more recent introduction of the Southern Hemisphere *B. exitiosa* to California and Tunisia. Because of the geographic disjunction of these sequences, anthropogenic means likely facilitated the dispersal of these exceptions.

Although *B. exitiosa* sequences found in New Zealand were predominantly in this group, sequences from northern New Zealand *O. stentina* belonged to both the Southern Hemisphere and Cosmopolitan groups while sequences from southern New Zealand *O. chilensis* exclusively belonged to the Southern Hemisphere group. Consequently, there were significant differences between these 2 sampling areas. Sampling location and host, therefore, influences the geographic patterns we see, highlighting the importance of exploring more locations and hosts. Oysters have been intentionally transplanted to novel locations worldwide since Roman times (Andrews 1980), resulting in the introduction of pathogens to new locales and to naïve, native hosts (Bishop et al. 2006). *Crassostrea gigas* was introduced to northwestern New Zealand in 1958 from Japan or Tasmania, Australia (Ruesink et al. 2005), and is hypothesized to be a reservoir for *B. exitiosa* (Lynch et al. 2010). Perhaps this is when the Cosmopolitan *B. exitiosa* was introduced to northern New Zealand. However, until *B. exitiosa* ITS region rDNA sequences from *C. gigas* and other locations in New Zealand are obtained, we cannot speculate further.

With respect to all other sequences found in the Southern Hemisphere group, it appears that gene flow is occurring between New Zealand, Australian, and Argentinean populations of *B. exitiosa*. One likely natural mechanism is rafting of *B. exitiosa*-infected oysters on surface currents, such as the Antarctic Circumpolar Current, which has been suggested as a dispersal mechanism for *O. chilensis* from New Zealand to Chile (Ó Foighil et al. 1999, Donald et al. 2005). Providing a means of transport for the host would also conceivably allow transport of the parasite *B. exitiosa.* To further validate this hypothesis, additional samples from Chile and from the African coasts would need to be examined.

California *Bonamia exitiosa*

Seventy-one *B. exitiosa* sequences found in *O. lurida* from California were unique to this sampling location and host, with the exception of the single sequence which clustered with the Southern Hemisphere (Figs. 1 & 2). Other than the possible recent introduction of the Southern Hemisphere *B. exitiosa* into California, it appears that there is and has been little connectivity between California *B. exitiosa* populations and those of other regions. Based on gene diversity ($h = 0.99$) and the structure of the network, the recent introduction of a majority of *B. exitiosa* to California is unlikely. A recent introduction would likely show a network dominated by a single sequence that was very closely related or identical to sequences from the geographic area from which it was introduced rather than the patterns observed in this study.

Restricted dispersal of this particular *B. exitiosa* lineage could be a result of both natural and anthro-

pogenic influences. Historically, *O. lurida* ranged from Baja California (Mexico) to Sitka, Alaska (USA) (Dall 1914), which would define the natural range of this *B. exitiosa* group if it is in fact host specific, and there is currently limited anthropogenic movement of this severely depleted oyster species. Additionally, the East Pacific Barrier, which is 5400 to 7300 km of uninterrupted open water (Grigg & Hey 1992), could also prevent the natural dispersal via rafting of both host and parasite, as has been demonstrated for other fauna (Grigg & Hey 1992).

The introduction of *B. exitiosa* into this region was likely facilitated by anthropogenic means. In Elkhorn Slough, California, alone, 38 of 58 known marine invasive species were likely introduced through oyster culture (Wasson et al. 2001). Based on the number of pairwise differences and pairwise Φ_{ST} values in this dataset, the California group appears to be most closely related to the western Atlantic group, suggesting that the California *B. exitiosa* may have originated from the western Atlantic coast (or vice versa). However, it would also be important to explore other regions and hosts. Again, *C. gigas*, one of the most cosmopolitan macroscopic marine invertebrates, was introduced to the US West Coast in 1902 (Ruesink et al. 2005) and should be considered in future efforts to better understand its potential role in the dispersal of *B. exitiosa*.

Cosmopolitan and western Atlantic *Bonamia exitiosa*

The Cosmopolitan group represents sequences found in all sampling locations except in California. This group may represent dispersal over some unknown time period of a lineage particularly adaptable to new hosts and environments, or it may reflect recent and extensive anthropogenic dispersal. Mechanisms of distribution probably vary, but anthropogenic means seem most likely given the disjunct geographic distribution of these samples. The analysis of *B. exitiosa* ITS region rDNA also reveals a more loosely defined cluster of sequences that are closely related to the Cosmopolitan group, but appear to be restricted to the western Atlantic coast (North Carolina, South Carolina, Florida, and even Argentina), found in wild *O. puelchana* and *O. stentina*, and experimental *Crassostrea ariakensis*.

One host that appears to be present in all locations, except California, is *O. stentina.* A phylogenetic study of *Ostrea* species found *O. stentina*, *O. equestris*, and *O. aupouria* to be synonymous (Shilts et al. 2007). All are known hosts of *B. exitiosa* (Hill et al. 2014), and occur in nearly every geographic region from which *B. exitiosa* has been detected: New Zealand/Australia, the southeastern USA, and the Mediterranean Sea. Each of the oyster species synonymized by Shilts et al. (2007) was described in the 1800s: *O. stentina* in 1826, *O. equestris* in 1834, and *O. aupouria* (= *Ostreola virescens*) in 1868 (Cook 2010). Thus, this oyster species has been established in its various locales for a minimum of 142 yr, making it possible for *B. exitiosa* to have been established for at least this long. Therefore, introduction via natural or anthropogenic means in recent decades cannot fully explain the distribution of this single host and this parasite.

Furthermore, the diversity of *B. exitiosa* ITS region rDNA sequences seen in North Carolina, South Carolina, and Florida is indicative of a non-recent introduction and/or multiple introductions. The former contradicts the hypothesis proposed by Bishop et al. (2006) that recent anthropogenic dispersal via ballast water may explain how *B. exitiosa* came to be in North Carolina. Without a molecular clock and additional genetic data of the hosts themselves, it is difficult to say exactly when this distribution occurred. However, it is plausible that ships during the Age of Exploration (ca. 1400–1800s) colonized by a small oyster, such as *O. stentina*, could have provided transport of *B. exitiosa*.

Natural dispersal of the parasite also may be occurring in the populations of the western Atlantic coast. If a continuous host population exists, it is possible that the western Atlantic coast *B. exitiosa* is dispersing through direct transmission. With oyster populations in close proximity, hydrodynamics and topographical features may also affect the distribution of the parasite through the water column (Cranfield et al. 2005). Natural co-dispersal with a host or hosts is also a possibility given the geographical proximity of the populations.

O. stentina has not been reported from California, so the presence of *B. exitiosa* may reflect a limited invasion event. Perhaps *B. exitiosa*-infected *O. stentina* were introduced transmitting the parasite to the native oyster, *O. lurida*, but did not establish populations. Alternatively, *O. stentina* may be present cryptically.

Origins of *Bonamia exitiosa*

With increasing observations of *B. exitiosa* around the world, the geographic origin of the parasite is

becoming less clear. Because *B. exitiosa* is present in archival histological material of *O. chilensis* from Foveaux Strait, New Zealand, dating to 1964 (Hine & Jones 1994), it was hypothesized that the parasite is enzootic to this region (Hine 1996, Corbeil et al. 2006). However, only the Southern Hemisphere *B. exitiosa* was found in this host, and this group is not as diverse as the Cosmopolitan group based on our dataset. The highest diversity was observed in *B. exitiosa* found in Tunisian *O. stentina*, suggesting a Mediterranean origin. On the other hand, *Bonamia* spp. SSU rDNA phylogenies have a basal *Bonamia* sp. in Hawaii (Hill et al. 2014), suggesting an origin at lower latitudes. Exploration of more tropical locations from additional non-commercial host species may provide further insight to the derivation of *B. exitiosa.*

FUTURE WORK

To better understand how *Bonamia exitiosa* came to achieve its current distribution, it is essential to understand how its hosts were distributed. The distribution of hosts, particularly the widely distributed host *Ostrea stentina*, has heavily influenced the dispersal and current biogeographic patterns of the parasite. It is obvious based on this dataset that *B. exitiosa* has a complex history that includes many introductions, both recent and historical. These findings emphasize the need for additional sampling to fill in geographic gaps (e.g. Africa, Asia, and Europe; Abollo et al. 2008, Narcisi et al. 2010, Longshaw et al. 2013) as well as additional potential hosts (e.g. *Crassostrea gigas*). Additional sampling would also allow calculation of more accurate distance estimates, further resolve the relationships among sampling areas, and perhaps find other evidence of recent dispersal of the parasite.

These hypotheses should further be tested by developing multiple genetic loci of oyster hosts and *B. exitiosa,* as well as the development of a molecular clock. This would further elucidate phylogeographic patterns and dispersal timing of the various hosts and the parasite and perhaps lead to insight into the question of origin.

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