Richness and distribution of tropical oyster parasites in two oceans

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SUMMARY

Parasites can exert strong effects on population to ecosystem level processes, but data on parasites are limited for many global regions, especially tropical marine systems. Characterizing parasite diversity and distributions are the first steps towards understanding the potential impacts of parasites. The Panama Canal serves as an interesting location to examine tropical parasite diversity and distribution, as it is a conduit between two oceans and a hub for international trade. We examined metazoan and protistan parasites associated with ten oyster species collected from both Panamanian coasts, including the Panama Canal and Bocas del Toro. We found multiple metazoan taxa (pea crabs, *Stylochus* spp., *Urastoma cyrinae*). Our molecular screening for protistan parasites detected four species of *Perkinsus* (*Perkinsus marinus, Perkinsus chesapeaki, Perkinsus olseni, Perkinsus beihaiensis*) and several haplosporidians, including two genera (*Minchinia, Haplosporidium*). Species richness was higher for the protistan parasites than for the metazoans, with haplosporidian richness being higher than *Perkinsus* richness. *Perkinsus* species were the most frequently detected and most geographically widespread among parasite groups. Parasite richness and overlap differed between regions, locations and oyster hosts. These results have important implications for tropical parasite richness and the dispersal of parasites due to shipping associated with the Panama Canal.

Key words: Panama Canal, corridor, isthmus, biogeography, protist, species diversity.

INTRODUCTION

Parasite species richness and interaction strength exhibit a range of geographic patterns, which result from a variety of ecological, epidemiological and evolutionary forces (Poulin *et al*. 2011; Morand, 2015). Moreover, host characteristics (e.g. species richness, abundance, density and geographic range) and the specificity of the parasite are thought to shape parasite biogeography (Nunn *et al*. 2005; Torres *et al*. 2006; Krasnov *et al*. 2008; Poulin *et al*. 2011). For example, it is generally assumed that parasite species richness is high in the tropics, matching the high host diversity there (Poulin, 2004, 2007; but see Torchin *et al*. 2015). In a review of biotic interactions over latitudes, Schemske *et al*. (2009) found many factors associated with parasitism are negatively correlated with latitude, including parasite species richness, rates of parasitism, infection intensity and genetic variation of the host. Unfortunately, they also concluded that the limited data regarding latitudinal variation of parasites and their interactions did not permit a robust test of general patterns. For marine systems in particular, analyses of parasite diversity are constrained due to the lack of parasite surveys and taxonomic resolution for many tropical parasites (Poulin, 2010).

To begin to fill this gap, we measured and compared parasite richness, including protistan and metazoan parasites, in the tropical waters along the coasts of two ocean basins in Panama. We focused on commonly occurring hosts, examining a group of bivalves generally considered oysters within Ostreidae, Isognomonidae and Pteriidae, for multiple reasons. First, as in many other global regions, oysters are abundant, accessible and diverse along both coasts of Panama. Second, oysters are ecologically important in coastal habitats (Kemp *et al*. 2005; Coen *et al*. 2007). Third, compared with other taxonomic groups, these bivalves are relatively well-studied from the perspective of parasites and pathogens, including identification of parasite taxa and knowledge regarding the effects of parasites on individual hosts (Villalba *et al*. 2004; Burreson and Ford, 2004; Aguirre-Macedo *et al*. 2007). Though few studies have examined parasite richness associated

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with oysters residing in tropical locations (but see Aguirre-Macedo *et al.* 2007; Moss *et al.* 2008; Cáceres-Martínez *et al.* 2012), there is a significant baseline of knowledge and identification methods to draw upon from temperate latitudes. In short, oysters provide a useful model system to examine marine parasite distribution and richness in the tropics.

The Panamanian isthmus is bordered by two oceans with very different abiotic factors and vast differences in the flora and fauna on the two coasts (Jones, 1972). The Pacific side has higher tidal amplitude, more variable salinity, more seasonal upwelling and is more greatly impacted by El Niño Southern Oscillation (ENSO) events than the Caribbean side (Glynn, 1972; Lessios, 2008). These abiotic factors cause differences in the diversity and abundance of macroorganisms, including bivalves, in the two oceans. Older comparisons of molluscan taxa between oceans led researchers to believe that molluscs were more diverse on the Pacific side, which was likely associated with greater sampling intensity there (Olsson, 1972). More recent surveys of bivalves revealed that richness is generally higher in the Caribbean, but that individual species are usually more abundant on the Pacific side (Smith *et al.* 2006; Marko and Moran, 2009). Additionally, the high volume of international shipping associated with the Panama Canal for the last century makes this area a potential 'hotspot' for biological invasions (Ruiz *et al.* 2009), influencing the diversity and distribution of both hosts and parasites. Thus, the two sides of the isthmus offer very different environments and native communities with opportunities via ship-mediated transfers for marine species, some of which can invade habitats near both entrances of the Panama Canal and use it to disperse across ocean basins.

To begin to resolve broad-scale patterns of biogeography and diversity for oyster parasites within the coastal waters of Panama, we used morphological and molecular data to identify metazoan and protistan parasites associated with ten oyster species collected from multiple locations along the Pacific and Caribbean coasts, including both sides of the Panama Canal and the Bocas del Toro archipelago. In addition to recording all observable metazoan parasites and symbionts, we specifically screened for notable protistan parasites, specifically those in the genus *Perkinsus* and in the phylum Haplosporidia, which are well-known and cause disease in oyster populations. Our goals were to (1) measure and compare the parasite richness and host association of the protistan and metazoan parasites, (2) characterize parasite species distributions among different locations and regions along both coasts and (3) compare differences in parasite richness and frequency of occurrence between ocean basins. We predicted a high diversity of protistan and metazoan parasite species associated with oysters on both coasts, with greater overlap of species between the Canal regions (Pacific-Canal *vs.* Caribbean-Canal) than between the two regions along the Caribbean coast (Caribbean-Canal *vs* Bocas del Toro), due to the potential for ship-mediated parasite dispersal across the Canal.

MATERIALS AND METHODS

Sampling scheme

We collected oysters from a variety of intertidal and subtidal habitats (e.g. mangrove rhizophores, rocks, docks, pilings) at multiple locations for each of three separate regions, which were the Pacific and Caribbean coasts near the Panama Canal and at Bocas del Toro (see supplementary material (ESM) Table 1). Sampling occurred during the Panamanian dry season (December 2011 through March 2012) and in primarily high salinity sites (>20 ppt) based on the assumption that parasite prevalence would be highest under these conditions. Maps showing the sampling locations and proportions of protistan parasites detected were generated using ArcGIS 10·2·2 for Desktop (Esri, Redlands, California). Within each geographic region (i.e. Pacific-Canal, Caribbean-Canal and Bocas del Toro), our aim was to sample three oyster species from three separate locations. We attempted to find locations with sufficient numbers of all three species co-occurring; however, this was not always possible. Within each location, ten oysters of each species were collected from five sites (with sites being ~5–10 m apart), resulting in 50 individuals collected per location, and this was repeated at three locations to yield 150 individuals per region (10 individuals × 5 sites/location × 3 locations = 150 individuals per species per region, where available) for each species. From this pool, ~30 individuals were screened per location, resulting in ~90 individuals screened for protistan parasites per region for each oyster species. To determine the presence of metazoan parasites, a maximum of six oysters of each species from each site was collected (6 individuals × 5 sites × 3 locations = 90 individuals per species per region) following the same sampling stratification as the collection for protistan parasites.

Upon collection, oysters were placed in coolers on ice and kept at ~4 °C in the laboratory for no more than 72 h. The oysters were tentatively identified in the field based on morphology. The majority of shells were thoroughly cleaned, dried, labelled with unique identification numbers and retained as vouchers.

Microscopy screening for Metazoan parasites

Oysters were shucked and immediately dissected. More specifically, major bivalve tissues and organs were mounted on large glass slides, squashed and

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Table 1. Sampling regions and locations, including the total abundance (in parentheses) of individual parasites or symbionts collected per species per location and the prevalence (as a percentage) of each metazoan parasite or symbiont associated with each bivalve species within each location

Region	Location	Host name	Number sampled	*Stylochus* spp.	Pea crabs	*Urastoma cyprinae*	Unidentified cyst	Unidentified trematode
Pacific-canal	Flamenco Marina	*Crassostrea columbiensis*	40	0%	0%	0%	0%	0%
	Bique Intertidal	*Crassostrea columbiensis*	41	**7·3% ± 8·0 (8)**	0%	0%	0%	0%
		Saccostrea palmula	29	**17·2% ± 13·7 (13)**	**55·2% ± 18·1 (26)**	0%	0%	0%
		Striostrea prismatica	30	**76·7% ± 15·1 (87)**	0%	0%	0%	0%
	Punta Chame	*Crassostrea columbiensis*	25	0%	0%	0%	0%	**4% (1)**
	Veracruz	*Striostrea prismatica*	29	**34·5% ± 17·3 (44)**	0%	0%	0%	0%
	Punta Culebra	*Saccostrea palmula*	31	**3·2% ± 6·2 (1)**	**12·9% ± 11·8 (4)**	0%	0%	0%
Caribbean–canal	Fort Sherman	*Crassostrea rhizophorae*	30	0%	**13·3% ± 12·2 (4)**	0%	0%	0%
		Isognomon sp.	35	0%	0%	0%	0%	0%
	Galeta	*Crassostrea rhizophorae*	8	0%	0%	0%	0%	0%
		Isognomon sp.	20	0%	0%	0%	0%	0%
		Saccostrea sp.	4	0%	**25% ± 42·4 (1)**	0%	0%	0%
	Rio Alejandro	*Crassostrea* sp.	21	0%	0%	0%	**5% (1)**	0%
		Isognomon sp.	30	0%	0%	0%	0%	0%
		Saccostrea sp.	24	0%	0%	0%	0%	0%
	Samba Bonita	*Crassostrea* sp.	30	0%	**6·7% ± 9·0 (2)**	0%	0%	0%
		Isognomon sp.	30	0%	0%	0%	0%	0%
		Saccostrea sp.	4	0%	0%	0%	0%	0%
Bocas del Toro	Punta Caracol	*Crassostrea rhizophorae*	30	0%	**20% ± 14·3 (6)**	**30% ± 16·4 (49)**	**20% (59)**	0%
		Isognomon sp.	30	0%	0%	0%	**13·3% (5)**	0%
		Saccostrea sp.	30	0%	0%	**3·3% ± 6·4 (3)**	**3·3% (1)**	0%
	Solarte	*Crassostrea rhizophorae*	30	0%	0%	0%	**10% (10)**	0%
		Isognomon sp.	30	0%	0%	0%	0%	0%
		Saccostrea sp.	30	0%	0%	0%	0%	0%
	STRI Dock	*Crassostrea rhizophorae*	30	0%	**23·3% ± 15·1 (8)**	0%	0%	0%
		Isognomon alatus	36	0%	0%	**2·8% ± 5·4 (1)**	0%	0%
	Casa Blanca Verde	*Saccostrea* sp.	30	0%	**3·3% ± 6·4 (1)**	**3·3% ± 6·4 (1)**	0%	0%
		Totals	**737**	**153 (20·7%)**	**52 (7·0%)**	**54 (7·3%)**	**76 (10·3%)**	**1 (0·1%)**

Prevalence values greater than zero are shown in bold. Confidence intervals (95%) are included for prevalence calculations for the three identified metazoans. Note that as we did not genetically confirm the identities of oysters screened for metazoan infections, we only list *Crassostrea* sp. in the two sites where both *Crassostrea* species were found.

then visually screened for metazoan parasites at 10–40× magnification using a dissecting microscope. The identity and number of metazoan parasites and symbionts was recorded. Vouchers of all metazoans found were preserved in 95% ethanol for potential future use. When possible, photos of parasites were taken during dissections for identification. Due to the low number of samples processed, we calculated confidence intervals for the prevalence estimates obtained.

Bivalve species determination

The methods used to determine the species identities of the oysters are described in Pagenkopp Lohan *et al*. (2015). Briefly, we amplified and sequenced a fragment of the mitochondrial cytochrome oxidase I (COI) gene using the primers jgLCO1490/jgHCO2198 from Geller *et al*. (2013). Final host identification was determined using Bayesian and Maximum Likelihood (ML) phylogenetic analyses combined with morphological criteria. Due to the large number of oysters in this study, we only molecularly confirmed a subset ($n = 5$–10 individuals per species per location) of the oysters sampled. We assumed all remaining oysters that were morphologically similar or only found in a single sampling location were the same species. The exception was for the *Crassostrea* species in the Caribbean, as it was impossible to use morphological and/or ecological criteria to separate *Crassostrea virginica* from *Crassostrea rhizophorae*.

We created a restriction enzyme digest that could distinguish *C. virginica* from *C. rhizophorae*. After amplifying the COI fragment mentioned above, we digested 13·2 μL of the polymerase chain reaction (PCR) product with 6 U of restriction enzyme *Sal*I-HF (New England Biolabs, Ipswich, MA), and 1 × NE buffer (New England Biolabs) in a total volume of 15 μL for 30 min at 37 °C, then 20 min at 65 °C. An aliquot of the digested product (8 μL) was electrophoresed on an agarose gel (4% w/v) stained with GelRed (Phenix Research, Candler, NC) and visualized under ultraviolet (UV) light. The PCR product from *C. virginica* did not have the *Sal*I-HF restriction site and remained undigested, whereas the PCR product from *C. rhizophorae* was cleaved into two fragments (~350 and 300 bp each). To confirm the accuracy of the digestion, any samples that appeared undigested (i.e. were identified as *C. virginica*) were directly cycle-sequenced (see below) for confirmation.

Metazoan parasites: DNA extraction, PCR amplification and sequencing

To molecularly determine the species identities of a subset of the pea crabs collected from the two coasts, we sampled 1–2 walking legs or half an individual crab, depending on size. Following an overnight digestion with proteinase K, we extracted genomic DNA using a DNEasy Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocol. All extractions completed within the same day included a blank extraction, which served as a negative extraction control for PCR. We used the same primer set, jgLCO1490/jgHCO2198 from Geller *et al*. (2013), and amplification protocols as for the oysters (see Pagenkopp Lohan *et al*. 2015). We directly cycle-sequenced all amplified fragments (see below).

Protistan parasites: DNA extraction, PCR amplification and sequencing

To screen for the presence of protistan parasites, we sampled pieces of gill, mantle and digestive gland from each individual and preserved them in 95% ethanol. Following an overnight digestion with proteinase K, we extracted genomic DNA from all three tissues sampled, which were pooled into a single extraction, to increase the likelihood of parasite detection using a Qiagen Biosprint Kit (Qiagen) following the manufacturer's protocols for animal tissues. All extractions completed within the same day included a blank extraction, which served as a negative extraction control for PCR.

We used two molecular assays to screen for protistan parasites. First, we used the primers PerkITS85/PerkITS750 (Casas *et al*. 2002), a genus-specific primer set that amplifies ~700 bp fragment of the first internal transcribed spacer region (ITS1) of the ribosomal gene complex (rDNA) of parasites within the genus *Perkinsus*. PCR reagents consisted of 1× GeneAmp 10× PCR Gold Buffer (150 mM Tris-HCl, pH 8·0; 500 mM KCl; Applied Biosystems, Carlsbad, CA), 1·5 mM $MgCl_2$, 0·2 mM each nucleotide, 0·5 μM each primer, 0·2 mg mL^{-1} bovine serum albumin (BSA; New England Biolabs), and 0·025 units μL^{-1} of AmpliTaq Gold with water to a final volume of 20 μL. Thermocycling was carried out using a Peltier Thermo Cycler DNA Engine Tetrad 2 (Bio-Rad, Hercules, CA) with an initial denaturation of 94 °C for 10 min, 40 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min and a final elongation of 72 °C for 5 min. Second, we used the generic primer set HAPF1/HAPR3 (Renault *et al*. 2000) to amplify a ~350 bp fragment of the ribosomal small subunit RNA (SSU rRNA) gene of haplosporidians. PCR reagents consisted of 1× GeneAmp 10× PCR Gold Buffer (150 mM Tris-HCl, pH 8·0; 500 mM KCl; Applied Biosystems), 1·5 mM $MgCl_2$, 0·2 mM each nucleotide, 0·5 μM each primer, 0·4 mg mL^{-1} BSA (New England Biolabs), and 0·03 units μL^{-1} of AmpliTaq Gold with water to a final volume of 20 μL. Thermocycling was carried out using a Peltier Thermo Cycler DNA Engine Tetrad 2

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(Bio-Rad) with an initial denaturation of 94 °C for 10 min, 10 cycles of 94 °C for 30 s, 54 °C for 30 s with a decrease of 1 °C per cycle, 72 °C for 1 min, then 35 cycles of 94 °C for 30 s, 44 °C for 30 s, 72 °C for 1 min, and a final elongation of 72 °C for 5 min. For each screening assay, an aliquot of PCR product (5 μL) was electrophoresed on an agarose gel (2% w/v) stained with GelRed (Phenix Research) and visualized under UV light. All amplified fragments from oysters, pea crabs and parasites were bidirectionally cycle-sequenced using the Big Dye Terminator Cycle Sequencing Kit v3·1 (Applied Biosystems, Inc.) and the amplicons were sequenced on an ABI 3130 Sequencer (Applied Biosystems, Inc.).

Phylogenetic analyses

Sequences were edited using Sequencher 5·1 (Gene Codes Corporation, Ann Arbor, MI). Only unique sequences, defined as those that differed by either a single base pair or gap, were used in the phylogenetic analyses. Identical sequences and sequences that only differed due to ambiguities were concatenated into contigs with other identical sequences or, in the case of ambiguities, either the next closest sequence or the contig containing the largest number of sequences (if more than one sequence or contig was a potential match). Phylogenetic analyses were then performed using the consensus sequence from each of the unique contigs.

A subset of sequences for all *Perkinsus* spp., haplosporidian species or clades, and pea crabs from published sources were obtained from GenBank and used to generate phylogenetic trees to determine the parasite species detected in Panama. Sequences within each taxa (*Perkinsus* spp., haplosporidians and pea crabs) were aligned in Geneious 7·1·8 (Biomatters, Ltd., San Francisco, CA) with the MAFFT (Katoh *et al*. 2002, Katoh and Toh, 2008) plug-in, using either the default parameters or the E-INS-I algorithm, then manually adjusted if needed. Terminal gaps were removed. For the haplosporidian alignment, sequences that were extremely different from all the others were removed from the alignment and the sequences were re-aligned. The alignment for the pea crab dataset was 505 bp. The alignment for the *Perkinsus* dataset was 632 bp. The alignment for the haplosporidian dataset was 308 bp. JModeltest 2·1·4 (Darriba *et al*. 2012) was used to determine the best substitution models for each alignment based on Akaike Information Criterion corrected values using the appropriate number of available substitution models for Bayesian and ML analyses. Phylogenetic trees were generated for all three alignments in Geneious 7·1·8 using the suggested substitution models implemented in MrBayes 2·0·6 (Ronquist and Huelsenbeck, 2003) with the default parameters and PhyML for the haplosporidian and *Perkinsus* alignments (Guindon *et al*. 2010) using the NNI topology search option with 1000 bootstrap replicates. As the sequences used in these analyses represent only a portion of a single gene, including a hypervariable region of the SSU rRNA gene, we did not use these data to infer evolutionary relationships between clades, but solely to determine the clades that included sequences from this study for parasite identification. Additionally, confidence intervals for the frequency estimates for both protistan taxa were calculated.

RESULTS

Metazoan parasites and symbionts

We microscopically screened 737 oysters and identified multiple metazoan taxa including pea crabs and turbellarians (*Stylochus* spp. and *Urostoma cyrinae*) (Table 1). While we found a number of cysts, none were morphologically identified at the time of sampling, though a few were later identified from photos as the metacestode stage of *Tylocephalum* sp. Pea crabs were the only metazoan parasite that occurred on both coasts (Table 1). The phylogenetic analyses revealed two distinct clades, indicating two species of pea crabs occurring on opposite sides of the isthmus (Fig. 1, GenBank Accession KU172681–KU172692). Comparison with unpublished sequence data (University of Louisiana Lafayette Zoological (ULLZ) Collection catalog numbers 9601 and 13298; Emma Palacios Theil and Darryl L. Felder, in prep.) identified these species as *Austinotheres angelicus* on the Pacific coast and *Zaops ostreum* on the Caribbean coast. *Austinotheres angelicus* infected only one species (*S. palmula*), while *Z. ostreum* infected at least three species (*C. rhizophorae, Saccostrea* sp., *Crassostrea* sp.).

There were slight variations in parasite richness between regions and locations. Within regions, Bocas del Toro had three species, while both the Caribbean-Canal and Pacific-Canal had two species. Within the locations the highest parasite richness was only three, observed at Punta Caracol. None of these observations account for unidentified cysts that may represent multiple species.

Metazoan parasites also showed variation in distribution among hosts and infection intensity. The micropredator *Stylochus* spp. was only observed in oysters on the Pacific coast and the heaviest infestations were always observed in *Striostrea prismatica* (Table 1). The number of worms per individual ranged from 0 to 12 worms at Bique Intertidal, 0–13 at Veracruz and 0–5 at Punta Culebra. The parasite *Urastoma cyprinae* was only detected in bivalves from Bocas del Toro and the heaviest infections were observed in *C. rhizophorae*. The infection

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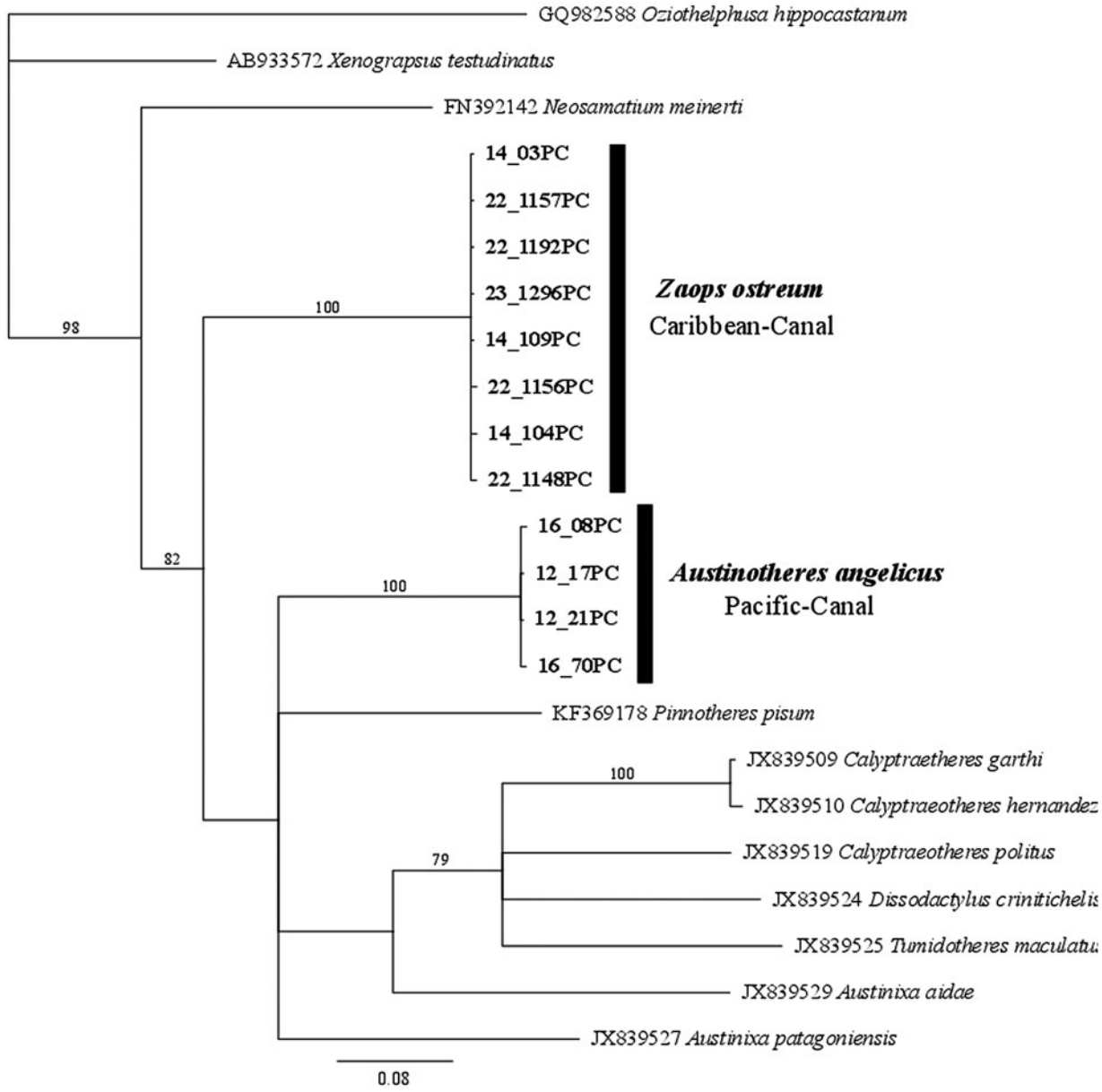

Fig. 1. Phylogram of pea crab COI sequences from this study (in bold) and GenBank sequences. The GTR + I + G substitution model was used for the Bayesian analysis and posterior probabilities are shown. See text for additional details.

intensities in a single host ranged from 0 to 17 at Punta Caracol, from 0 to 1 at STRI Dock and Casa Blanca/Verde.

Protistan parasites

We used genetic methods to screen 752 oysters for protistan parasites. We detected at least 16 different species of protistan parasites, all of which represent new host records for Panama (GenBank Accession KU172633 – KU172680). As we did not confirm infection status for these hosts, which require histological examination of the parasites in host tissue (Burreson, 2008), we report the frequency (or percentage) of detections (i.e. number of positive individuals). Based on the phylogenetic analyses using ITS1 sequence data, four species of *Perkinsus* were detected, including *Perkinsus marinus, Perkinsus chesapeaki, Perkinsus olseni* and *Perkinsus beihaiensis* (Fig. 2). Based on the phylogenetic analyses comparing the haplosporidian sequences from this study to those from previously published studies, two additional genera, *Haplosporidium* and *Minchinia*, were detected. Unfortunately, the majority of haplosporidian sequences could not be assigned to a particular genus due to the short length of the amplified fragment and the large number of unidentified haplosporidians in the literature (Fig. 3). Therefore, we conservatively report finding nine haplosporidian clades (possibly representing individual species), two species of *Minchinia*, and *Haplosporidium costale*. In some instances, clades are represented by a single sequence (e.g. Clade 1, *Minchinia* sp.

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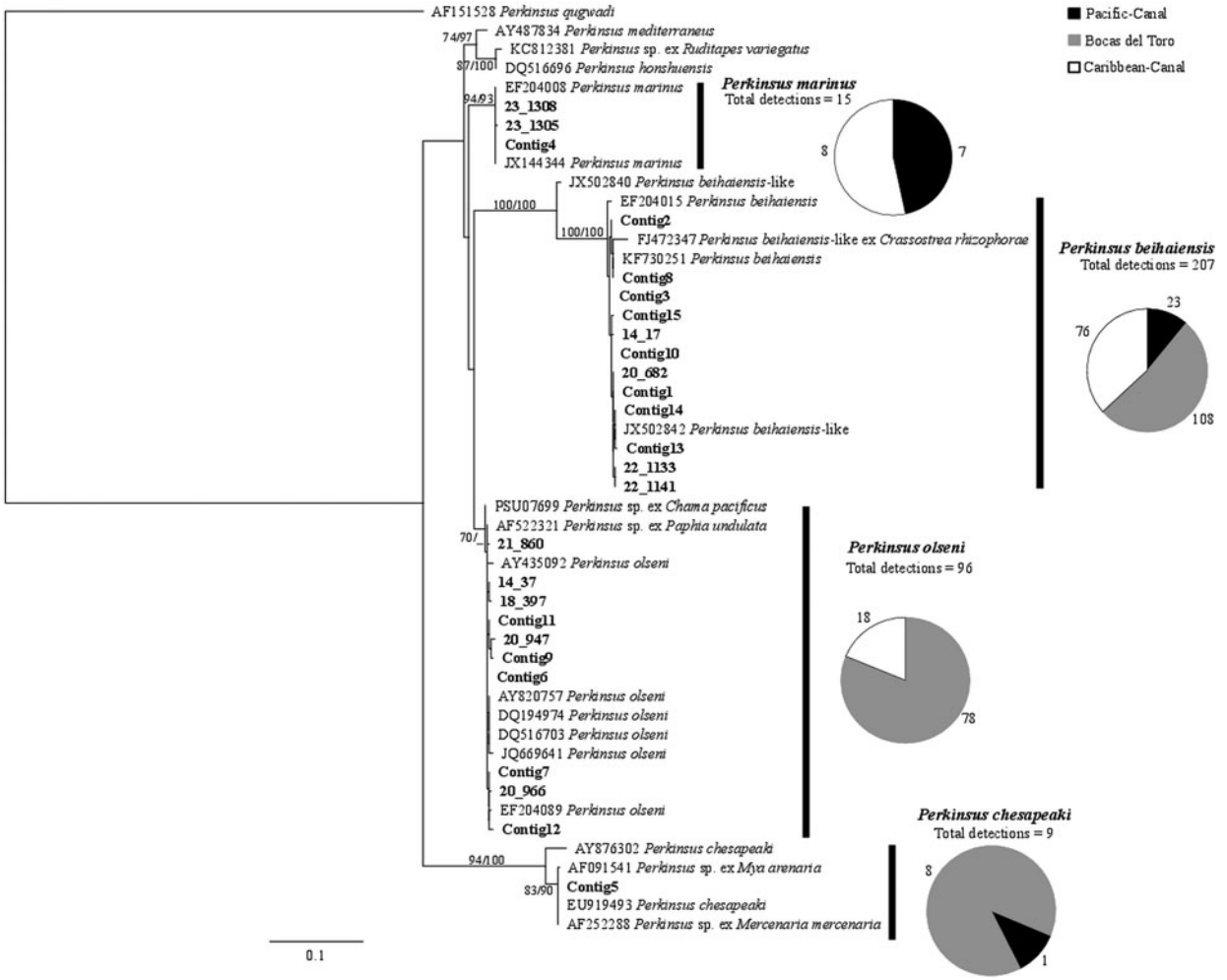

Fig. 2. Phylogram for *Perkinsus* spp. constructed using ITS1 sequences generated in this study (in bold) and GenBank sequences. The GTR + G substitution model for the Bayesian analysis and TPM3uf + G model for the Maximum Likelihood analysis produced highly similar topologies. Bootstrap values (>70) followed by posterior probabilities (>90) are included at the nodes of the topology produced by Maximum Likelihood. See text for additional details.

B) while other clades consist of multiple contigs each containing multiple identical sequences from different locations throughout Panama (ESM Table 2). Clade 6 was the most frequently detected ($n = 37$), followed by Clade 5 ($n = 32$), then *H. costale* ($n = 24$). These three clades were also the most geographically widespread (ESM Table 2).

Within the three regions of Panama, the richness of haplosporidians detected was higher than for the *Perkinsus* species; however, *Perkinsus* species were more frequently detected than haplosporidians (Fig. 4; ESM Tables S3 and S4). Comparing *Perkinsus* richness among regions, three species were detected in each of the three regions, though the species composition within regions was different (Fig. 2). For the haplosporidians, seven species were detected in the Pacific-Canal, while five species were detected in each of the other two regions (Fig. 3).

Overall, detection frequencies for *Perkinsus* species within regions were 3-fold higher along the Caribbean coast compared with the Pacific coast, ranging from 55·6% at Bocas del Toro to 45·1% at Caribbean-Canal, then decreasing to 17·5% at Pacific-Canal (ESM Table S3). On the other hand, detections for the haplosporidians were higher at the Canal regions compared with Bocas del Toro, with overall detection frequencies among all oysters screened ranging from only 9·7% at Bocas del Toro, to 27·4% at Caribbean-Canal and 22·6% at Pacific-Canal.

Examining species richness and detection frequency across locations, the richness of protistan parasites (combining *Perkinsus* spp. and haplosporidians) was highest at Bique Intertidal, where seven parasite species or clades were detected (Fig. 4). STRI Dock, the site with the highest diversity of potential hosts sampled, had the second highest richness of protistan parasites, with six species or clades detected. Oysters from Rio Alejandro and the STRI Dock had the highest detection frequencies. At Rio Alejandro, 39·4 and 67·0% of oysters screened were positive for haplosporidians or *Perkinsus* spp., respectively, and at STRI Dock 16·0 and 68·0% of oysters screened were positive

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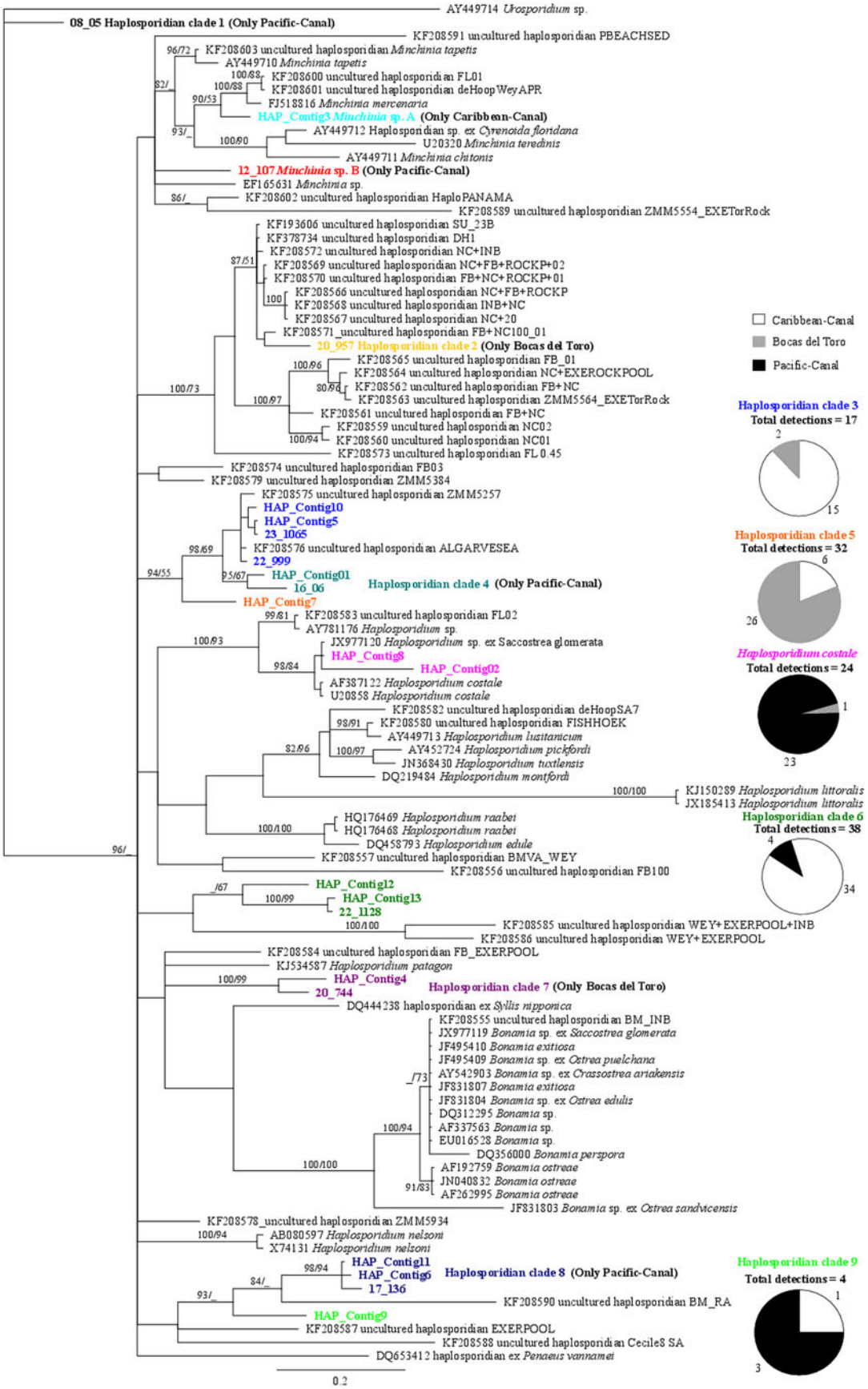

Fig. 3. Phylogram for haplosporidians constructed using SSU sequences generated in this study (in bold) and GenBank sequences. The HKY + G substitution model for the Bayesian analysis and K80 + G model for the Maximum Likelihood analysis produced highly similar topologies. Posterior probabilities (>80) followed by bootstrap values (>60) are included at the nodes of the Bayesian topology. See text for additional details.

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Table 2. Total number of oyster species collected and processed for protistan parasites, with the percentage and total number of detections (in parentheses) of each *Perkinsus* species (A), and multiple genera (i.e. the number of individuals that were positive for at least one *Perkinsus* species and one haplosporidian), and haplosporidians (B), which is calculated as the per cent contribution of each parasite species per host to the total number of parasite detections per parasite species (column)

Ocean basin	Host species	Total sampled	*P. beihaiensis*	*P. chesapeaki*	*P. marinus*	*P. olseni*	Both taxa
A							
Caribbean	*Isognomon* sp.	161	**36·2% (75)**	0·0% (0)	0·0% (0)	**35·4% (34)**	**38·6% (27)**
	Isognomon alatus	31	**1·5% (3)**	0·0% (0)	0·0% (0)	**27·1% (26)**	**20·0% (14)**
	Crassostrea rhizophorae	175	**35·3% (73)**	0·0% (0)	**40·0% (6)**	**1·0% (1)**	**4·3% (3)**
	Crassostrea virginica	9	0·0% (0)	0·0% (0)	**13·3% (2)**	0·0% (0)	0·0% (0)
	Saccostrea sp.	138	**14·5% (30)**	**77·8% (7)**	0·0% (0)	**2·1% (2)**	**24·3% (17)**
	Dendostrea frons	30	**1·5% (3)**	**11·1% (1)**	0·0% (0)	**8·3% (8)**	0·0% (0)
	Pinctada imbricata	31	0·0% (0)	0·0% (0)	0·0% (0)	**26·0% (25)**	**5·7% (4)**
Pacific	*Striostrea prismatica*	55	**2·9% (6)**	0·0% (0)	**6·7% (1)**	0·0% (0)	**1·4% (1)**
	Saccostrea palmula	61	**6·8% (14)**	0·0% (0)	0·0% (0)	0·0% (0)	**4·3% (3)**
	Crassostrea columbiensis	61	**1·5% (3)**	**11·1% (1)**	**40·0% (6)**	0·0% (0)	**1·4% (1)**
	Total	**752**	**207**	**9**	**15**	**96**	**70**

Ocean basin	Host species	Total sampled	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6	Clade 7	Clade 8	Clade 9	*Minchinia* sp. A	*Minchinia* sp. B	*H. costale*
B														
Caribbean	*Isognomon* sp.	161	0·0% (0)	0·0% (0)	**52·9% (9)**	0·0% (0)	**53·1% (17)**	**5·3% (2)**	0·0% (0)	0·0% (0)	0·0% (0)	**100·0% (6)**	0·0% (0)	0·0% (0)
	Isognomon alatus	31	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	**43·8% (14)**	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)
	Crassostrea rhizophorae	175	0·0% (0)	0·0% (0)	**5·9% (1)**	0·0% (0)	0·0% (0)	**15·8% (6)**	**25·0% (1)**	0·0% (0)	**25·0% (1)**	0·0% (0)	0·0% (0)	**4·2% (1)**
	Crassostrea virginica	9	0·0% (0)	0·0% (0)	**5·9% (1)**	0·0% (0)	0·0% (0)	**2·6% (1)**	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)
	Saccostrea sp.	138	0·0% (0)	0·0% (0)	**35·3% (6)**	0·0% (0)	0·0% (0)	**65·8% (25)**	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)
	Dendostrea frons	30	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)
	Pinctada imbricata	31	0·0% (0)	**100% (1)**	0·0% (0)	0·0% (0)	**3·1% (1)**	0·0% (0)	**75·0% (3)**	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)
Pacific	*Striostrea prismatica*	55	0·0% (0)	0·0% (0)	0·0% (0)	**66·7% (2)**	0·0% (0)	**2·6% (1)**	0·0% (0)	**100·0% (5)**	**50·0% (2)**	0·0% (0)	**100·0% (1)**	0·0% (0)
	Saccostrea palmula	61	**100% (1)**	0·0% (0)	0·0% (0)	**33·3% (1)**	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	**25·0% (1)**	0·0% (0)	0·0% (0)	**83·3% (20)**
	Crassostrea columbiensis	61	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	**7·9% (3)**	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	0·0% (0)	**12·5% (3)**
	Total	**752**	**1**	**1**	**17**	**3**	**32**	**38**	**4**	**5**	**4**	**6**	**1**	**24**

Prevalence values greater than zero are shown in bold.

for haplosporidians or *Perkinsus* spp., respectively (Fig. 4; ESM Tables S3 and S4).

Although less diverse, *Perkinsus* species tend to be more geographically widespread and more frequently detected than haplosporidians. *Perkinsus* spp. were detected in 43·5% ($n = 327$) of oysters screened, while haplosporidians were only detected in 18·1% ($n = 136$) (Fig. 3; ESM Table S4). Among all the protistan parasites, *P. beihaiensis* was the species most frequently detected (27·5%, $n = 207$), followed by *P. olseni* (12·8%, $n = 96$), then haplosporidian Clade 6 (5·1%, $n = 38$) (Figs. 2 and 4). A number of haplosporidian clades were only detected a single time including *Minchinia* sp. B, Clade 1, and Clade 2 (Figs 3 and 4).

Parasite species appeared unevenly detected among host species. Examining the detection frequency by host association, *P. beihaiensis* was most frequently detected from *Isognomon* sp. (36·2%, $n = 75$) and *C. rhizophorae* (35·3%, $n = 73$) (Table 2A). *Perkinsus olseni* was most frequently detected from *Isognomon* sp. (35·4%, $n = 34$) and *Isognomon alatus* (27·1%, $n = 26$) (Table 2A). *Perkinsus marinus* was most frequently detected from *C. rhizophorae* (40·0%, $n = 6$) and *P. chesapeaki* was most frequently detected from the Caribbean *Saccostrea* sp. (77·8%, $n = 7$) (Table 2A). When examining detection per host species, *Perkinsus* spp. had extremely high detection frequencies from *I. alatus* (93·5%), *Pinctada imbricata* (80·6%) and *Isognomon* sp. (67·7%). *Perkinsus* spp. detections were lowest from *S. prismatica* (12·7%) and *Crassostrea columbiensis* (16·4%) (Table 2A).

The detection frequencies of haplosporidians were also variable among hosts. The majority of haplosporidian detections were from *I. alatus* (45·2% of all individuals from this species, $n = 14$), *Saccostrea palmula* (37·7%, $n = 23$), *Saccostrea* sp. (22·4%, $n = 31$) and *Isognomon* sp. (21·1%, $n = 34$) (Table 2B). Of the two most frequently detected clades, Clade 6 was most frequently detected from the Caribbean *Saccostrea* sp. (65·8%, $n = 25$) and *C. rhizophorae* (15·8%, $n = 6$) (Table 2B). Clade 5 was most frequently detected from *Isognomon* sp. (53·1%, $n = 17$) and *I. alatus* (43·8%, $n = 14$) (Table 2B). Two haplosporidian clades were detected multiple times but only from a single oyster species, including *Minchinia* sp. A., which was only detected from *Isognomon* sp., and Clade 8, which was only detected from *S. prismatica* (Table 2B).

As the same individual hosts were screened for both protistan taxa, we were able to determine instances where both protistan taxa (i.e. at least one *Perkinsus* spp. and one haplosporidian clade) were detected. Overall, we detected both protistan taxa from 9·3% ($n = 70$) of the oysters screened (Table 2A). The majority of multiple detections were observed along the Caribbean coast, with 15·5% in Caribbean-Canal, 8·6% in Bocas del Toro and only 2·8% in the Pacific-Canal. Within locations, the highest instances of multiple detections occurred at Rio Alejandro (22·3%) and Punta Caracol (11·3%) (Table 2A). When examining host associations, the highest instances of multiple detections occurred in *Isognomon* sp. (38·6%) and *Saccostrea* sp. (24·3%) (Table 2A).

DISCUSSION

We found several parasitic taxa, including a number of new species records, in Panamanian waters. We found three metazoan parasites and one micropredator, including two different pea crab species separated by the Panamanian isthmus with each capable of infecting multiple hosts. Additionally, molecular screening for protistan parasites revealed four *Perkinsus* species, making this study the first record of four co-occurring *Perkinsus* species, which are all new records for Panamanian waters. Three of the four species were observed in two of three regions, with the highest detection frequencies (45·1–55·6%) occurring in Caribbean regions. Finally, we found at least 12 genetically distinct haplosporidians, which likely represent 12 separate species. The majority of haplosporidians appear novel and all are new records for Panamanian waters. Seven of the 12 haplosporidian clades were isolated to a single region, with detections of haplosporidians highest (>20% detection) in the Canal regions.

Importantly, this study documents occurrence and association with particular host species but does not assess the nature of these associations, including whether the protistan parasite taxa are infecting the various hosts or are incidental associations. We currently lack knowledge about the ecology and effects of these parasites in Panama. Further research is needed to determine which bivalve species are hosts, how competent each host is for the parasite in question, the full life cycle of each parasite, effects on these and other bivalves and the dispersal potential of the transmissive stages.

Parasite richness

Our results support the prediction that many previously undetected parasites are present in Panamanian waters. Overall, our analysis revealed relatively high protistan parasite richness in tropical oysters from both Panamanian coasts compared with available data in temperate locations (Villalba *et al.* 2004; Burreson and Ford, 2004; Moss *et al.* 2008; Arzul and Carnegie, 2015). Previously, only three of the seven known *Perkinsus* species (*P. marinus, P. beihaiensis, P. olseni*) had been detected in tropical waters, with *P. beihaiensis* being the only one exclusively found in tropical waters (Villalba *et al.* 2004; Moss *et al.* 2008). Our findings support previous suggestions that the absence of records for *Perkinsus* in

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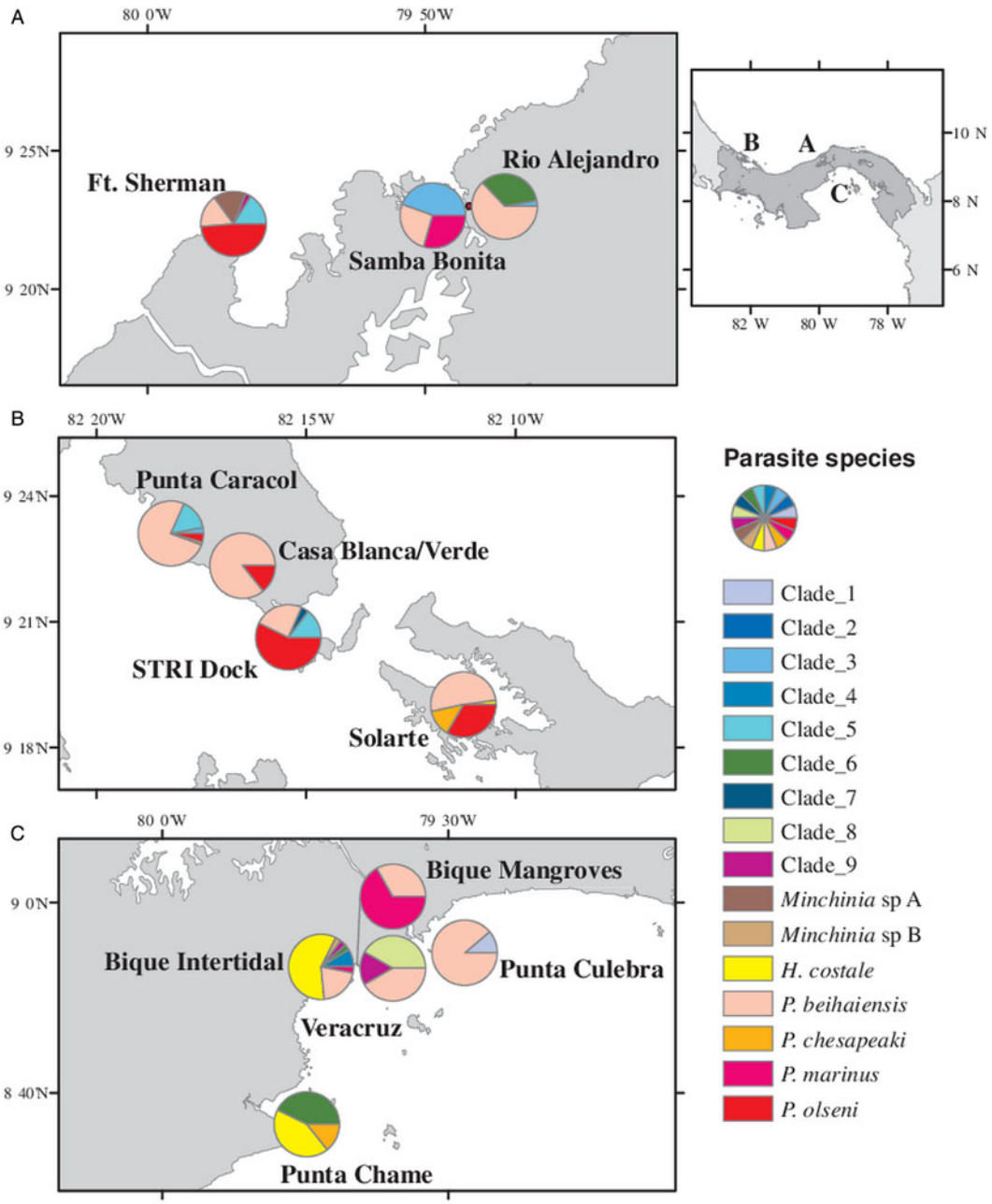

Fig. 4. Map of locations sampled in three regions of Panama including (A) the Caribbean-Canal, (B) Bocas del Toro and (C) the Pacific-Canal. Each colour in the pie charts represents a presumptive parasite species detected in each location. The per cent shading of the pie graphs indicates the proportion of each parasite species or clade detected in each location. Samples sizes are in ESM Tables S3 and S4.

tropical waters is likely due to sampling bias, as the majority of studies have been in temperate regions, rather than due to a lack of occurrence (Villalba *et al*. 2004; Moss *et al*. 2008); however, these differences may also be due to methodological differences between studies conducted in temperate and tropical regions. Finally, we note that our analysis provides a minimum estimate of tropical parasite diversity. The relatively small sample sizes of hosts that were screened for metazoan parasites, which had low prevalence values, could lead to an underestimation of the prevalence and richness of metazoan parasites in these waters.

Unfortunately, the level of taxonomic resolution currently available to determine *Perkinsus* species does not exist for all haplosporidians. While there are >30 described species of haplosporidians, many have only been observed a single time and location (Burreson and Ford, 2004; Arzul and Carnegie, 2015). Recent work demonstrated a high level of previously unknown diversity within the haplosporidians, comprised of a number of clades that likely represent novel species (Hartikainen *et al*. 2014). Additionally, our approach likely underestimated the haplosporidian species, as it is possible that multiple species were lumped into a single clade due to

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the small gene fragment used. Thus, we consider our results to be a conservative estimate of haplosporidian richness, which still demonstrated a relatively high richness in Panamanian waters compared with temperate locations (Burreson and Ford, 2004; Arzul and Carnegie, 2015).

Regional comparisons of richness

While we expected to find substantial parasite species overlap between the Canal sites, due to the high degree of shipping traffic through the Canal and possible homogenization of parasite assemblages on Atlantic and Pacific sides, this was not the observed pattern. Instead, the patterns differed greatly depending on the organisms in question, with some parasites isolated to one ocean (e.g. pea crabs, *U. cyprinae*), one was spread across all regions (*P. beihaiensis*), and still others with more disjunct patterns between locations and regions (*P. chesapeaki*). When examining parasites across regions, more regional overlap was observed for protistan parasites compared with metazoan parasites. For example, only one metazoan (*Z. ostreum*) was shared across regions, while four protistan parasites occurred in both the Pacific-Canal and Caribbean-Canal regions (Fig. 4). Four protistan species were found between both Caribbean regions, two were shared between the Pacific-Canal and Caribbean, and seven were only detected in a single region. These results likely indicate a complex combination of factors influencing the biogeographic patterns, including the anthropogenic spread of some protistan parasites, which are able to survive and likely establish in each region and either a lack of spread or inability to survive in the different regions for other species.

There are many potential factors that could contribute to the establishment of parasites in the different regions. First, host density and diversity are positively correlated with overall parasite species richness (Poulin, 2004; Lindenfors *et al.* 2007), so the availability of competent hosts could influence parasite distributions. Though we do not know of any published surveys comparing the distribution and abundance of oysters in the families Ostreidae, Isognomonidae, or Pteriidae in Panamanian waters, our sampling efforts, which were directed towards the most abundant species on both coasts, recorded ten species, including two new records (*Saccostrea* sp. and *Isognomon* sp.; Pagenkopp Lohan *et al.* 2015). Higher host richness was observed on the Caribbean side, but the density of species within locations was generally higher on the Pacific side (Pagenkopp Lohan and Hill-Spanik, personal observation). Additional sampling to determine the density and diversity of potential hosts and confirmation of infection for each host is required to assess this hypothesis.

Second, host specificity is an important factor in determining parasite distributions. Previous research has shown that specialist species are more common in tropical latitudes (Krasnov *et al.* 2008; Poulin *et al.* 2011). Additionally, parasites with low host specificity are more likely to host-switch (Holt *et al.* 2003), be successful invaders (Taraschewski, 2006) and be more geographically widespread (Krasnov *et al.* 2005). Thus, we may expect higher species richness in the tropics due to a high degree of parasite specialization accompanying high host diversity. Alternatively, given the shipping volume into the Panama Canal, we may expect a high diversity of generalist parasites as, after anthropogenic activities concentrated them in that area, they would be more apt to find a competent host in order to establish and survive.

For the metazoans, all three parasite species are known host generalists. *Urastoma cyprinae* is known to infect the largest number of hosts in at least five countries (Fleming *et al.* 1981; Goggin and Cannon, 1989; Murina and Solonchenko, 1991; Cáceres-Martínez *et al.* 1998; Canestri Trotti *et al.* 1998; Aguirre-Macedo *et al.* 2007), while the two pea crabs species are comparatively more host specific and geographically isolated (Campos, 2002; Manning, 1993). These broader observations match the results of this study, with *U. cyprinae* infecting more hosts than the two pea crab species.

Based on the current literature, *Perkinsus* species appear to be less host specific and more widespread in general. Including the results from this study, *P. olseni* is currently the least host specific of the *Perkinsus* species, as it has been detected in 17 countries from 27 host species representing five Orders and six Families (Villalba *et al.* 2004). Including this study, *P. marinus* has been detected in four countries from 16 host species representing three Orders and five Families (Villalba *et al.* 2004; Reece *et al.* 2008). Including this study, *P. chesapeaki* has been detected in five countries from 14 host species representing four Orders and eight Families (Villalba *et al.* 2004; Dang *et al.* 2015). Finally, including the results of this study, *P. beihaiensis* has been detected in four countries from 13 species, over half of which are from this study alone, representing three Orders and three Families (Moss *et al.* 2008; da Silva *et al.* 2014). While we did not confirm infection status of the oysters, we expected *P. olseni* to be detected from the most host species, based on the low specificity and global distribution from previous studies. To the contrary, we found that *P. beihaiensis* was detected from the greatest number of hosts.

Unfortunately, determining any information about the host specificity of the haplosporidians is presently impossible, as most could not be identified to species level. The only species we could identify, *H. costale*, has been previously detected in three countries from five species, all from the family Ostreidae (Wood and Andrews, 1962; Wang *et al.*

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2010). Our data add another three potential ostreid hosts. With the extremely limited information about hosts and geographic range for these species, it appears that many are host specialists and geographically isolated (Burreson and Ford, 2004; Arzul and Carnegie, 2015).

The difference in host specificity between *Perkinsus* and haplosporidian species appears to be an important factor determining their diversity and distributions. The lack of specificity among the *Perkinsus* species may be the reason that these species were the most geographically widespread and most frequently detected throughout Panamanian waters. The high specificity of the haplosporidians may explain why they were more diverse, but less widespread and detected at lower frequencies.

In conclusion, though mass mortalities are the most conspicuous impact of parasites, they can have additional community/ecosystem wide impacts that are no less profound, such as changing species compositions and interactions (e.g. competition, predation) (Prenter *et al*. 2004). The high parasite diversity found in Panamanian waters, which includes many new records for these waters, emphasizes our lack of knowledge regarding the diversity, abundance and effects of parasites in tropical waters. Characterizing the diversity and distribution of host generalist parasites, in particular, around major shipping corridors and hubs such as the Panama Canal is crucial, given the potential for these pathogens to be globally dispersed through shipping. Additionally, the variation observed in parasite distributions suggests other mechanisms are influencing parasite dispersal and establishment. While anthropogenic dispersal is occurring, multiple factors (e.g. host specificity, host distribution) are contributing to the observed biogeographic patterns and heterogeneity of parasite assemblages between regions.

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SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0031182015001900

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