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# The distribution and host-association of a haemoparasite of damselfishes (Pomacentridae) from the eastern Caribbean based on a combination of morphology and 18S rDNA sequences

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ABSTRACT

Coral reefs harbor the greatest biodiversity per unit area of any ecosystem on earth. While parasites constitute the majority of this biodiversity, they remain poorly studied due to the cryptic nature of many parasites and the lack of appropriate training among coral reef ecologists. Damselfishes (Pomacentridae) are among the most abundant and diverse fishes on coral reefs. In a recent study of blood parasites of Caribbean reef fishes, the first ever apicomplexan blood parasites discovered in damselfishes were reported for members of the genus *Stegastes*. While these blood parasites were characterized as "*Haemohormidium*-like", they appear to be distinct from any other known apicomplexan. In this study, we examined host associations, geographic distributions, and provide further insights on the phylogenetic affiliation of this parasite. A combination of morphological characteristics and 18S rDNA sequences suggest that this parasite may be the same species at multiple sites and occurs from the southern to the northern extreme of the eastern Caribbean, although it appears rare in the north. At present it appears to be limited to members of the genus *Stegastes* and infects all life history stages. It is most common in benthophagous species that occur in high population densities and appears basal to a major monophyletic clade containing species of coccidia, distinct from the Piroplasmida, the order to which *Haemohormidium* spp. have been assigned. These findings suggest a possible fecal-oral mode of transmission.

## 1. Introduction

Near-shore scleractinian coral reefs harbor the greatest biodiversity found in the world's oceans (e.g., Roberts et al., 2002), and in fact contain more species per square meter than any other ecosystem on the planet (Knowlton et al., 2010). This high biodiversity contained within a relatively small area facilitates a multitude of complex interactions between components of the biotic and abiotic community (Dornelas et al., 2006). Parasites compose the majority of biodiversity on coral reefs (Rhode, 1992, 1999; Poulin and Morand, 2000; Muñoz et al., 2007; Knowlton et al., 2010), and provide a key link in coral reef ecosystems, providing both a food source and selective pressure on hosts that influence the behavior of the coral reef inhabitants (Hudson et al., 2006). Along with providing key ecological links in coral reefs, parasites also cause and/or act as vectors for disease (Lefévre and Thomas, 2007).

Most research on parasitic diseases in coral reef systems has focused on diseases of the corals themselves as a major cause of coral decline (e.g., Harvell et al., 2004; Correa et al., 2009). Research on diseases of fishes has mainly focused on species that are of economic or recreational importance, and/or diseases impacting the aquaculture industry (Arkoosh et al., 1998; Johnson et al., 2004; Masson et al., 2013). This research has been further biased towards bacterial and fungal infections affecting large top-trophic level fish (Cahill, 1990; McVicar, 1997). Given that diseases can have a large impact on population structure and thus knock-on effects at the community or ecosystem level, a broader understanding of potential disease-causing organisms in coral reef fishes seems important.

Apicomplexan hemoparasites are obligate parasites of many species of vertebrates (Davies and Johnston, 2000). Apicomplexans can exist within their host with relatively little impact or can cause catastrophic damage resulting in death. The majority of blood-borne apicomplexans

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require two hosts to complete their development. Asexual development, which leads to the formation of gamont stages in the peripheral blood, occurs in a vertebrate (intermediate) host, and sexual development, initiated by the uptake of gamont stages, occurs in a haematophagous invertebrate (definitive) host. Transmission of infective sporozoite stages from the infected invertebrate host occurs either through inoculation as in the case of the haemosporidia (e.g. species of *Plasmodium*) and piroplasms (e.g. species of *Babesia*), and some haemogregarines (e.g. species of *Haemogregarina*), or through ingestion of the infected invertebrate as in the case of most haemogregarines (e.g. species of *Hepatozoon*). Haemococcidia, however, such as species of *Lankesterella* and *Schellackia*, complete their development in their vertebrate host, invertebrates acting only as paratenic or mechanical hosts when ingested by the vertebrate (O'Donoghue, 2017). The vast majority of work on the phylum Apicomplexa has focused on *Plasmodium* and other genera of socioeconomic importance (Wozniak et al., 1994; Bejon et al., 2006; Sant'Anna et al., 2008; Ogedengbe et al., 2013; Heddergott et al., 2012) in terrestrial systems. Much less is known about apicomplexan parasites in coral reef systems or in marine fishes.

Members of the family Pomacentridae are small-to medium-sized fishes that exhibit a circumtropical distribution and include some subtropical and warm temperate species (Allen, 1991; Helfman et al., 2009). They include herbivores, planktivores, and omnivores that inhabit all areas from shoreline to deep-reef structures (Allen, 1991; Helfman et al., 2009). Some species defend permanent multipurpose territories while in others only the males are territorial when defending nests. Members of this family are present in high numbers on reefs, and are prey for larger predators (e.g., Greenfield and Johnson, 1990; Wilson and Meekan, 2002; Mumby et al., 2012).

In the Caribbean, pomacentrids are represented by members of the genera *Abudefduf*, *Chromis*, *Stegastes*, and *Microspathodon*. The most common species of *Abudefduf* (*A. saxatilis*) and *Chromis* (*C. multilineata*) are midwater shoalers that spend their time feeding on zooplankton during the day and retire to the reef at night (e.g., Randall, 1968; Allen, 1991). In contrast, *Abudefduf taurus* is solitary and inhabits shallow, high surge areas. Both sexes of species of *Stegastes* and *Microspathodon* maintain permanent territories and occupy a wide range of shallow coral reef habitats (Waldner and Robertson, 1980; Itzkowitz et al., 1995).

As in other systems where top-level predators have been removed, parasitic diseases often replace them as the primary regulators of populations (Packer et al., 2003; Lafferty et al., 2008; Raffel et al., 2008). Thus, identifying actual or potential disease-causing organisms and how they are transmitted becomes essential to understanding coral reef community dynamics.

In a recent survey of hemoparasite biodiversity of reef-associated fishes of the eastern Caribbean, Cook et al. (2015) sampled 1298 individual fish from 6 eastern Caribbean islands, representing 27 families, 57 genera and 103 species. In all, members of 14 species from 8 families were infected with 8 distinct types of blood parasites, 6 of which were apicomplexan. These included a newly discovered intraerythrocytic parasite that was tentatively referred to as *Haemohormidium*-like and was common in adults of three species of *Stegastes* damselfishes (Pomacentridae) including *S. adustus*, *S. diencaeus* and *S. leucostictus* (Cook et al., 2015). This blood parasite was rare or absent in three other species of *Stegastes* and was absent in *A. saxatilis* and both Caribbean *Chromis* spp. sampled. However, variation among *Stegastes* and apparent absence in *A. saxatilis* may have been attributable to small sample sizes and/or sampling from a single site. In a subsequent study, Renoux et al. (2017) developed an apicomplexan DNA barcoding system, targeting the 18S rDNA gene, to detect infections of the *Haemohormidium*-like parasites in *Stegastes* spp. Phylogenetic analysis of this parasite by Renoux et al. (2017) placed it at the base of a major monophyletic clade containing species of coccidia, suggesting it to be more closely related to this group than to the piroplasms, the group to which the Haemohormidiidae have been assigned pending molecular support (see O'Donoghue, 2017). As a follow-up to the work of Cook et al. (2015) and Renoux et al. (2017), the aim of the current study was to determine the geographic distribution and host-association of this parasite in damselfishes in the eastern Caribbean. Specifically, we: 1) further quantify which damselfish species and life history stages are infected by the *Haemohormidium*-like blood parasite, increasing the sample size for under-sampled species and including juvenile life history stages; and 2) further elucidate the geographic distribution and phylogenetic affiliation of this blood parasite in the eastern Caribbean.

## 2. Materials and methods

### 2.1. Host blood collection

This study was conducted between May 2013 and August 2016. Fish used in this study were collected on nearshore reefs from 0 to 7 m depth by free divers or scuba divers using modified cast nets or large monofilament hand nets. In order to further assess host associations among Caribbean damselfishes, and life history associations among *Stegastes* species, we sampled a total of 627 damselfish from sites at or near where infected fish had previously been found in at least one species in addition to two new sites (Fig. 1). These sites were: Great Lameshur Bay, St. John, United States Virgin Islands (USVI; 18.33° N, 64.73° W), two sites (Brewers Bay, Fortuna Bay) on St. Thomas (18.33° N, 64.91° W), USVI; White Bay, Guana Island, British Virgin Islands (BVI; 18.50° N, 64.63° W); Culebra, Puerto Rico (18.30° N, −65.30° W); La Parguera, Puerto Rico (17.97° N, −67.04° W); and Frederiksted St. Croix, USVI (17.71° N, −64.87° W). Collections from these sites included 39

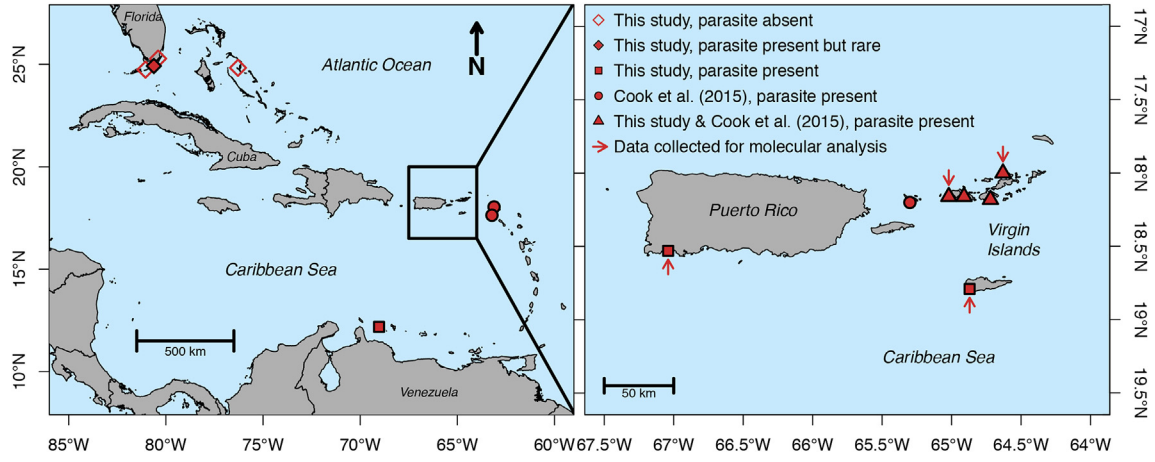


**Fig. 1.** Map of the Eastern Caribbean region showing collection sites for the current study and Cook et al., 2015.

*Microspathodon chrysurus*, 23 *Abudefduf saxatilis*, along with 167 juvenile and 398 adult *Stegastes*. At all sites we endeavoured to collect at least 10 individuals from at least two locally abundant *Stegastes* spp., including at least 10 juveniles (except at St. Croix, where only adults were targeted).

To further assess the geographic distribution of the *Haemohormidium*-like parasite, we sampled an additional 49 *Stegastes* from Eleuthera, The Bahamas (25.07° N, 76.12° W); 117 *Stegastes* from among Marathon, Key Largo, and Middle Key in the Florida Keys, USA (24.71° N, 81.05° W); and 67 *Stegastes* from Curaçao, Netherlands Antilles (12.19° N, 69.03° W). At these sites, we preferentially targeted those species known to be frequently parasitized. Finally, to identify if the blood parasite found at various sites was the same species or a complex of closely-related species, a third round of samples was collected for molecular analysis from 4 sites including Brewers Bay, Guana Island, La Parguera, and southwest St. Croix. These samples (n = 271) were collected from May-August 2016 and also focused on species known to be frequently infected including 85 *S. adustus*, 84 *S. diencaeus* and 46 *S. planifrons*, as well as the less commonly infected 22 *S. leucostictus*, and 11 *S. variabilis*.

Blood samples were collected within 24 h of capture following Cook et al. (2015). The sampling procedure was authorized by Arkansas State University IACUC approval #326673-1. Fish were anesthetized using a 1:20 dilution of clove oil solution (clove oil solubilized in ethanol) in fresh seawater. Once a fish was anesthetized, it was removed from the clove oil solution, placed in a dry cloth, and blood (< 0.1 cc) was collected from the caudal artery. Duplicate blood smears were made for each fish on labelled, frosted, glass slides. For the subset of fish used for molecular analysis, an additional volume of blood was preserved immediately in 100% molecular grade ethanol as per methods outlined in Renoux et al. (2017). Blood smears were fixed using absolute methanol, and stained using Giemsa stain, modified solution (Sigma Aldrich) prior to screening.

### 2.2. Quantification of blood parasite

#### 2.2.1. Screening of blood smears for the Haemohormidium-like parasite

Thin blood smears were screened using a 100× oil immersion objective, and micrographs and measurements of parasites were taken on a calibrated Nikon Eclipse E800 compound microscope (Nikon, Amsterdam, Netherlands) using the Nikon NIS-Elements microscope imaging software program D3.2 (Nikon). The morphometrics of parasites were subsequently compared to those of the *Haemohormidium*-like parasite described by Cook et al. (2015). For molecular analysis, only blood from fish with high levels of infection was used. This was done according to Renoux et al. (2017) and was based on the number of parasites per 500 erythrocytes; intensities of ≥1 infection per 500 erythrocytes were used.

#### 2.2.2. Statistical analysis of blood parasite infection in damselfish

For each study site and species, the total number of fish positive for blood parasites was divided by the total number of fish sampled to calculate the proportion of fish infected (infection prevalence). Confidence intervals for infection prevalence were calculated using the Wilson procedure with a correction for continuity (Wilson, 1927; Newcombe, 1998). We compared prevalence with binomial logistic regressions using a generalized linear mixed effects model (GLMM) with *host species* as a categorical fixed effect, nested within *study site* as a random effect. We limited this analysis to adult-size fish to avoid any effects of life history stage on infection rate (see life history comparison below). This analysis allowed us to control for among-site variation, and was performed for Brewers Bay, Fortuna Bay, Lameshur Bay, La Parguera, Frederiksted, and White Bay, where multiple *Stegastes* species (*S. adustus*, *S. diencaeus*, *S. leucostictus*, *S. partitus*, *S. planifrons*, and *S. variabilis*) were collected during 2013 (for the first five sites), and 2015 (Frederiksted), Supplemental samples collected from White Bay in 2016 were combined with 2013 samples to achieve adequate sample sizes for calculation of prevalence at this site. To simultaneously test the null hypotheses of no difference in infection prevalence among host species, we corrected p-values and confidence intervals for post-hoc Tukey comparisons with *multcomp* (Hothorn et al., 2008), a package in the statistical software R v3.1.2 (R Core Team, 2017). Our regressions were also constructed in R, with the package *lme4* (Bates et al., 2015).

### 2.3. DNA extraction, PCR and phylogenetic analysis of 18S rDNA

Fishes from the supplemental samples for molecular analysis (Supplement Table 3) and identified microscopically as infected with the *Haemohormidium*-like parasite with intensities of ≥1 infection per 500 erythrocytes were preferentially used for DNA extraction following a rapid DNA extraction method as detailed in the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa). Molecular characterisation of the *Haemohormidium*-like parasite was performed via PCR amplification, amplifying approximately the full 18S rRNA gene using forward primer EF (5′-GAAACTGCGAATGGCTCATT-3′) and reverse primer ER (5′-CTTGCGCCTACTAGGCATTC-3′) (Kvičerová et al., 2008). Conditions for PCR were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles, entailing a 95 °C denaturation for 30 s, annealing at 55 °C for 30 s with an end extension at 72 °C for 2 min, and following the cycles a final extension of 72 °C for 10 min.

All PCR reactions were performed with volumes of 25 µl, using 12.5 µl Thermo Scientific DreamTaq PCR master mix (2×) (2× DreamTaq buffer, 0.4 mM of each dNTP, and 4 mM MgCl2), 1.25 µl of each primer (10 µM), and at least 25 ng of DNA. PCR grade nuclease free water (Thermo Scientific, Vilnius, Lithuania) was used to make up final reaction volume. Reactions were undertaken in a Bio-Rad C1000 Touch™ Thermal Cycler PCR machine (Bio-Rad, Hemel Hempstead, UK). An agarose gel (1%) stained with gel red was used to visualise resulting amplicons under UV light. Two PCR products from each sample were sent to a commercial sequencing company (Inqaba Biotechnical Industries (Pty) Ltd. Pretoria, South Africa) for purification and sequencing in both directions. Quality of resultant sequences was assessed using Geneious Ver. 7.1 (http://www.geneious.com, Kearse et al., 2012) before consensus sequences were generated from both forward and reverse sequence reads. Sequences were identified using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/), and deposited in the NCBI GenBank database under the accession numbers: MH401637, MH401638, MH401639, MH401640, MH401641, MH401642 or MH401637-42.

For the phylogenetic analysis sequences generated of the *Haemohormidium*-like parasite from the different species of damselfish and from the different sites were compared. Comparative sequences of coccidia (with reference to the findings of Renoux et al., 2017) with *Adelina dimidiata* (GenBank: DQ096835) as outgroup (following Barta et al., 2012; Xavier et al., 2018), were downloaded from GenBank and aligned to the sequences generated within this study. Sequences were aligned using the Clustal W alignment tool (Thompson et al., 1994) implemented in Geneious Ver. 7.1. The alignment consisted of 43 sequences and was 1100 nt in length, with the exception of six sequences (MF468290, MF468291, MF468292, MF468293, MF468323, MF468328) being ~500 nt. These shorter sequences were included as they represent species of coccidia recently isolated from marine fish hosts by Xavier et al. (2018), two of these falling with a *Haemohormidium*-like parasite isolated by Renoux et al. (2017) (see Xavier et al., 2018). To infer phylogenetic relationships of the aligned dataset a Bayesian inference (BI) method was used. A model test was preformed to determine the most suitable nucleotide substitution model, according to the Akaike information criterion (AIC) using jModelTest 2.1.7 (Guindon and Gascuel, 2003; Darriba et al., 2012). The best model identified was the General Time Reversible model with estimates of invariable sites and a discrete Gamma distribution (GTR + I + Γ). The BI analysis was performed using MrBayes software (ver. 3.2.6)

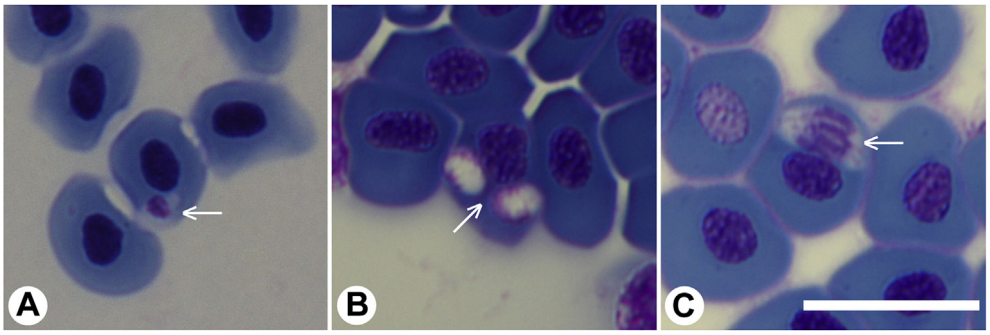


**Fig. 2. Peripheral blood stages of the *Haemohormidium*-like parasite infecting species of *Stegastes*.** Giemsa stained light micrographs of the *Haemohormidium*-like parasite as observed in the peripheral blood of *Stegastes diencaeus* from St Thomas, eastern Caribbean (Genbank accession number MH401641). A. rare possible trophozoite stage. B. possible meront stages undergoing transverse binary fission. C. possible meront stages undergoing longitudinal binary fission. Scale bar = 10 μm.

(Ronquist et al., 2012) run on the CIPRES portal (Miller et al., 2010). Markov chain Monte Carlo (MCMC) chains were run for 10,000,000 generations, log-likelihood scores were plotted, and only the final 75% of trees were used to produce the consensus trees by setting the 'burn in' parameter at 2500.

## 3. Results

### 3.1. Species and life history stage

#### 3.1.1. Presence of blood parasites among damselfishes

A summary of infections among life history stages and species at sites used for species comparison is presented in Supplement Table 1. The intraerythrocytic *Haemohormidium*-like parasite found in this study was morphologically comparable to that described by Cook et al. (2015) (see Cook et al., 2015 Fig. 1a-e and present study Fig. 2a-c). Besides rare possible trophozoite stages (Cook et al., 2015 Fig. 2a) and possible meront stages of the parasite that appear to be undergoing transverse binary fission (Fig. 2b), the most common and characteristic stage of this parasite was what has been provisionally identified as a dividing meront stage with two to three slender nuclei (rarely four nuclei) (Fig. 2c). This stage measured 6.4 ± 0.4 μm (mean ± SD; range 5.6–7.6) × 1.9 ± 0.6 μm (mean ± SD; range 0.8–3.3) (n = 35) in the present study, compared to 5.7 × 1.5 μm (n = 10) in Cook et al. (2015).

No blood parasites were found in *Microspathodon chrysurus* (n = 39) or *Abudefduf saxatilis* (n = 23), even though these fish were collected from sites where the infection was common in *Stegastes* during this and/or a previous study (Cook et al., 2015). At localities where adult and juvenile *Stegastes* were sampled, blood parasites were found in both. These included *S. leucostictus, S. planifrons* and *S. variabilis* from White Bay, Guana Island; *S. diencaeus, S. leucostictus,* and *S. planifrons* from Lameshur Bay, St. John; and all 6 *Stegastes* species from St. Thomas. From La Parguera, both *Stegastes adustus* and *Stegastes leucostictus* juveniles harbored blood parasites. The smallest individual sampled in this study measured 2.6 cm, and the smallest that harbored blood parasites measured 2.9 cm. Of the five species-site combinations where sufficient numbers (n ≥ 10) of juveniles and adults of the same species were collected from the same site, four had blood parasites that were more prevalent in adults than juveniles.

Among the six *Stegastes* spp. at the six study sites with sufficient sampling (adults only), *S. adustus* had the highest proportion infected at 76.0% (95% CI 67.4–83.0%), followed by *S. planifrons* at 60.0% (95% CI 48.8–70.3%), *S. diencaeus* at 54.3% (95% CI 42.0%–66.1%), *S. leucostictus* at 25.5% (95% CI 14.4–40.6%), *S. variabilis* at 14.3% (95% CI 7.1–25.9%), and *S. partitus* at 5.4% (95% CI 1.4–15.8%) (Fig. 3). Infection prevalences of *S. adustus*, *S. planifrons*, and *S. diencaeus* were each significantly greater than those of *S. leucostictus*, *S. variabilis*, and *S. partitus* (Table 1; GLMM: all pairwise comparisons with Tukey adjusted $p < 0.05$). However, there were no significant differences in infection prevalence among the three species with higher prevalences (*S. adustus*, *S. planifrons*, and *S. diencaeus*; Table 1; GLMM: all pairwise comparisons with Tukey-adjusted $p > 0.05$), nor among the three species with lower prevalences (*S. leucostictus*, *S. variabilis*, and *S. partitus*; Table 1; GLMM: all pairwise comparisons with Tukey-adjusted $p > 0.1$). However, the lack of statistically significant differences between *S. partitus* and *S. leucostictus* and *S. variabilis* appears driven by one site in which three of four (75%) of *S. partitus* were infected (the only three infected fish among all adult *S. partitus* collected).

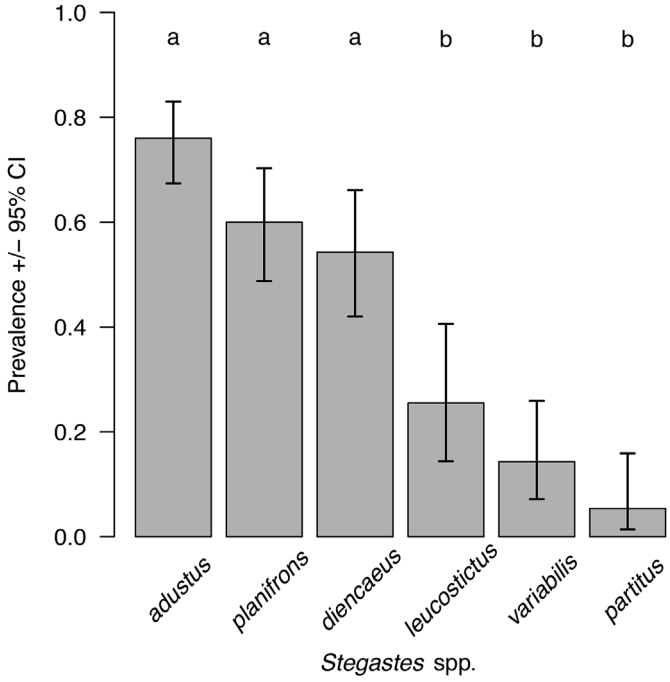


**Fig. 3. Prevalence of infection differences among six *Stegastes* spp., averaged across six study sites.** 95% confidence intervals calculated using the Wilson procedure with continuity corrections. Different lower-case letters above each bar indicates a significant ($p \leq 0.05$) difference between species, as indicated by a binomial logistic regression (GLMM results shown in Table 1).

#### 3.1.2. Geographic range of blood parasites in Stegastes of the eastern Caribbean

Blood parasites were found in one or more *Stegastes* individuals at nine of the sites sampled (Fig. 1). This included White Bay (Guana Island), St. John, St. Thomas (both sites), St. Croix, Puerto Rico (both sites), Curaçao, and Key Largo. Interestingly, only two individuals (one *S. planifrons* and one *S. variabilis*) were infected from the Florida Keys (= 1.7%), and none of the 49 fish sampled (23 *S. partitus* and 26 *S. diencaeus*) from Eleuthera were infected. A summary of infections at sites used for supplemental geographic comparison is presented in Supplement Table 2.

**Table 1**
Simultaneous tests for general linear hypotheses from a binomial logistic regression (GLMM) of infection prevalence as a function of host species (fixed effect) nested within study site (random effect).

| Comparison | Estimate* | Std. Error | z | p** |
|---|---|---|---|---|
| *S. diencaeus - S. adustus* = 0 | −1.015 | 0.364 | −2.789 | 0.055 |
| ***S. leucostictus - S. adustus* = 0** | −2.275 | 0.457 | −4.976 | **< 0.001** |
| ***S. partitus - S. adustus* = 0** | −4.022 | 0.647 | −6.216 | **< 0.001** |
| *S. planifrons - S. adustus* = 0 | −0.758 | 0.338 | −2.244 | 0.206 |
| ***S. variabilis - S. adustus* = 0** | −2.967 | 0.445 | −6.665 | **< 0.001** |
| ***S. leucostictus - S. diencaeus* = 0** | −1.260 | 0.450 | −2.798 | **0.053** |
| ***S. partitus - S. diencaeus* = 0** | −3.006 | 0.670 | −4.488 | **< 0.001** |
| *S. planifrons - S. diencaeus* = 0 | 0.258 | 0.368 | 0.700 | 0.981 |
| ***S. variabilis - S. diencaeus* = 0** | −1.952 | 0.460 | −4.245 | **< 0.001** |
| *S. partitus - S. leucostictus* = 0 | −1.746 | 0.726 | −2.405 | 0.145 |
| ***S. planifrons - S. leucostictus* = 0** | 1.518 | 0.455 | 3.333 | **0.010** |
| *S. variabilis - S. leucostictus* = 0 | −0.692 | 0.526 | −1.314 | 0.766 |
| ***S. planifrons - S. partitus* = 0** | 3.264 | 0.655 | 4.985 | **< 0.001** |
| *S. variabilis - S. partitus* = 0 | 1.055 | 0.717 | 1.471 | 0.668 |
| ***S. variabilis - S. planifrons* = 0** | −2.209 | 0.450 | −4.907 | **< 0.001** |

*Natural log of estimates is the multiplicative change in the odds of infection between 2 spp.
**P-values adjusted with Tukey contrasts for multiple comparisons of means.
**Bold** text indicates significant comparison.

### 3.2. Molecular identification and phylogenetic analysis

Amplicons (> 1300 nt) of the *Haemohormidium*-like parasite were retrieved from 3 of the 5 (60%) infected damselfish species that formed part of the subset collected for the molecular analysis including *S. adustus*, *S. diencaeus* and *S. planifrons* from 4 of the 6 (67%) sites including Guana Island, La Parguera (Puerto Rico), St. Croix, and St. Thomas (Fig. 4). According to the 18S rRNA gene, parasite isolates represent either the same parasite species or two closely related species. Those isolated from *Stegastes* spp. from Guana Island (GenBank: MH401637-9) had a 2 nt difference (both insertions) from those of the other three sites (GenBank: MH401637-9). Isolates of these three sites compared with those of a *Haemohormidium*-like parasite isolated by Renoux et al. (2017) from a *S. adustus* (KT806397) and *S. diencaeus* (KT806398) from St John. The *Haemohormidium*-like parasite was basal to a major monophyletic clade containing species of coccidia, a finding comparable to that of Renoux et al. (2017). Furthermore, amplicons retrieved in this study and in Renoux et al. (2017) formed a monophyletic clade with that of apicomplexans of unknown identity retrieved during a molecular survey from tissues of the liver of *Solea senegalensis* (MF468328) and the heart of *Pagrus caeruleostictus* (MF468323), both species of fish collected from the Northeast Atlantic (see Xavier et al., 2018).

## 4. Discussion

Apicomplexan parasites of amphibians, reptiles and mammals are often characterized molecularly using the 18S rRNA gene. However, apicomplexans of fishes are almost exclusively identified morphologically, by comparing peripheral blood stages and their vectors (Davies and Johnston, 2000; Renoux et al., 2017). Here we combined morphological and molecular approaches. The distinctive morphological characteristics of this *Haemohormidium*-like species, particularly its small size and 'meront' stage development, support its identification in the six *Stegastes* species inhabiting the reefs of the eastern Caribbean, as the same species reported by Cook et al. (2015). Further evidence of this parasite's presence in our samples is provided through molecular sequence data: highly similar sequences isolated from the two most frequently infected damselfish species, *S. adustus* and *S. diencaeus,* at four of our sites (five sites, if including those from Renoux et al. (2017)). If our morphological and molecular assessment is correct that this is the same parasite across sites and species, then the parasite has a wide geographic distribution and low host-specificity within the *Stegastes* genus; we have not yet detected it in any other Caribbean pomacentrids taken from the same sites, including *Chromis* spp. (n = 61, Cook et al., 2015), *Abudefduf saxatilis* (n = 31, Cook et al., 2015 and this study), and *Microspathodon chrysurus* (n = 45, Renoux et al., 2017 and this study).

Based on morphological data alone, this *Haemohormidium*-like blood parasite appears to occur from the southernmost to the northernmost parts of the eastern Caribbean region. Outside of the Caribbean in the subtropical western Atlantic, the parasite was not found at our site in the Bahamas and was extremely rare in the Florida Keys. This may be because northern sites experience cool conditions in winter, which may reduce parasite and/or vector populations. We have yet to sample sites in the western Caribbean. The only other apicomplexan blood parasite of marine fishes recorded to date with a wide distribution and low host-specificity is the haemogregarine *Haemogregarina* (sensu lato) *bigemina* Laveran and Mesnil, 1901. *H. bigemina* has been recorded infecting fishes from 34 families across the world, but this distribution is based on morphology alone and has not yet been confirmed with molecular approaches (Davies et al., 2004; Cook et al., 2015).

Using parasite morphology alone, Cook et al. (2015) recorded similar *Haemohormidium*-like parasites as in the present study, except in another two families of Eastern Caribbean fishes. This included two labrid species, *Nicholsina usta usta* (n = 2 infected of 4 sampled) and *Scarus taeniopterus* (n = 1 infected of 6 sampled), and one blennid *Ophioblennius macclurei* (n = 9 infected of 14 sampled). However, the majority of infections reported by Cook et al. (2015) were from *Stegastes* spp. It would thus appear that this parasite may be genus-specific and the infections seen in the species of Labridae and Blenniidae an opportunistic case of host-switching or a different species entirely. Molecular analysis later revealed that the *Haemohormidium*-like parasite that infected *O. macclueri* was a different species than the one in *Stegastes* spp., even though the parasites were morphologically indistinguishable (Renoux et al., 2017).

The prevalence of this parasite in *Stegastes* spp. may be partially attributable to the variable feeding behaviors and ecologies within the genus. We found parasites in individuals as small as 3 cm in length. The highest prevalence, as mentioned above, was seen in *S. adustus* (nearly 80%) followed by *S. planifrons* and *S. diencaeus* (50–60%), then *S. leucostictus* and *S. variabilis* (20–25%), and *S. partitus* which was rarely infected. These differences track differences in social structure, feeding habits, and population density (Waldner and Robertson, 1980). The first three species are benthophagous, occupy hard reef structure with high algal growth, and occur in colonies of conspecifics that reach highest densities (Ferreira et al., 1998). While *S. leucostictus* and *S. variabilis* are also benthophagous, they tend to occur on rubble substrate and have larger territories, and thus occur in lower population densities. In contrast to the other five species, *S. partitus* is primarily planktivorous. A parasite's mode of transmission is tied to host behavior. The benthophagous nature and high-density colonies of *S. adustus* facilitates exposure of the parasite to a number of new hosts on a continual basis. Similarly, if host behavior exposes the parasite to a wide variety of potential hosts, selection is inclined to favor host switching, that will in turn lead to a decrease in the host specificity of the parasite (Dick and Patterson, 2007), potentially explaining the wide distribution of this parasite, particularly in multiple species of *Stegastes*.

The variation in infection prevalence among *Stegastes* combined with the phylogenetic relationship of this blood parasite to other Apicomplexa (basal position relative to that of known coccidia species) suggests that it may be transmitted via an oral-fecal route via oocysts. Species of coccidia that do not demonstrate blood-borne stages form infective stages (oocysts), which are disseminated into the environment along with the excretion of waste, particularly feces. These sporozoite-containing oocysts are infective upon ingestion by an appropriate host (Kheysin, 1972). Species of *Stegastes* appear to defecate primarily outside territorial boundaries (M. Nicholson and P. Sikkel, unpublished

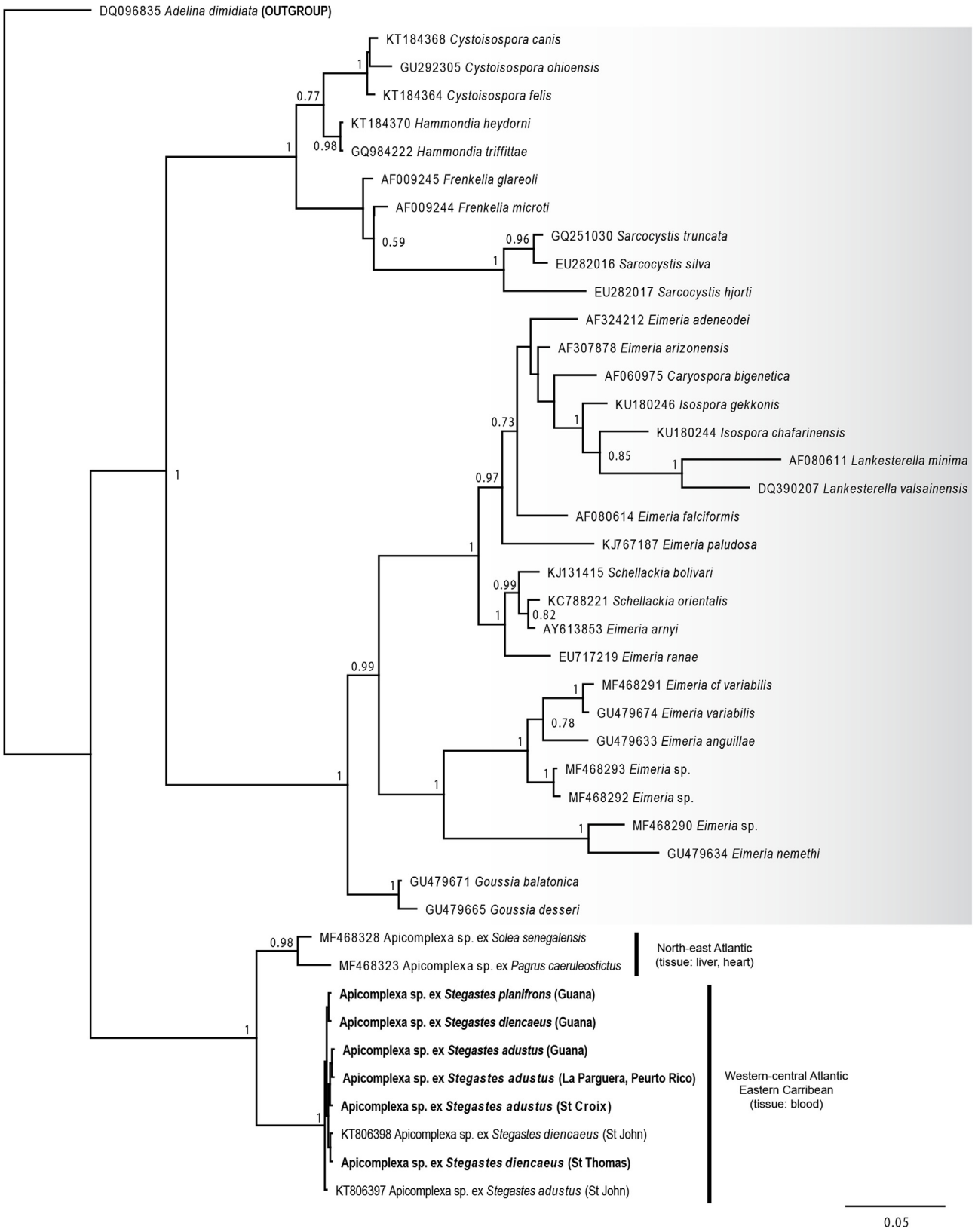


**Fig. 4. Phylogenetic analysis of the Haemohormidium-like parasite based on 18S rDNA sequences**. Bayesian inference (BI) analysis showing the phylogenetic relationships for 8 Haemohormidium-like parasite isolates, 6 from the present study (GenBank: MH401637-42) (in bold) and 2 from Renoux et al. (2017), isolated from three species of Stegastes including Stegastes adustus, Stegastes diencaeus and Stegastes planifrons, from 5 sites in the eastern Caribbean. Comparative sequences representing known coccidia, with Adelina dimidiata (DQ096835) as outgroup, were downloaded from the GenBank database. Nodal support values > 50% are represented on the tree.

data), leading to a higher likelihood of ingesting feces for benthophagous species that live in dense colonies. However, the majority of coccidia do not show peripheral blood stages, with the exception of two genera: *Lankesterella* and *Schellackia*, in which the sporozoites are encountered in the blood cells (Megía-Palma et al., 2014). Also, merozoite stages of species of the genus *Isospora* (formerly regarded as a species of *Atoxoplasma*) have been recorded infecting blood cells. Therefore, a second route of transmission may be ingestion by blood-feeding invertebrates that in turn act as paratenic transport hosts, infecting new vertebrate hosts upon being eaten (O'Donoghue, 2017). In the Caribbean, the blood-feeding gnathiid isopod *Gnathia marleyi* is commonly found to infest over 20 different species of bony fishes, including species of *Stegastes* (Farquharson et al., 2012; Coile and Sikkel, 2013; Jenkins et al., 2017). As such, there is the potential for this ectoparasite to act as a paratenic host of the *Haemohormidium*-like parasite. *Stegastes* have been observed to consume gnathiids (PC Sikkel unpublished data). However, if this is the case and the parasite infecting *Stegastes* spp. is not genus-specific, we would have expected to find infections in fishes that feed primarily on small invertebrates, especially in those for which gnathiids appear to form part of the diet. Artim et al. (2017) recorded gnathiids from the gut contents of five genera of microcarnivorous fishes. Two of these genera of fishes, *Haemulon* and *Holocentrus*, were sampled by Cook et al. (2015) with no record of the *Haemohormidium*-like parasite at sites where this parasite is common in species of *Stegastes*. It is, however, still possible that *G. marleyi* does act as a route of infection of the *Haemohormidium*-like parasite. Desser et al. (1990) demonstrated experimentally that a leech could act as the vector of the haemococcidian *Lankesterella minima* (Chaussat, 1850), providing evidence that species of this genus may not only use the ingestion of parasitized hosts for transmission, but also inoculation. As *G. marleyi* gnathiids, in contrast to leeches, are commonly encountered infesting damselfishes in the eastern Caribbean (PC Sikkel unpublished data), a third route of transmission involving inoculation of the *Haemohormidium*-like parasite by gnathiids needs to be considered.

Currently, based on 18S rDNA, the *Haemohormidium*-like parasite is distinct from known genera of the coccidia, as well as from genera of the piroplasms for which there are available molecular data (this study; Renoux et al., 2017). Unfortunately, it is not possible at this time to compare this parasite on a molecular level to species of *Haemohormidium*, as no molecular data have been provided for known species of this genus. However, based on morphology, the parasite of the present study does not share the typical characteristics of the type species of *Haemohormidium cotti* Henry, 1910 (Davies, 1995; Cook et al., 2015; Renoux et al., 2017). Until this parasite's uncertain taxonomic identity has been resolved, we suggest referring to it as Apicomplexa sp. The phylogenetic position of this parasite may be better resolved in the future with the addition of molecular samples of other taxa of fish apicomplexan blood parasites, including known *Haemogregarina* spp. of fishes, and the use of other molecular markers such as mitochondrial (mtDNA) in combination with the 18S rRNA gene (Ogedengbe et al., 2015). Future research should further elucidate the transmission pathway of this parasite under laboratory conditions. This will include screening parasitized fishes for other developmental stages of the parasite, and investigating gnathiids as potential hosts/vectors by studying gnathiid loads on the different species of *Stegastes*, as well as examining gnathiids for parasite development.

## Conflicts of interest

None.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2018.05.004.

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