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DEVELOPING AN APICOMPLEXAN DNA BARCODING SYSTEM TO DETECT BLOOD PARASITES OF SMALL CORAL REEF FISHES

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ABSTRACT: Apicomplexan parasites are obligate parasites of many species of vertebrates. To date, there is very limited understanding of these parasites in the most-diverse group of vertebrates, actinopterygian fishes. While DNA barcoding targeting the eukaryotic 18S small subunit rRNA gene sequence has been useful in identifying apicomplexans in tetrapods, identification of apicomplexans infecting fishes has relied solely on morphological identification by microscopy. In this study, a DNA barcoding method was developed that targets the 18S rRNA gene primers for identifying apicomplexans parasitizing certain actinopterygian fishes. A lead primer set was selected showing no cross-reactivity to the overwhelming abundant host DNA and successfully confirmed 37 of the 41 (90.2%) microscopically verified parasitized fish blood samples analyzed in this study. Furthermore, this DNA barcoding method identified 4 additional samples that screened negative for parasitemia, suggesting this molecular method may provide improved sensitivity over morphological characterization by microscopy. In addition, this PCR screening method for fish apicomplexans, using Whatman FTA preserved DNA, was tested in efforts leading to a more simplified field collection, transport, and sample storage method as well as a streamlining sample processing important for DNA barcoding of large sample sets.

Members of the phylum Apicomplexa are obligate intracellular parasites of many vertebrate and invertebrate hosts. Morphological and molecular research on parasitic members of this phylum have focused almost exclusively on parasites that inhabit predominately terrestrial vertebrates (i.e., humans, livestock, poultry, zoo animals, and reptiles) (Wozniak et al., 1994; Ujvari et al., 2004; Bejon et al., 2006; Sant'Anna et al., 2008; Heddergott et al., 2012; Ogedengbe et al., 2013; Cook et al., 2014). The paucity of research on apicomplexans of other species, which constitutes the majority of vertebrate biodiversity, creates a large gap in our understanding of these organisms.

Thus far, what little work that has been done on detection and identification of apicomplexan parasites in marine fishes has been based on morphological characteristics using microscopy. This method is labor intensive, requires extensive training in identification techniques, and often results in subjective assignments and difficulty in correctly identifying parasitemias (Davies and Johnston, 2000). Advances in more-precise molecular identification techniques have enabled increased sensitivity and specificity for discrimination of a parasite from its respective host and for classification of apicomplexans (Wozniak et al., 1994; Perkins and Keller, 2001; Harris et al., 2011; Barta et al., 2012; Maia et al., 2012). However, the development of molecular techniques for the study of apicomplexans in fishes has not been reported.

DNA barcoding has emerged as a powerful, rapid, molecular-based method with significant contributions to both taxonomic and biodiversity research (Hebert et al., 2002; Hajibabaei et al., 2007; Weigt et al., 2012; Pereira et al., 2013). Organisms can be accurately identified at the species-level by this process, which uses polymerase chain reaction (PCR) to amplify short DNA fragments corresponding to standardized regions of the genome with associated discriminatory sequence variations that are then subject to DNA sequencing. These sequences are then compared to a public DNA database of all the possible sequence matches or to a defined reference library populated with DNA barcoding sequences of voucher specimens (e.g., BOLD Systems v3; http://www.boldsystems.org/).

Nuclear (e.g., 16S rRNA, 18S rRNA, ITS regions), mitochondrial (e.g., cytochrome *b*, mitochondrial control regions), and some chloroplastic genes have all been used in barcoding eukaryotic organisms (Patwardhan et al., 2014). The more-slowly evolving gene of the mitochondrial genome, cytochrome *c* oxidase I (COI), is acknowledged as the "gold standard" for species identification and DNA barcoding of animals (Hebert et al., 2003). However, for parasite identification, discovery, and diversity research the nuclear 18S small subunit (SSU) rRNA gene has been more-commonly used (e.g., Wozniak et al., 1994; Perkins and Keller, 2001; Harris et al., 2011; Maia et al., 2012; Netherlands et al., 2014).

The 18S rDNA has been reported as a reliable marker gene for parasite identification as it contains both highly conserved and variable regions and typically is present as multiple chromosomal copies, providing the increased sensitivity especially important in discriminating often low numbers of blood-borne parasites amidst large amounts of "contaminating" host DNA (Perkins and Keller, 2001; Waldenström et al., 2004; Berry et al., 2008; Maia et al., 2012; Haanshuus et al., 2013). Evolution progresses much slower in the 18S rRNA gene relative to COI, making it potentially a more valuable marker for resolving at higher taxonomic levels (Tang et al., 2012). Currently there are no published primers specific to fish-parasitic apicomplexans targeting this diagnostic region, although several 18S rDNA primers of closely related apicomplexans have been reported (Wozniak et al., 1994; Perkins and Keller, 2001; Harris et al., 2011; Barta et al., 2012; Maia et al., 2012).

Recent work from our group in the eastern Caribbean has identified a new apicomplexan inhabiting species of blennioids

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(Blenneidae) and *Stegastes* damselfishes (Pomacentridae) (Cook et al., 2015). The finding of apicomplexan blood parasites in blennoids was not surprising, given their presence in other blennoid fishes (Davies et al., 2004). However, the finding of an apicomplexan in *Stegastes* damselfish was surprising because damselfishes, especially members of *Stegastes*, are among the best-studied marine fishes, with research spanning multiple decades (e.g., Waldner and Robertson, 1980; Itzkowitz et al., 1995; Sikkel et al., 2006). Morphologically, this newly discovered blood parasite in *Stegastes* damselfishes bears some resemblance to those found in Caribbean blennoids and is common in all eastern Caribbean *Stegastes* except the bicolor damselfish, *Stegastes partitus*. It, along with that of the Caribbean blenny blood parasite, has been tentatively identified based on morphology as *Haemohormidium*-like by Cook et al. (2015).

The aim of this study was to establish a reliable and robust molecular diagnostic for detecting apicomplexans in advanced ray-fin (actinopterygian) fishes, with an initial focus on Caribbean *Stegastes* damselfishes. Novel primers identifying the 18S rRNA gene for fish apicomplexans were developed that selectively detect parasitic DNA found in low numbers in host fish blood. Whatman FTA® technology (GE Life Sciences, Pittsburgh, Pennsylvania) was also tested for DNA archiving and for sensitivity for molecular detection of the target apicomplexan in providing an improved method for future sample collection in the field.

MATERIALS AND METHODS

Host blood collection

Blood samples were taken from damselfishes and blennoids collected from the U.S. Virgin Islands as part of a larger study on host and geographic distribution of damselfish and blenny apicomplexans in the Caribbean (P. Sikkel et al., unpubl. data). Blood samples from fish (<0.1 cc) were obtained by drawing blood from the caudal artery via syringe with a 26–30-ga needle whereafter a single drop of blood was placed on a glass slide, smeared, and fixed in absolute methanol using methods outlined in Cook et al. (2015). In addition to creating blood smears for each specimen in the field, 1–2 droplets of whole blood were immediately preserved in 100% molecular grade ethanol, shipped on ice, and stored at –80 C for molecular analysis. Blood samples from a subset of parasitized individuals (n = 11) were collected, deposited, dried, and archived (room temperature, airtight pouch with desiccant packet) on Whatman FTA paper (GE Life Sciences). In addition to blood, heart and spleen tissue was collected and DNA was extracted from select *Stegastes adustus* and *Stegastes diencaeus* individuals that had screened positive microscopically (see below) for the blood parasite to test the new fish apicomplexan primers.

Microscopy analysis for parasite-infected fish blood

Samples (n = 53) from St. John, U.S. Virgin Islands collected during the summer of 2013 were used for this study. This included samples from frequently-infected *Stegastes* spp. (*Stegastes adustus*, *Stegastes diencaeus*, *Stegastes leucostictus*, *Stegastes planifrons*, and *Stegastes variabilis*: n = 8, 12, 1, 11, 1, respectively), a rarely-infected *Stegastes* species (*Stegastes partitus*, n = 5), yellowtail damselfish (*Microspathodon chrysurus*, n = 6), which have not been found to be infected, and from 1 blennoid species from the genus *Ophioblennius* (Family Blenneidae, n = 9) known to harbor a *Haemohormidium*-like blood parasite. Blood smears from these fish were processed and stained with Giemsa Modified Solution (Sigma-Aldrich, St. Louis, Missouri). Blood smears were then screened (×100 magnification) using a Nikon e800 (Nikon, Melville, New York) for at least 30 min as proposed by Davies and Merrett (2000) for determining the infection status. Parasite densities were established based on number of parasites per 500 host fish erythrocytes. The *Ophioblennius* used in this study were the same individuals as those reported by Cook et al. (2015) to be infected by a *Haemohormidium*-like blood parasite.

DNA extraction of fish blood

DNA was extracted from whole blood samples preserved in molecular grade ethanol (200 proof) of 53 fish targeted in this study using the QiaAMP DNA Mini Kit (no. 51306; Qiagen, Valencia, California) with modifications to the manufacturer's instructions. Briefly, an initial centrifugation step to pellet blood and remove contaminating solvent was followed by partially drying the sample to evaporate any trace ethanol. In processing small amounts of blood (1–2 droplets or dried blood scrapings), 200 µl of phosphate-buffered saline was used to dissolve the sample prior to addition of the lysis buffer (200 µl) and 1.25 µg/ml proteinase K (ProK). Samples were incubated in a 56 C water bath for 1 hr and DNA purified in accordance with kit instructions. Following this column-based purification, nucleic acids were eluted in nuclease-free water to enable concentration in a SpeedVac at low heat setting (Savant, ThermoFisher, Wilmington, Delaware). DNA was quantified by UV spectrophotometry using the Nanodrop ND1000 spectrophotometer (Thermo-Fisher) and DNA integrity verified by agarose gel electrophoresis (1% agarose, IXTAE [40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3]). Similar methods were used to isolate DNA from heart and spleen tissue from select fish positive for blood parasites (n = 8, *S. adustus* and n = 10, *S. diencaeus*). All extracted DNA samples were stored at –20 C for future analysis.

Whatman FTA processing for fish blood samples

To determine if a more streamlined method from field collection of parasitized fish blood could be developed, a subset of infected fish blood samples verified by microscopy was also collected on Whatman FTA archive paper (n = 11). Specifically, using a modification of the manufacturer's recommended procedure, blood samples (~10 µl) were deposited on a Whatman FTA 96 sample archive card. Following complete drying of the sample (~24 hr), FTA cards were maintained at room temperature in a sealed pouch with desiccant until analysis. To process the DNA embedded on FTA cards, a 2.0-mm punch encompassing the blood sample was removed, cut in half, transferred to a 1.5-ml microfuge tube, and subjected to a series of washes. The initial washes (2 times, 5 min in 200 µl FTA Wash solution) to remove all FTA chemicals were followed with 3 (2 min) rinses in 200 µl 1X TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) buffer. All FTA disc processing steps were carried out with agitation on a platform shaker. Processed FTA discs with the DNA template entrapped were air dried for ~30 min to minimize liquid carryover and then immediately transferred to individual tubes containing PCR master mix and subjected to analysis. A subset

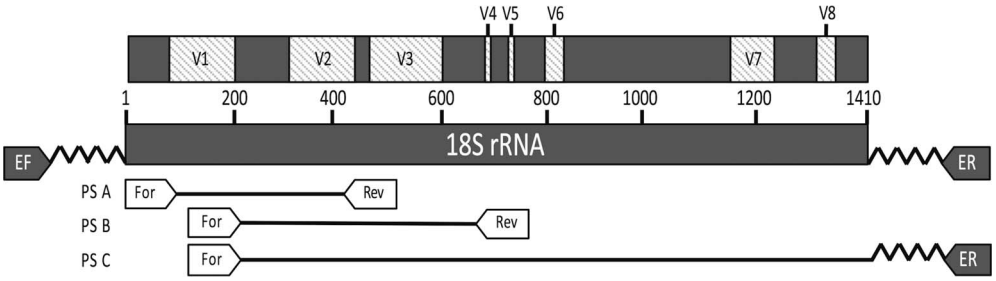

FIGURE 1. Fish apicomplexan 18S rRNA gene map and diagnostic primers. Avian apicomplexan 18S rDNA primer set (EF/ER) amplified a region of an approximate 1,500-base pair (bp) fragment from whole blood of the redlip blenny (*Ophioblennius macclurei*). Within this large segment, internal primers were designed to target variable regions. Variable regions (V1-V8) were established by aligning all query 1,500-bp fragments generated with the 18S rDNA fragment from *Toxoplasma gondii* (GenBank L37415). Based on alignment, a series of fish apicomplexan diagnostic primers were developed and tested including Primer set A (PsA), Primer set B (PsB), and Primer set C (PsC) amplifying regions of 500 bp, 550 bp, and 1,100 bp, respectively.

of FTA preserved samples were tested that included 11 fish blood samples that microscopically screened positive for blood parasites (7 *Stegastes* and 4 *Ophioblennius*) and 13 blood samples from microscopically confirmed parasite-negative individuals (*Stegastes partitus*).

Fish apicomplexan primer design strategy

In the absence of any fish-specific apicomplexan gene sequences in the public databases, previously published primers amplifying an avian apicomplexan (*Eimeria tenella*) 18S rRNA gene were selected (Kvičerová et al., 2008); avian apicomplexan-specific 18S rDNA, forward primer (EF; 5′-GAAACTGCGAATGGCTCATT-3′) and general reverse primer (ER; 5′-CTTGCGCCTACTAGGCATTC-3′). The PCR products generated using these avian apicomplexan primers with fish blood DNA were verified by electrophoresis on a 1.5% agarose/1X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) gel, visualized with ethidium bromide staining, and gel images were captured using a VersaDoc Imaging System, Model 4000 (Bio-Rad Laboratories, Hercules, California). Select PCR products were either directly purified or resolved electrophoretically and gel extracted using the corresponding QIAquick PCR Purification or Gel Extraction kit platform (Qiagen). Forward and reverse strands of PCR amplicons were completely sequenced (DNA Sequencing and Genotyping Facility, University of Chicago, Chicago, Illinois) using avian apicomplexan 18S rDNA primers and internal primers.

All sequencing files were imported into Geneious, version 8.1.6 (http://www.geneious.com, Kearse et al., 2012), enabling access to a variety of alignment and sequence analysis tools. Briefly, sequences were initially aligned using default parameters for MUSCLE (Edgar, 2004) alignment, accessed through the Geneious software, to other apicomplexan parasite 18S rRNA genes available in the public databases to establish all sequences to be fish apicomplexan homologs of this gene. Visual inspection of each of the sequences was performed to identify possible polymorphic regions (gray; Fig. 1) to avoid in designing hemogregarine-specific primers. Primer3 (Geneious, version 8.1.6) was used in generating 3 initial primer sets that targeted the fish apicomplexan 18S rDNA (designated primer sets [Ps] A, B, and C) that are detailed in Table I. Additional primers used in this study also included DNA barcoding primers amplifying the cytochrome *c* oxidase I (COI) gene specific for fish hosts (Hellberg et al., 2010; Fish Barcode of Life Initiative [FISH-BOL] 2017).

TABLE I. Fish and apicomplexan diagnostic primers used and developed in this study. Avian apicomplexan 18S rDNA primer set (EF/ER) amplified a region of an approximate 1,500-base pair (bp) fragment from whole blood of the redlip blenny, *Ophioblennius macclurei*. Within this large segment, internal primers were designed to target variable regions established by aligning the 1,500-bp fragment with a similar sized 18S rDNA fragment from *Toxoplasma gondii* (GenBank L37415). Fish Apicomplexan diagnostic primers targeting the 18S rRNA gene were developed and tested and included Primer set A (PsA), Primer set B (PsB), and Primer set C (PsC). For verification of DNA quality–quantity, fish DNA barcoding primers targeting the mitochondrial cytochrome *c* oxidase I (COI) gene (COI F/R and Mini COI F/R) were used and amplified 700-bp and 205-bp amplicons, respectively.

Primer Name	Direction	Primer Sequence (5′→3′)	Usage	Gene	Reference
EF	Forward	GAACTGCGAATGGCTCATT	PCR/sequencing	18S	Kvičerová et al., 2008
ER	Reverse	CTTGCGCCTACTAGGCATTC	PCR/sequencing	18S	Kvičerová et al., 2008
PsA-F	Forward	TCGGCAAATACCCGACTTTT	PCR/sequencing	18S	This study
PsA-R	Reverse	CACCAGACTTGCCCTCCAAT	PCR/sequencing	18S	This study
PsB-F	Forward	CGGGTAACGGGGAATTAGGG	PCR/sequencing	18S	This study
PsB-R	Reverse	TGCTGCAGTATTCAAGGCAA	PCR/sequencing	18S	This study
PsC-F	Forward	CGGGTAACGGGGAATTAGGG	PCR/sequencing	18S	This study
PsC-R	Reverse	CTTGCGCCTACTAGGCATTC	PCR/sequencing	18S	This study
COI-F	Forward	TCAACYAATCAYAAAGATATYGGCAC	PCR/host verification	CO1	Baldwin et al., 2009; FISH-BOL, 2017
COI-R	Reverse	ACTTCYGGGTGRCCRAARAATCA	PCR/host verification	CO1	Baldwin et al. 2009; FISH-BOL, 2017
Mini CO1 F	Forward	CCAGCACCHTCTAAYATYTCAGT	PCR/host verification	CO1	Hellberg et al., 2010
Mini CO1R	Reverse	AAGAAAGATGCYCCGTTRGC	PCR/host verification	CO1	Hellberg et al., 2010

PCR amplification

To test the specificity of the lead primer set B (PsB), blood samples from n = 43 microscope confirmed-positive fish were screened by PCR using PsB as well as fish COI primers. To validate the specificity and selectivity of the fish apicomplexan PsB, fish blood from non-parasitized (microscopically determined) bicolor damsels (*S. partitus*, n = 5) and yellowtail damsels (*M. chrysurus*, n = 6) was also screened. GoTaq Hot Start Polymerase kit (no. M7401, Promega, Fitchburg, Wisconsin) was used for all PCR analyses and performed on the Veriti 96 Well Thermal Cycler (Applied Biosystems, Life Technologies, Grand Island, New York). Briefly, all PCR reactions (20 µl) were carried out in reactions with 1X GoTaq Flexi Buffer, 1.5 mM $MgCl_2$, 10 µM of each forward and reverse primers, 0.2 mM dNTPs, and 1.25 U GoTaq® Hot Start polymerase (Promega). DNA template (50 ng) was used for both host fish COI and parasite 18S rDNA amplifications unless otherwise indicated. Thermocycle conditions were: 98 C for 2 min; 25 cycles of 96 C for 20 sec; 55 C (COI primers) or 60 C (parasite primers) for 20 sec; 72 C for 40 sec; and a final extension at 72 C for 7 min; post-PCR products were stored at –20 C until further processing. PCR reactions were resolved by agarose gel electrophoresis as previously described. Select PCR products were purified using QIAquick PCR Purification Kit (Qiagen, no. 28106), sequenced as previously described.

Parasite 18S rDNA sequence identification and phylogenetic analysis

Parasite 18S rDNA amplicons were assembled using Geneious version 8.1.6, and chromatogram-based contiguous sequences were generated, trimmed, and manually corrected for ambiguous base calls. A Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information [NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi], Bethesda, Maryland) was performed to identify sequences of apicomplexans that compared most closely to the present material. Apicomplexan sequences of known and unidentified genera and species sharing the highest identity with the present material were chosen based on a query length of 1,458 base pairs (bp) with query covers of no less than 99% and an identity ranging from 84 to 94%. Additionally, representative sequences of no less than 1,000 bp of known species of apicomplexans were chosen from the Piroplasms, Adeleids, and Coccidia (Eimeriidae and related families as well as the Sarcocystidae) (with reference to Barta et al., 2012). Sequences were downloaded from GenBank, aligned to the sequences generated in this study using the MUSCLE alignment tool (Edgar, 2004) implemented through Geneious version 8.1.6., and manually trimmed. Uncorrected pair-wise distances (p-distance) were determined with the MEGA7 bioinformatics software program (Kumar et al., 2016) for an alignment of 1,504 nt containing 14 of these apicomplexan sequences and the 3 sequences of the present material. For the phylogenetic analysis, only known genera and species were included in the alignment of 1,057 bp, which consisted of 46 sequences. The alignment was cleaned from problematic alignment blocks using Gblocks 0.91 (Castresana, 2000), implemented in SEAVIEW v3.2 (Galtier et al., 1996), resulting in a final alignment of 995 bp. A blood parasite, one species of *Plasmodium*, *Plasmodium falciparum* (GenBank: M19172), was used as the outgroup (chosen with reference to Barta et al., 2012).

To infer phylogenetic relationships, a Bayesian inference (BI) and maximum likelihood (ML) method was used. A comprehensive model test was performed to determine the most suitable nucleotide substitution model per the Akaike information criterion (AIC) using jModelTest 2.1.7. (Guindon and Gascuel, 2003; Darriba et al., 2012). The best model identified with the best AICc score was the General Time Reversible (GTR) model with estimates of invariable sites and a discrete Gamma distribution (GTR + I + Γ). BI analysis was performed using MrBayes software (ver. 3.2.6) (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 2,500. The ML analysis was implemented from within Geneious version 8.1.6 using PhyML version 3.0. (Guindon and Gascuel, 2003). The alpha-parameter selected was the GTR model, with support assessed using 1,000 rapid bootstrap inferences. Resulting trees were combined in a 50% majority consensus tree.

RESULTS

The apicomplexan blood parasites reported here are the same parasites identified and described by Cook et al. (2015) as a *Haemohormidium*-like parasite (also see fig. 1a–e in Cook et al., 2015) and in detailed discussion in the Supplement of the same paper (http://www.int-res.com/articles/suppl/m533p001_supp.pdf.).

Among published apicomplexan primers (http://tolweb.org/Apicomplexa), the only primer set that was successful, the avian *Eimeria* 18S rRNA gene primer set (EF/ER; Table I), amplified an expected ~1.5 kbp fragment for a select subset of apicomplexan-infected fish blood samples. For the fish blood samples analyzed in this initial screening, both forward and reverse complete reads of the 18S rDNA amplicons were generated and aligned to 18S rDNA sequences (NCBI BLAST). Novel parasite 18S rRNA full-length gene sequences from blood samples of 3 fish species, *Ophioblennius macclurei*, *Stegastes adustus*, and *Stegastes diencaeus*, were identified and representative sequences submitted to NCBI (GenBank: KT806396, KT806397, KT806398, respectively). Based on the confidence in the identification criteria used for these microscopically-confirmed infected voucher specimens (for morphological description see Cook et al., 2015), the primers developed herein are identified as targeting the fish apicomplexan 18S rRNA gene.

Alignment of sequences from 2 *O. macclurei* specimens with 18S SSU rDNA sequences from *Toxoplasma gondii* (GenBank: L37415) enabled identification of the variable and conserved regions within the fish apicomplexan 18S rRNA gene (Fig. 1). A set of diagnostic 18S rDNA primers were designed (Primer3) to incorporate several of the variable regions of this fish apicomplexan gene in efforts to selectively discriminate between related species (Table I). Figure 2A and 2B shows that representative microscopically-confirmed samples demonstrating all fish apicomplexan primers generated the predicted amplicon sizes PsA (500 bp), PsB (550 bp), and PsC (1,100 bp). However, only primer set B (PsB) showed consistent, robust amplification product signal with no cross-reactivity to host fish DNA when tested against both parasite infected (n = 8) and non-infected (n = 3) fish blood samples (data not shown). For all subsequent analyses, PsB was

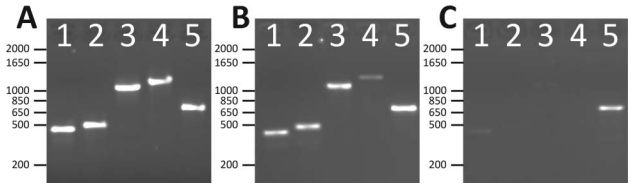

FIGURE 2. Diagnostic primer development strategy for Apicomplexan parasites in fish blood. Primers identified in Figure 1 were tested using microscopically-confirmed parasitized (parasite positive) and non-parasitized (parasite negative) fish blood DNA samples. Lanes 1–3 in all panels tested diagnostic primer sets A, B, and C respectively. The degenerative apicomplexan primers were used to detect a full-length 18S rDNA fragment (lane 4). Fish-specific cytochrome *c* oxidase I (CO1) primers in validating host DNA as a surrogate measure for parasite DNA integrity (lane 5). (**A**) A parasite-positive dusky damsel *Stegastes adustus* sample also screened positive by PCR. (**B**) A microscopic parasite-negative sample (longfin damsel, *Stegastes diencaeus*) screened positive by PCR. (**C**) A bicolor damselfish designated microscopic parasite-negative did not amplify with most primer sets tested (PsB, PsC, or EF/ER); however, minimal cross-reactivity was observed with PsA. PsB was chosen lead primer set for fish apicomplexan study.

designated the lead primer set for diagnostic species identification of fish apicomplexans.

The COI universal finfish primers (~650 bp amplicon; Baldwin et al., 2009; www.fishbol.org) served as a quality indicator of extracted DNA for each fish blood sample. Of the 53 specimens subject to PCR screening, 50 (90.9%) amplified for host COI DNA, indicating ~10% of the fish blood samples may have been compromised in collection, storage, or both. Of those samples screening positive by microscopy for the parasite, 37 out of 41 individuals (90.1%) could be successfully detected with our new fish apicomplexan primer set. These primers are capable of detecting very low parasite levels (low as 1 out of 500 erythrocytes [0.002%] determined by microscopy). However, of the 4 microscopic-confirmed infected samples that were not detected by PCR, 3 corresponded to samples with very low infection rates (1/500 erythrocytes) (Table II).

In addition to these primers detecting parasite in fish blood, in the infected fish where we also extracted spleen and liver tissue the PCR successfully amplified both parasite and fish DNA targets for both tissues types (data not shown). Select amplicons were confirmed by DNA sequencing to validate the specificity of the fish apicomplexan primers for detection of the blood-borne parasite in fish tissues (data not shown). DNA from the microscopically-determined negative samples of *M. chrysurus* (n = 6; not known to harbor the blood parasite) and *S. partitus* (n = 5; only infrequently harbor blood parasites) samples did not amplify with PsB primers (Fig. 2C). In addition, no-template controls performed with PCR reactions for each primer set revealed no contamination in the system (data not shown). It is worth noting that 2 of the 41 microscope-confirmed fish blood samples detected positive by fish apicomplexan PCR could not be amplified with the standard fish COI primers.

Amplicons of ~1.1 kbp to 1.3 kbp for the 18S rRNA gene of the *Haemohormidium*-like parasite were obtained from infected *O. macclurei* (GenBank: KT806396), *S. adustus* (GenBank: KT806397), and *S. diencaeus* (GenBank: KT806398) using primer sets EF and ER. In the phylogenetic analysis (Fig. 3) the *Haemohormidium*-like parasite fell at the base of a major monophyletic clade containing species of Coccidia, distinct from the monophyletic clade containing members of the order Piroplasmida (p-distance = 0.09–0.11) (see Table III) as well as from the major monophyletic clade containing species of *Haemogregarina* (p-distance = 0.09). Furthermore, the *Haemohormidium*-like parasite did not cluster with known coccidian blood parasites such as species of *Lankesterella* and *Schellackia* (p-distance = 0.11–0.12, 0.08–0.09, respectively). The *Haemohormidium*-like parasite found parasitizing the *O. macclurei*, even though morphologically indistinguishable from that parasitizing both the *S. adustus* and *S. diencaeus*, appears to be a different species based on the phylogenetic analysis and sequence data (28 SNPs in an alignment of 1,413 bp). Interestingly, per the evolutionary divergence estimates, the *Haemohormidium*-like parasite was most-closely related to an unidentified symbiont of coral (GenBank AF238264) (p-distance = 0.03–0.04) and thereafter an unidentified species of coccidian (GenBank HM117908) (p-distance = 0.05–0.06) parasitizing both the marine goatfish *Mulloidichthys* species and bluestripe snapper *Lutjanus kasmira*.

TABLE II. Comparative assessment of parasite-infected fish blood sample using fish apicomplexan molecular analysis vs. standard microscopic analysis. Fish blood samples with parasite load (n = 42) verified by microscopy, at different levels (1–5 infected erythrocytes/500 erythrocytes), were designated "+" for the presence of parasite. Samples showing absence of parasite by microscopy (n = 11) were designated "–". DNA extracted from these same samples was used in determining the lowest level of parasitemia detectable using the PCR-based diagnostic developed in this study. Samples amplified successfully using primer set B indicated presence (+) of parasite DNA (18S rDNA; PsB). Fish DNA present in samples confirmed using primer set targeting the universal fish host gene cytochrome oxygenase I gene (COI).

Species	+/–	Parasite/500	n	Parasite DNA (Ps B)	Host DNA (COI)
Stegastes adustus	+	1–2	8	6 (75%)	8 (100%)
Stegastes diencaeus	+	1–4	12	11 (91.6%)	10 (83%)
Stegastes leucostictus	+	1	1	1	1
Stegastes planifrons	+	1–5	11	8 (72.7%)	9 (81.8%)
Stegastes variabilis	+	1	1	–	1
Stegastes partitus	–	–	5	–	5
Microspathodon chrysurus	–	–	6	–	6
Ophioblennius macclurei	+	1–3	9	8 (88.9%)	8 (88.9%)

In our assessment of Whatman FTA paper for streamlining both collection and analysis of parasite-infected fish blood samples, we detected both the parasite target gene (PsB) and the host fish gene (CO1) amplicons (Fig. 4) in pre-determined infected fish blood samples (n = 11). Of the 11 confirmed microscopically and by conventional PCR to be positive for parasites, 8 (73%) of the FTA preserved samples amplified with our parasite 18S primers while all (100%) of the 13 parasite-negative individuals confirmed by FTA method. The large COI amplicon size (~700 bp) used to confirm presence of host fish DNA on FTA card was only detected in 40% of the samples.

DISCUSSION

Considering the labor-intensive process required in screening reef fish blood samples for parasite infection using microscope-based methods, and the large sample sets required for a typical

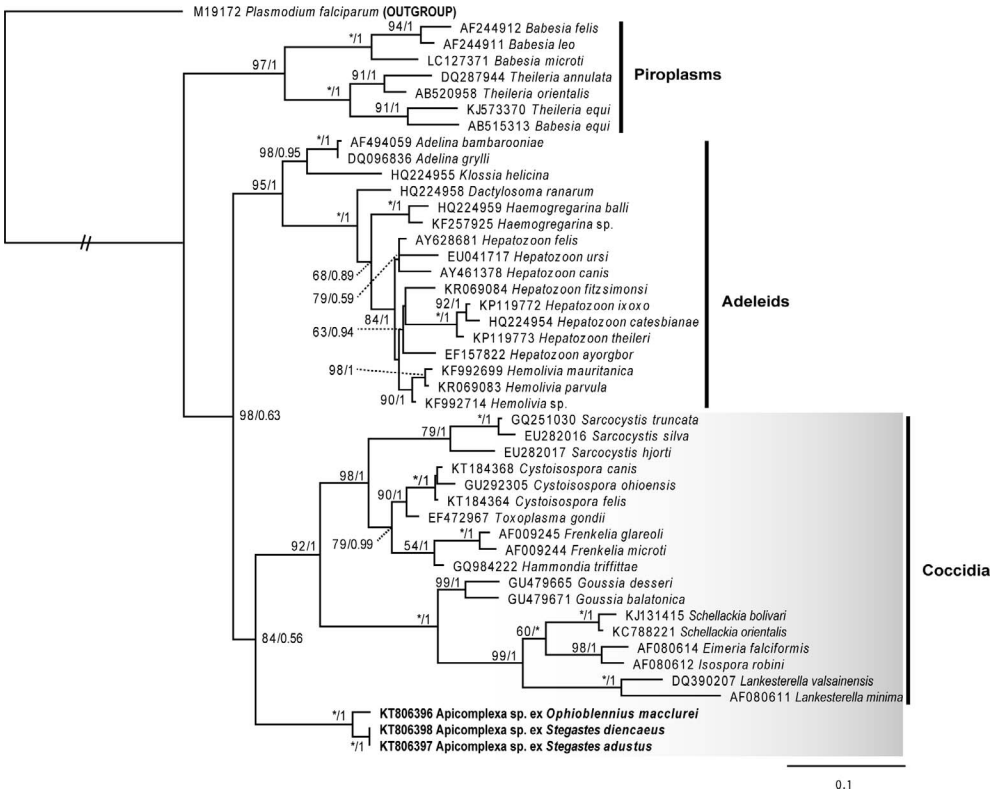

FIGURE 3. Phylogenetic analysis of the *Haemohormidium*-like parasite based on 18S rDNA sequences. Maximum likelihood (ML) and Bayesian inference (BI) analysis showing the phylogenetic relationships for 3 *Haemohormidium*-like parasite isolates from *Ophioblennius macclurei* (GenBank KT806396), *Stegastes diencaeus* (GenBank KT806398), and *Stegastes adustus* (GenBank KT806397), respectively, in bold; 7, 17, and 18 representative species of the piroplasms, adeleids, and coccidia (highlighted), respectively; *Plasmodium falciparum* was used as the outgroup. All comparative sequences were downloaded from the GenBank database. Tree topologies for both the BI and ML trees were congruent; the nodal support values (ML/BI) > 50% are represented on the ML tree, * denotes values of 100%.

study (1,000+ blood samples/collection site), this study aimed to develop a molecular-based diagnostic to more rapidly confirm presence of apicomplexan parasites in fish blood. The 18S SSU RNA gene, widely used for classification of apicomplexan parasites (Morrison, 2009), was targeted in initial efforts aimed at establishing a fish apicomplexan-specific molecular marker gene.

These fish apicomplexans are a newly discovered taxa (Cook et al., 2015), and molecular identification at higher taxonomic levels is often needed for these initial efforts (Collins and Cruickshank, 2012). The 18S rRNA gene has in fact been used for species discrimination in classifying many tetrapod apicomplexans; however, species-specific identification using this gene target has proven unreliable in cases where divergent paralogs of this gene in the same species is present (El-Sherry et al., 2013). While 18S rDNA sequences may provide a useful triage tool for initial sorting into "species-like units" as additional species and larger sample sizes are generated, alternative DNA barcoding marker strategies may be warranted. For example, modification of the 18S rDNA primers to target the more-discriminatory variable regions of this marker gene may be needed for species discrimination (Pawlowski et al., 2012; Wu et al., 2015). Conversely, other genes including the more-commonly used mitochondrial gene cytochrome *c* oxidase I (COI) gene marker may prove to be more robust for species identification of fish apicomplexans, as shown for a subgroup of turkey parasites (El-Sherry et al., 2013). Although taxonomic resolution potential can vary among organismal groups (Huang et al., 2008; Ward, 2009), historically for animals the COI barcoding marker gene is associated with higher resolution at species level and is also useful at revealing intraspecific variation (http://www.barcodeoflife.org/). Finally, with emergence over the past decade of the many rapid and powerful nucleic acid sequencing techniques, the concept of single-loci DNA barcoding for species delimitation may be supplanted with systems using multiple loci for DNA-based taxonomy (Collins and Cruickshank, 2013).

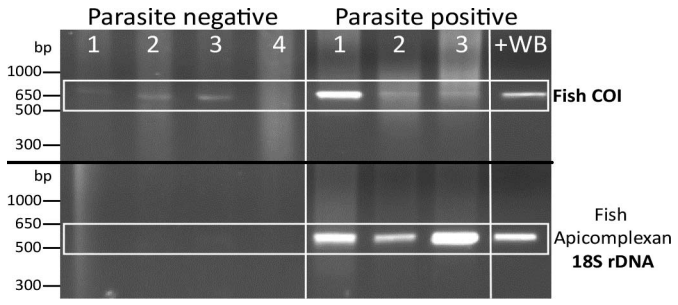

FIGURE 4. PCR amplification of fish blood parasites preserved using the Whatman FTA archiving card method. FTA samples from selected parasite-negative and -positive fish blood samples were collected, processed, and PCR amplified with CO1 primers for host fish verification (~650–700 base pairs [bp]) and fish apicomplexan primer set B (550 bp). A parasite-positive sample, processed using the conventional DNA extraction methods for fish whole blood (+WB), serves as positive controls for both primers in this analysis.

Species of the genus *Haemohormidium* have been assigned to the order Piroplasmida (Phylum: Apicomplexa) along with species of the genera *Theileria* and *Babesia* (Davies, 1995; Barta et al., 2012). However, this placement remains uncertain (see Davies and Johnston, 2000). Because no molecular data presently exist for species of *Haemohormidium*, species of *Theileria* and *Babesia* were used for comparison in the phylogenetic analysis.

TABLE III. Matrix showing the uncorrected pair-wise distances (p-distance) based on 18S rDNA sequences for the three isolates of the *Haemohormidium*-like parasite from *Ophioblennius macclurei* (GenBank KT806396), *Stegastes adustus* (GenBank KT806397), and *Stegastes diencaeus* (GenBank KT806398), respectively, compared to the 7 apicomplexan sequences (first 7 after the present material) sharing the highest identity with this parasite based on the results of the Basic Local Alignment Search Tool (NCBI). Additionally, sequences representing the coccidia, adeleids, and pirosplasms were also included in the analysis.

Group (Family)	GenBank accession no.	Organism	Uncorrected pair-wise distances (p-distance)															
Unknown	KT806396	*Haemohormidium*-like parasite ex. *Ophioblennius macclurei*																
Unknown	KT806397	*Haemohormidium*-like parasite ex. *Stegastes adustus*	0.01															
Unknown	KT806398	*Haemohormidium*-like parasite ex. *Stegastes diencaeus*	0.01	0.00														
Unknown	AF238264	Unidentified symbiont	0.04	0.03	0.03													
Coccidia (unknown)	HM117908	Unidentified *Coccidia* sp.	0.06	0.05	0.05	0.06												
Coccidia (Sarcocystidae)	KF309699	*Sarcocystis* sp.	0.07	0.06	0.06	0.07	0.05											
Coccidia (Sarcocystidae)	KJ746531	*Besnoitia besnoiti*	0.08	0.07	0.07	0.07	0.05	0.03										
Coccidia (Sarcocystidae)	XR001974106	*Toxoplasma gondii*	0.07	0.06	0.06	0.06	0.04	0.03	0.01									
Coccidia (Sarcocystidae)	GQ899206	*Neospora caninum*	0.07	0.06	0.06	0.06	0.04	0.03	0.01	0.00								
Coccidia (Sarcocystidae)	KT184370	*Hammondia heydorni*	0.07	0.06	0.06	0.06	0.04	0.03	0.01	0.00	0.00							
Coccidia (Lankesterellidae)	AF080611	*Lankesterella minima*	0.12	0.11	0.11	0.11	0.09	0.11	0.11	0.10	0.10	0.10						
Coccidia (Schellackiidae)	KC788221	*Schellackia orientalis*	0.09	0.08	0.08	0.08	0.06	0.08	0.07	0.07	0.07	0.07	0.05					
Adeleids (Adeleidae)	AF494059	*Adelina bambarooniae*	0.06	0.06	0.06	0.06	0.06	0.08	0.08	0.07	0.07	0.07	0.12	0.09				
Adeleids (Haemogregarinidae)	HQ224959	*Haemogregarina balli*	0.09	0.09	0.09	0.09	0.10	0.11	0.11	0.11	0.11	0.11	0.15	0.12	0.07			
Adeleids (Haemogregarinidae)	KF257925	*Haemogregarina* sp.	0.09	0.09	0.09	0.09	0.09	0.10	0.11	0.10	0.10	0.10	0.14	0.11	0.07	0.01		
Piroplasms (Theileriidae)	DQ287944	*Theileria annulata*	0.11	0.10	0.10	0.10	0.10	0.11	0.11	0.11	0.11	0.11	0.14	0.12	0.10	0.13	0.12	
Piroplasms (Babesiidae)	LC127371	*Babesia microti*	0.10	0.09	0.09	0.10	0.09	0.10	0.11	0.11	0.11	0.11	0.14	0.11	0.10	0.12	0.12	0.07

However, it is clear from this analysis, as well as from the divergence estimates (p-distance = 0.9–0.11), that the *Haemohormidium*-like parasite infecting the two species of *Stegastes* and one species of *Ophioblennius* from this study cannot be assigned to the order Piroplasmida. This finding is not surprising with reference to the study by Cook et al. (2015), who discussed their concern over this parasite species not conforming to the typical morphology described for the type species of the genus, *Haemohormidium cotti* Henry, 1910.

Cook et al. (2015) described the *Haemohormidium*-like parasite to have dividing stages separating into 3 possible gamont or merozoite stages. Another parasite genus of marine fishes that demonstrates this type of intraerythrocytic division is *Haemogregarina* sensu lato, such as *Haemogregarina bigemina* Laveran and Mesnil, 1901 (Davies et al., 2004). However, as no molecular data presently exist for these haemogregarines of fishes, we included in the analysis 2 species of *Haemogregarina* from freshwater terrapins (GenBank HQ224959, KF257925). However, similar to the findings regarding the piroplasms, according to the phylogenetic analysis and divergence estimates the present material was not closely related to species of *Haemogregarina* either, supporting Burreson (1989), Smit et al. (2006), and Cook et al. (2015) views that this *Haemohormidium*-like parasite cannot be readily identified as a haemogregarine.

Interestingly, according to the evolutionary divergence estimates, the *Haemohormidium*-like parasite was most-closely related to an unidentified symbiont of coral collected off the coastal areas of Panama and an unidentified species of coccidian parasitizing marine fish species caught off the various coastal areas of Hawaii. Typically in the life cycle of coccidia, coccidian oocysts are eliminated into the environment from the host organism through the excretion of waste products such as feces. Oocysts sporulate forming sporozoites within, which are infective to the new host after ingestion of the oocyst (Kheysin, 1972). With this in mind, it is possible that the unidentified symbiont of coral may be another species of coccidian, which contaminated samples used in a study focusing on the coral symbiont genus *Symbiodinium* (see Toller et al., 2001). Potentially, the *Haemohormidium*-like parasite may be a similar species of coccidia, particularly as it clusters with the larger coccidian parasite clade as sister to other Eimeriorina species. The molecular data for the species of coccidium parasitizing both the marine fish species from Hawai'i (GenBank HM117907 and HM117908) was based on a study by Work et al. (2003). Interestingly, the placement of this species, which clustered in a well-supported clade with *Goussia janae* (a fish-host species of *Goussia*) in the phylogenetic analysis of Whipps et al. (2012), was similar to that of the *Haemohormidium*-like species in the present study. In both studies, the marine fish coccidium and the *Haemohormidium*-like parasite, respectively, were basal to the combined groups of the Sarcocystids and Eimeriids. However, even though the coccidium, which is likely a species of *Goussia* (Whipps et al., 2012) described by Work et al. (2003), was found to be extraintestinal, no blood stages of the parasite were found. Coccidian parasite genera that are known to demonstrate intraerythrocytic peripheral blood stages include haemococcidia *Lankesterella* and *Schellackia* (Megía-Palma et al., 2014). Nonetheless, even though the phylogenetic position of the *Haemohormidium*-like parasite in this study is suggested at present to be a species of coccidia, it did not cluster with species of either of these haemococcidian genera.

Currently it is not possible to assign this *Haemohormidium*-like parasite to any of the known apicomplexan genera. Possibly in the future, with the addition of molecular samples, including those species of marine fish *Haemogregarina* and known *Haemohormidium* such as *H. cotti*, as well as with ultrastructural and life cycle studies, the placement of this *Haemohormidium*-like parasite will be better resolved.

While sample processing issues using the Whatman FTA cards for parasite-infected fish blood clearly need to be optimized, our results offer promise for this method in providing fish parasite DNA detection with a method that is faster, easier, and more cost-effective than conventional sample collection and DNA extraction used in field studies. This low level of amplification of the fish blood COI marker, as well as the parasite 18S gene, was likely due to degraded template. Issues with fish blood quickly clotting and sample build-up on the surface of the FTA paper could be observed on some samples, which most likely resulted in incomplete deactivation of nucleases and degraded DNA. Furthermore, PCR background signal was quite notable and validates that the FTA DNA template was likely compromised during the archiving process, leading to lower-quality DNA templates. Optimization might include design of new DNA quality control primers (universal fish COI primers; amplicon ~650–700 bp) to better match the amplicon size generated using the fish apicomplexan Ps B (~550 bp). Fish COI minibarcode primers previously reported generating a ~500 bp amplicon (Hellberg et al., 2010) were tested in this study; however, no amplification was observed (data not shown). Alternative COI primers more selective for Caribbean reef fish (e.g., Leray et al., 2013) and which produce a smaller amplicon may provide a more-robust COI barcode primer set for future studies. Strategies including redesign of the PsB, re-PCR of the initial PCR product, use of a nest-PCR protocol (e.g., Hellgren et al., 2004), and real-time PCR (e.g., Tran et al., 2014) may enable more-robust detection for samples with low infection rates with limited DNA target in the future.

This is the first study to report the design and testing of specific diagnostic PCR primers for molecular taxonomy of apicomplexans inhabiting coral reef fishes. The work reported here introduces new techniques and diagnostic primers in the identification and detection of apicomplexan blood parasites in damselfish and blennies in the Eastern Caribbean and the use of Whatman FTA paper to successfully preserve and amplify parasite DNA. The diagnostic primers and techniques described here will provide a solid base for future work in fish apicomplexans, extending into other families of coral reef fishes that harbor these blood parasites.

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