*Zootaxa* 5048 (1): 099–117
https://www.mapress.com/j/zt/


**Article**

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.5048.1.5
http://zoobank.org/urn:lsid:zoobank.org:pub:833420E8-275A-4845-8059-6AFF195CCC70

# Using DNA barcoding to identify host-parasite interactions between cryptic species of goby (*Coryphopterus:* Gobiidae, Perciformes) and parasitic copepods (*Pharodes tortugensis:* Chondracanthidae, Cyclopoida)

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## Abstract

Previous work, using morphological characters, identified a generalist copepod parasite (*Pharodes tortugensis*) at high prevalence on two common gobies (C*oryphopterus glaucofraenum* and *C. dicrus*) in the British Virgin Islands (BVI). DNA barcoding subsequently revealed *C. glaucofraenum* to be three morphologically similar species (*C. glaucofraenum*, *C. venezuelae* and *C. tortugae*), casting doubt on host identities in the BVI and the classification of the parasite as a single species. Mitochondrial cytochrome c oxidase subunit I (COI) data from 67 gobies in the BVI showed that, in addition to *C. dicrus*, host gobies were a mix of *C. glaucofraenum* and *C. venezuelae,* while *C. tortugae* was unexpectedly absent from the study area. COI data (n = 70) indicated that the copepod infecting all three hosts was a single species, almost certainly *P. tortugensis*. The pharodes-coryphopterus interaction has a strong impact on host dynamics in the BVI, and a revised understanding of these dynamics must account for any differences among the three newly confirmed hosts in transmission of, and susceptibility to, the shared parasite. No other infected hosts were discovered at our sites, but *P. tortugensis* is reportedly widespread and infects 12 additional host species elsewhere. Further DNA barcoding is thus needed to test whether *P. tortugensis* is truly a widespread generalist, or instead represents a group of more specialized cryptic species.

**Key words:** British Virgin Islands, Caribbean, COI sequences, host-specificity, new geographic record

## Introduction

Species are basic units of study for ecologists, and much of ecological theory specifies how species interact with one another as parasites and hosts, predators and prey, competitors and facilitators. The classification of species is, however, constantly evolving as taxonomists identify new species and reevaluate the relationships of those already identified. Traditional methods of classifying species, based on shared morphological features, are increasingly augmented by genetic methods that identify species using standardized regions of DNA (DNA barcoding) (Marshall 2005). DNA barcoding has revealed many cryptic species that lack obvious phenotypic differences, and so were previously classified as one taxon (Trontelj & Fišer 2010). Improvements in taxonomy can thus help clarify the identities of species that participate in ecological interactions (Bickford *et al.* 2007).

Host-specificity, the extent to which parasites infect different host species (Poulin *et al.* 2011), is a fundamental feature of host-parasite interactions and accurately identifying the participants in host-parasite interactions has wide-ranging implications. Ecological implications range from the accuracy of biological diversity estimates to predicting the transmission of specific diseases (Poulin 2014). For example, attempts to control a pathogenic parasite may be thwarted if an unrecognized host species serves as a reservoir for the parasite even if it is extinct in recognized hosts (Besansky 1999; Haydon 2002). In addition, the impacts of invasive parasites can escape detection if invaders are mistaken for native species or other invaders that are morphologically similar (Goedknegt *et al.* 2018). Lastly, tak-

*Accepted by J. Sparks: 16 Sept. 2021; published: 5 Oct. 2021*

ing advantage of host-parasite interactions for biological pest control typically relies on accurately characterizing a specialist relationship between the pathogen and its host (Bickford *et al.* 2007).

The discovery of cryptic parasite and host species (Nadler & De Leon 2011) has spurred re-evaluation of many host-parasite interactions (Banks & Paterson 2005; Costello 2016; de León & Nadler 2010). It has been argued that the number of hosts occupied by a given parasite is often underestimated (Costello 2016). Supporting this hypothesis are examples in which the range of hosts infected by parasites was higher than previously thought because a presumed single host was subsequently found to represent several cryptic species (e.g. Westram *et al.* 2011). On the other hand, there are also cases when a single generalist parasite believed to infect multiple hosts was, in fact, a complex of cryptic specialist parasite species each infecting a subset of the hosts (Poulin & Keeney 2008; e.g. Smith *et al.* 2006).

In this study, we clarify a host-parasite interaction in which both hosts and parasites include potentially cryptic species. The parasite is a copepod, *Pharodes tortugensis* Wilson, that was described using morphological characters (Ho 1971) and has, to the best of our knowledge, not been studied genetically. *P. tortugensis* infects the branchial chamber of fishes and was described from fishes in museum collections that included several small gobies and blennies from the western Atlantic, plus a few larger reef-associated fishes (Table 1). During field surveys of potential hosts in the British Virgin Islands (BVI) from 1994–2016, *P. tortugensis* was found only on three coryphopterus gobies that inhabit mixed sand and reef habitat (Table 1). The identification of *P. tortugensis* (Petrik-Finley 2005) was confirmed by the author of the species (Dr. Ju-she Ho, University of California Long Beach, personal communication 2002). Two common gobies, *Coryphopterus glaucofraenum* Gill and *C. dicrus* Böhlke & Robins were infected frequently (Finley & Forrester 2003; Petrik-Finley 2005). *Coryphopterus eidolon* Böhlke & Robins was also infected, but this goby is rare in the BVI and so the prevalence of infection was not estimated accurately (Petrik-Finley 2005).

Since these BVI field surveys, DNA barcoding has led to the discovery of new *Coryphopterus* species and the re-examination of others, including *C. glaucofraenum*, one of the species hosting *P. tortugensis* in the BVI (Baldwin *et al.* 2009; Baldwin & Robertson 2015; Thacker & Cole 2002; Victor 2007, 2008; Volk *et al.* 2020). These studies resolved longstanding debate over whether *Coryphopterus tortugae* (Jordan) and *Coryphopterus venezuelae* Cervigón were separate from *C. glaucofraenum* and supported the validity of each as distinct species (Böhlke & Robins 1960; Cervigón 1994; Garzón-Ferreira & Arturo Acero 1990; Thacker & Cole 2002). Victor (2008) also described a fourth species *Coryphopterus bol*, but subsequent work suggests *C. bol* may be a junior synonym of *C. venezuelae* (Baldwin *et al.* 2009; Baldwin & Robertson 2015). Although *C. glaucofraenum*, *C. tortugae* and *C. venezuelae* are distinct genetically and have slightly different markings, there remains uncertainty over whether they can be reliably identified in the field using visual markings (Robertson & Van Tassell 2019; Victor 2015). Their respective geographical distributions, habitat use and ecological interactions also require reconsideration (Baldwin & Robertson 2015; Greenfield & Johnson 1999; Robertson & Van Tassell 2019; Victor 2008, 2015).

Based on the taxonomic status of hosts and parasites prior to 2007, it was argued that ecologically important pharodes-coryphopterus interactions in the BVI involve one parasite (*P. tortugensis*) infecting two common hosts (*C. glaucofraenum* and *C. dicrus*) and one rare host (*C. eidolon*) (Finley & Forrester 2003; Forrester *et al.* 2019; Forrester & Finley 2006; Petrik-Finley 2005). The revised classification of *C. glaucofraenum* suggests that this host may represent up to three cryptic host species. This discovery also raises the possibility that, rather than being a single generalist parasite, *P. tortugensis* might actually consist of multiple cryptic parasite species, some of which could be more specialized than previously thought. The objective of this study was thus to combine DNA barcoding with analysis of markings visible in the field to clarify the identities of host and parasite species taking part in the pharodes-coryphopterus interaction in the British Virgin Islands.

## Methods

### Study sites and collection of specimens

Fish were collected at two fringing reef sites near Guana Island (18°28’N, 64°34’W) in the British Virgin Islands at water depths of 5–8 m. The two sites were selected to test for segregation of the gobies by habitat. (1) Harris Ghut comprises white coral sand with a rippled surface, interspersed with patches of coral and limestone reef. (2) White Bay West consists of finer muddy sand interspersed with limestone reef, coral rubble, and seagrass. The finer sediment at White Bay West, slightly higher turbidity, and its inner position within the bay indicates lower exposure to wave energy and currents than Harris Ghut (Folk 1980). Harris Ghut provides habitat hypothesized to be favoured by *C. tortugae*, whereas *C. glaucofraenum* is hypothesized to prefer the sheltered habitat found in White Bay West (Greenfield & Johnson 1999; Victor 2015).

We collected 145 gobies for analysis. Each goby was digitally photographed in its natural habitat by a diver prior to capture. Gobies were then collected using the anaesthetic Quinaldine and a hand net, placed in a clear plastic bag then photographed a second time while still underwater. Because *P. tortugensis* might be host-specific and infect some goby species, but not others, both infected and uninfected gobies were collected (n = 85 uninfected, n = 60 infected). Gobies infected with *P. tortugensis* can be diagnosed visually by divers because they have a distinctive distension of the operculum (Finley & Forrester 2003; Forrester *et al.* 2019; Petrik-Finley 2005). Our collections focused on individuals suspected to be *C. glaucofraenum*, *C. tortugae* and *C. venezuelae*, but we also collected a small sample of *C. dicrus* (n = 7) because it is also a common host of *P. tortugensis*. While collecting, we also searched for other gobies and blennies with distended opercula that might also be infected with *P. tortugensis* (Table 1), but none were encountered.

**TABLE 1.** Hosts of *P. tortugensis* in the BVI discovered using DNA barcoding in this study (BVI genetic ID) and previously using morphological characters (BVI morphological ID) (Petrik-Finley 2005), plus hosts identified using morphological characters in other areas (Horton *et al.* 2020). Listed are fish species on which *P. tortugensis* was found (yes) or not found (no), or no data available (-).

| Host Family | Host species | Host common name | **BVI genetic ID** | **BVI morphological ID** | **Other sites morphological ID** |
|---|---|---|---|---|---|
| Gobiidae | *Coryphopterus glaucofraenum* | bridled goby | yes | yes | - |
| Gobiidae | *Coryphopterus venezuelae* | sand-canyon goby | yes | yes | - |
| Gobiidae | *Coryphopterus dicrus* | colon goby | yes | yes | - |
| Gobiidae | *Coryphopterus eidolon* | pallid goby | - | yes | - |
| Gobiidae | *Coryphopterus personatus* | masked goby | - | no | - |
| Gobiidae | *Coryphopterus hyalinus* | glass goby | - | no | - |
| Gobiidae | *Gnatholepis thompsoni* | Goldspot goby | - | no | - |
| Gobiidae | *Tigrigobius multifasciatus* | Greenbanded goby | - | no | - |
| Gobiidae | *Bathygobius soporator* | frillfin goby | - | no | yes |
| Gobiidae | *Tigrigobius saucrus* | leopard goby | - | no | yes |
| Gobiidae | *Elacatinus chancei* | shortstripe goby | - | no | yes |
| Gobiidae | *Elacatinus evelynae* | sharknose goby | - | no | yes |
| Gobiidae | *Elacatinus horsti* | yellowline goby | - | no | yes |
| Gobiidae | *Elacatinus illecebrosus* | barsnout goby | - | - | yes |
| Blenniidae | *Hypleurochilus aequipinnis* | oyster blenny | - | - | yes |
| Blenniidae | *Scartella cristata* | Molly Miller | - | - | yes |
| Blenniidae | *Malacoctenus boehlkei* | diamond blenny | - | no | - |
| Blenniidae | *Malacoctenus macropus* | rosy blenny | - | no | - |
| Blenniidae | *Parablennius marmoreus* | seaweed blenny | - | no | - |
| Belonidae | *Ablennes hians* | flat needlefish | - | - | yes |
| Sparidae | *Calamus bajonado* | jolthead porgy | - | - | yes |
| Carcharhinidae | *Rhizoprionodon terraenovae* | Atlantic sharp-nose shark | - | - | yes |

After being photographed, gobies were euthanized using Quinaldine and preserved in 95% ethanol. Copepods were removed from parasitized gobies under a dissecting microscope. Consistent with previous work (Petrik-Finley 2005), female copepods were found attached to the ventral surface of the branchial chamber, whereas males and juveniles were found within the branchial chamber, on the gill arches and on the underside of the operculum. A typical infection consisted of one or two large females, plus a few smaller males and juveniles (mean = 4 copepods per goby, range = 1–17). All dissected copepods were preserved in 100 $\mu$L of 100% ethanol and stored at -20°C.

**Identifying gobies using visual markings**

Using published keys and guides to morphological characters and markings that distinguish *Coryphopterus* gobies (Baldwin & Robertson 2015; Robertson & Van Tassell 2019; Victor 2015), we selected three pigment markings that could be discerned by divers in the field and were visible on the photographs of the gobies (Table 2). Using the photographs taken in the field, each goby was identified to species using these three characters (hereafter referred to as its visual ID).

**TABLE 2.** Visual pigment markings used to identify the three morphologically similar gobies from photos and in the field on SCUBA. *Coryphopterus dicrus* is readily distinguishable from the other species, and so is not included.

| Location of pigment marks | *C. glaucofraenum* | *C. venezuelae* | *C. tortugae* |
|---|---|---|---|
| Behind opercle | Dark marking, two peaks, usually triangular | Dark marking, single peak, triangular or circular | Dark marking, single peak, triangular or circular |
| Base of pectoral fin | No pigment marking | Ventral marking, circular or rectangular, yellow or orange in colour | No pigment marking |
| Base of caudal fin | Two circular spots, colon-like, dark in colour | Variable; central bar or two colon-like spots or vertical dumbbell or C-shaped, dark in colour | Central bar, dark in colour |

We also tested whether *C. glaucofraenum* could be distinguished from *C. tortugae*, and possibly *C. venezuelae*, based on body shape. Garzón-Ferreira and Acero (1990) showed that the ratio of body depth to body length was higher in *C. glaucofraenum* (20.5–26.2%) than *C. tortugae* (*19.5–22.5%*), although the individuals they described as *C. tortugae* also included *C. venezuelae* (Victor 2008). Using photographs in which gobies were roughly perpendicular to the frame, we measured the standard body length (SL) and body depth, measured at the base of the dorsal fin spines, of each goby (using the image analysis software Fiji, Schindelin *et al.* 2012). Because infection alters body shape (Petrik-Finley 2005), body depth was measured only for uninfected gobies (n = 80). The distribution of body depths (as a % of SL) was compared among species using a Kruskal-Wallis test.

**DNA extraction, amplification and sequencing**

The right pectoral fin, caudal fin, or the right operculum were taken as tissue samples from gobies, and entire copepods were used for DNA extraction. DNA was extracted using the NucleoSpin® Tissue kits (Macherey-Nagel) following the manufacturer protocol. A 658 base pair region of the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using the primers LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al.* 1994). All PCR products were amplified using TaKaRa ExTaq in a final volume of 50 $\mu$L consisting of 37.75 $\mu$L purified water, 5 $\mu$L 10 X buffer solution, 4 $\mu$L dNTP, 1 $\mu$L of each primer ([50 $\mu$M]), 1 $\mu$L sample gDNA, and 0.25 $\mu$L ex-Taq DNA polymerase. Polymerase Chain Reactions (PCRs) were run in an Eppendorf 6325 Vapo.Protect MasterCycler Pro-S under the following thermal protocol: initial denaturation at 94℃ for 2 min, 38 cycles of denaturation at 94℃ for 30 s, annealing at 50℃ for 25 s, and extension at 72℃ for 30 s, with a final extension of 72℃ for 5 min. Goby samples that did not amplify using LCO/HCO primers were amplified using Fish F1 and Fish R1 (Ward *et al.* 2005) using the following PCR cycle: initial denaturation at 94℃ for 2 min, 33 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 60 s, with a final extension of 72℃ for 5 min. Copepod samples that did not amplify or were contaminated with fish DNA were amplified using Cope1489F and Cope2189R (Bucklin *et al.* 2010) using the following thermal protocol: initial denaturation at 94℃ for 2 min, 33 cycles of denaturation at 94℃ for 30 s, annealing at 45℃ for 30 s, and extension at 72℃ for 60 s, with a final extension of 72℃ for 5 min. PCR products were cleaned using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel) and Sanger sequencing was performed at the RI Genomics and Sequencing Center.

DNA from 67 gobies was sequenced, of which 39 were infected with copepods. Seventy copepods were sequenced from the 39 infected gobies. In order to test whether more than one parasite species could infect a single host, two copepods were sequenced from most gobies (n = 31), but for some (n = 8) just one copepod was sequenced.

Bidirectional reads were assembled (excluding the 5' and 3' primer regions) using Geneious (vrs9.1.8). BLAST searches were performed to confirm the identity of all sequences. The mtCOI barcodes were aligned using MAFFT (Katoh *et al.* 2019) with additional data from GenBank and the complete alignment was trimmed to 658 bp. The verified sequences were submitted to the National Center for Biotechnology Information (NCBI, see Appendix Table 1). Maximum likelihood phylogenetic analysis was conducted under the GTR+I+G model using IQ-Tree (Nguyen *et al.* 2015). Node support was calculated using 1000 nonparametric bootstrap replicates. Based on the phylogenetic analysis, each unknown sample was assigned a species identity (hereafter sequence ID; Appendix Table 1).

**Probability of a species being present but not sampled**

After identifying the species in our samples, we assessed how confident we could be in concluding that that other species not collected were truly absent. To make this estimate, we considered each sample a binomial trial in which an undetected species is found or not (Bland 2013). The observed proportion of undetected species ($p$) in our samples was thus zero, and we calculated the upper 95% confidence interval for this estimate of $p$ given our sample size (following McDonald 2014).

**Data availability**

The raw data used in this study, including digital images used for goby visual IDs, are archived online (https://doi.org/10.5061/dryad.h18931zjs). The GenBank Accession numbers are reported in Tables 4–5 and Appendix Table 1.

## Results

**Identity and habitat use of gobies present in the study area**

DNA barcoding revealed three distinct genetic lineages within our goby samples (Figure 1), each with low within-group sequence divergences (< 1.5 %) typical of intraspecific variation (Ward *et al.* 2009). The lineages matched those published for *C. glaucofraenum*, *C. venezuelae* and *C. dicrus* using neighbour-joining trees constructed with COI sequences (Baldwin *et al.* 2009; Baldwin & Robertson 2015; Victor 2008).

All but one of the 145 gobies in our field samples could be assigned a visual ID using the three characters in Table 2. There was 100% agreement between the visual IDs and sequence IDs of the 67 gobies identified using both methods (Table 3). *C. glaucofraenum* and *C. venezuelae* also tended to differ in body depth (Kruskal-Wallis test; $H = 20.62$, $p < 0.0001$). *C. venezuelae* were more slender than *C. glaucofraenum*, although there was not complete separation in body depths between the two species (Figure 2). The few *C. dicrus* measured (n = 3) ranged in body depth from 22.0–23.1%, and so overlapped in body depth with *C. glaucofraenum* (Figure 2).

**TABLE 3**. Match between assignment of gobies to species using COI sequences versus visual characters.

| | | Sequence ID | | | |
|---|---|---|---|---|---|
| | | *C. glaucofraenum* | *C. venezuelae* | *C. dicrus* | *C. tortugae* |
| Visual ID | *C. glaucofraenum* | 15 | 0 | 0 | 0 |
| | *C. venezuelae* | 0 | 45 | 0 | 0 |
| | *C. dicrus* | 0 | 0 | 7 | 0 |
| | *C. tortugae* | 0 | 0 | 0 | 0 |

We did not collect any *C. tortugae* and our sample provides reasonable confidence of its absence (estimated proportion of gobies that are *C. tortugae* = 0, 95% CI = 0 to 0.026, n = 145). A review of photographs taken during other studies since 1994 (n = 53) also revealed no *C. tortugae*, further supporting its absence (e.g., Finley & Forrester 2003; Forrester 1995, 1999; Forrester *et al.* 2011; Forrester & Finley 2006; Forrester & Steele 2000, 2004).

*C. venezuelae* and *C. glaucofraenum* (n = 138) appeared to segregate by habitat. Only *C. venezuelae* was observed at Harris Ghut, where the habitat was white sand and patchy coral reef, whereas a mix of *C. glaucofraenum* and *C. venezuelae* (73% and 27% respectively) were found at White Bay West, where the substratum was silty sand, rubble, and seagrass.

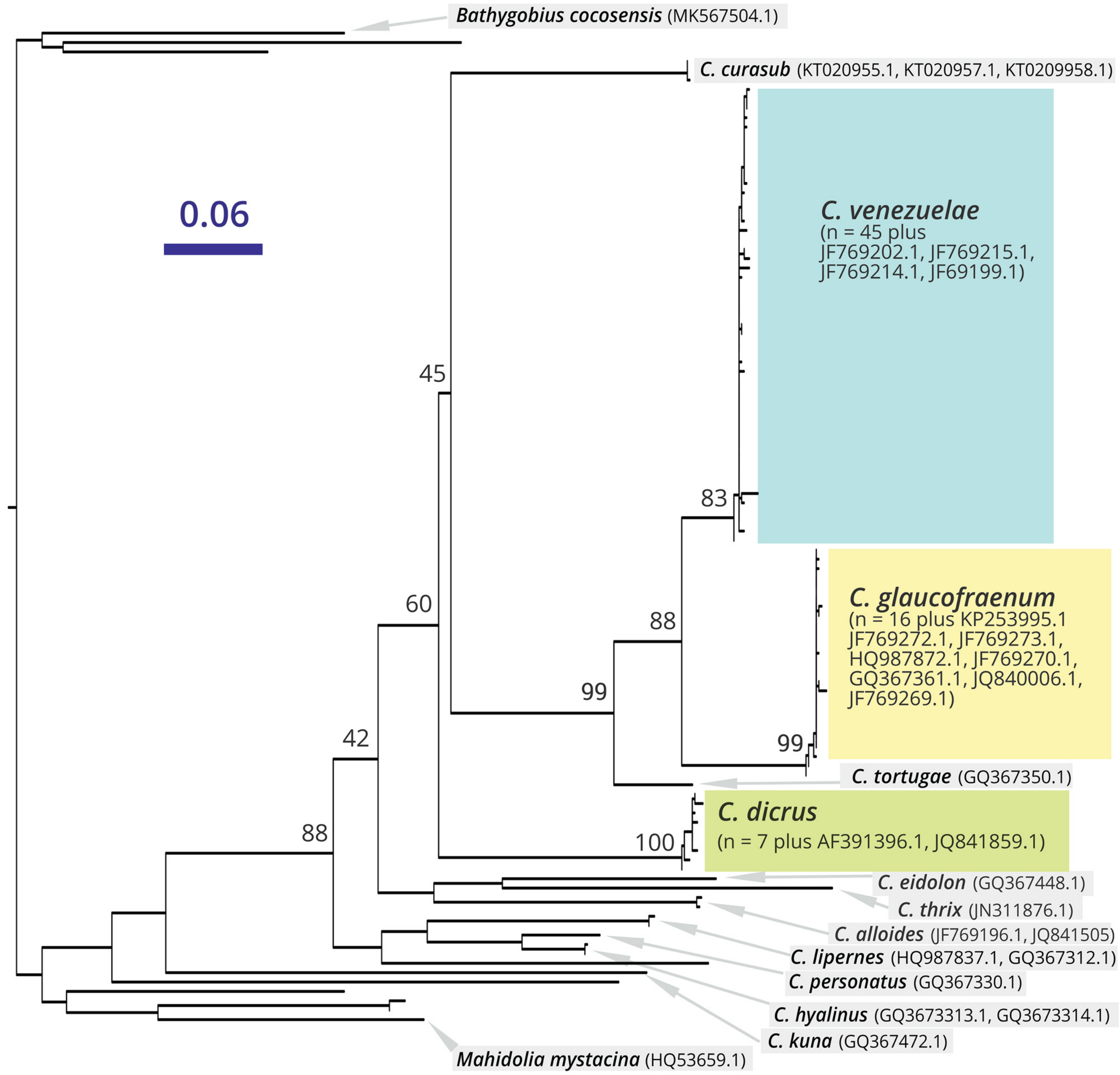


**FIGURE 1.** Maximum likelihood tree derived from COI sequences of our goby samples plus voucher sequences from all *Coryphopterus* species except *C. punctipectophorus*. Voucher sequences are identified by GenBank sequence ID. Sequences from several other goby species are included as outgroups (not all are identified in the figure; see Table 4 for a list). Support values for bipartitions are indicated, and divergence represented by scale bar = 6%.

**TABLE 4.** List of goby samples from previous studies included in Figure 1.

| GenBank Accession # | **Genus** | **Species** | **Voucher or isolate** | **Voucher #** |
|---|---|---|---|---|
| JF769196 | *Coryphopterus* | *alloides* | voucher | n7530bca160 |
| JQ841505 | *Coryphopterus* | *alloides* | voucher | BZLW8268 |
| JF769199 | *Coryphopterus* | *bol* | voucher | n762acn310 |
| JF769202 | *Coryphopterus* | *bol* | voucher | pr785acb245 |
| JF769214 | *Coryphopterus* | *bol* | voucher | n7530acn186 |
| JF769215 | *Coryphopterus* | *bol* | voucher | n7530acn187 |
| KT020955 | *Coryphopterus* | *curasub* | voucher | USNM 406373 |
| KT020957 | *Coryphopterus* | *curasub* | voucher | USNM 430037 |
| KT020958 | *Coryphopterus* | *curasub* | voucher | USNM 430019 |
| AF391396 | *Coryphopterus* | *dicrus* | isolate | CORYPUN |
| JQ841859 | *Coryphopterus* | *dicrus* | voucher | FCC8121 |
| GQ367448 | *Coryphopterus* | *eidolon* | voucher | NMNH Fish BZE4089 |
| GQ367361 | *Coryphopterus* | *glaucofraenum* | voucher | NMNH Fish BZE7769 |
| HQ987872 | *Coryphopterus* | *glaucofraenum* | voucher | cn10c69 |
| JF769269 | *Coryphopterus* | *glaucofraenum* | voucher | pr784bcg159 |
| JF769270 | *Coryphopterus* | *glaucofraenum* | voucher | pr784bcg195 |
| JF769272 | *Coryphopterus* | *glaucofraenum* | voucher | st307acgx260 |
| JF769273 | *Coryphopterus* | *glaucofraenum* | voucher | st307acx300 |
| JQ840006 | *Coryphopterus* | *glaucofraenum* | voucher | BZLW4116 |
| JQ840463 | *Coryphopterus* | *glaucofraenum* | voucher | BZLW5226 |
| KP253995 | *Coryphopterus* | *glaucofraenum* | voucher | FTP 12 |
| GQ367313 | *Coryphopterus* | *hyalinus* | voucher | NMNH Fish BZE4511 |
| GQ367314 | *Coryphopterus* | *hyalinus* | voucher | NMNH Fish BZE4512 |
| GQ367472 | *Coryphopterus* | *kuna* | voucher | NMNH Fish BZE6049 |
| GQ367312 | *Coryphopterus* | *lipernes* | voucher | NMNH Fish CUR8327 |
| HQ987837 | *Coryphopterus* | *lipernes* | voucher | pr784acl76 |
| GQ367330 | *Coryphopterus* | *personatus* | voucher | NMNH Fish BZE7163 |
| JN311876 | *Coryphopterus* | *thrix* | voucher | n7530bc157 |
| GQ367350 | *Coryphopterus* | *tortugae* | voucher | JVT77256 |
| FJ583288 | *Cryptocentrus* | *leptocephalus* | voucher | BIOUG CAN HLC 11903 |
| HQ536660 | *Cryptocentrus* | *leptocephalus* | isolate | C199 |
| MK567504 | *Bathygobius* | *cocosensis* | voucher | USNM FISH 442433 |
| MK572079 | *Brachygobius* | *nunus* | | |
| JQ349994 | *Fusigobius* | *sp.* | voucher | BOLD AAU4384 |
| MG450087 | *Lophogobius* | *cyprinoides* | voucher | BACQ |
| HQ536659 | *Mahidolia* | *mystacina* | isolate | C182 |
| HQ945926 | *Oligolepis* | *keiensis* | voucher | ADC10 |
| MH674047 | *Tridentiger* | *barbatus* | isolate | KL175 |

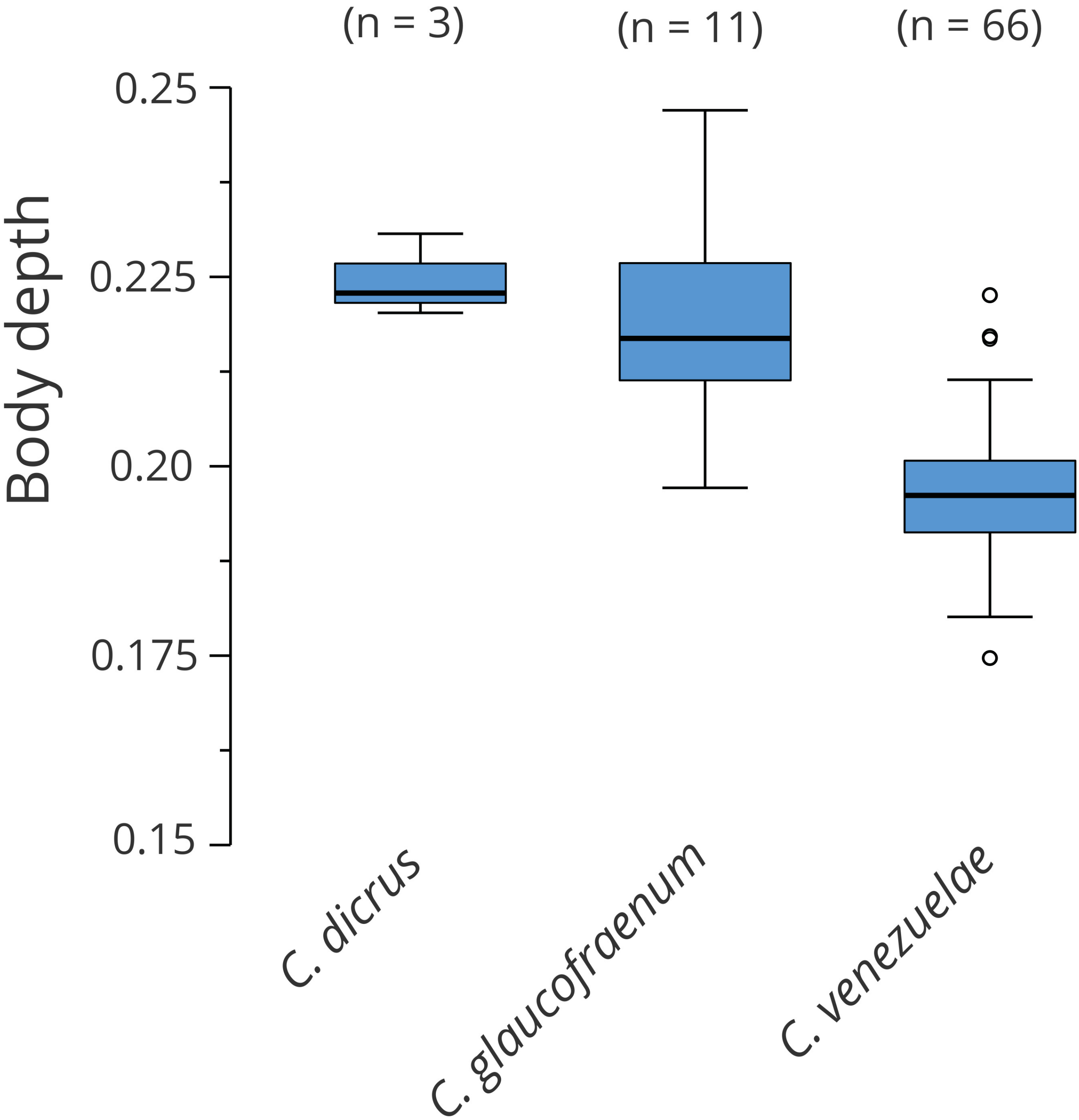


**FIGURE 2.** Differences in body depth between goby species. A boxplot of body depth (as a % of body length in SL) for the three gobies, with sample sizes in parentheses. For the boxplot: box boundaries represent 25th and 75th percentiles respectively; line inside box indicates the median, lower and upper error lines indicate 10th and 90th percentiles respectively, and circles show data falling outside 10th and 90th percentiles.

**TABLE 5.** List of copepod samples from previous studies included in Figure 3.

| GenBank Accession # | Family | Genus | Species | Voucher or accession ID # |
|---|---|---|---|---|
| KT030281 | Clausidiidae | *Conchyliurus* | *quintus* | LEGO-POE007 |
| KR049027 | Clausidiidae | *Hemicyclops* | *tanakai* | LEGO-POE050 |
| KR049025 | Clausidiidae | *Hemicyclops* | *gomsoensis* | LEGO-POE009 |
| MK370310 | Giselinidae | | *sp.* | 723DZMB |
| MN854870 | Ergasilidae | *Acusicola* | *sp. 1* | 774AcitAsL |
| MN854851 | Ergasilidae | *Acusicola* | *sp. 1* | 623AcitAsL |
| MF651988 | Ergasilidae | *Ergasilus* | *jaraquensis* | 193762 |
| KR049036 | Ergasilidae | *Ergasilus* | *wilsoni* | LEGO-POE014 |
| KR049037 | Ergasilidae | *Neoergasilus* | *japonicus* | LEGO-POE015 |
| KR049047 | Rhynchomolgidae | *Zamolgus* | *cavernularius* | LEGO-POE028 |
| MH374723 | Rhynchomolgidae | *Paradoridicola* | *sp. 1* | |
| GBCRO6094-19 | Anchimolgidae | *Prionomolgus* | *sp. 1* | MH374772 |
| GBCRO6097-19 | Anchimolgidae | *Prionomolgus* | *sp. 2* | MH374685 |
| GBCRO6118-19 | Anchimolgidae | *Schedomolgus* | *sp. 1* | MH374682 |
| GBCRO6186-19 | Anchimolgidae | *Schedomolgus* | *sp. 1* | MH374839 |
| KR049023 | Chondracanthidae | *Chondracanthus* | *distortus* | LEGOPOE006 |
| GBCRO110819 | Chondracanthidae | *Chondracanthus* | *distortus* | KR049023 |
| GBCRO111119 | Chondracanthidae | *Chondracanthus* | *zei* | KR049033 |
| KR049033 | Chondracanthidae | *Chondracanthus* | *zei* | LEGOPOE042 |
| BNSC59815 | Chondracanthidae | *Chondracanthus* | *lophii* | KT208406 |
| BNSC59515 | Chondracanthidae | *Chondracanthus* | *lophii* | KT209368 |
| KR049022 | Chondracanthidae | *Brachiochondria* | *pinguis* | LEGOPOE005 |
| MH242703 | Chondracanthidae | *Chondracanthus* | *irregularis* | BFHL2227 |
| MN138366 | Chondracanthidae | *Acanthochondria* | *rectangularis* | BMBM0758 |
| BNSCP09714 | Chondracanthidae | *Chondracanthus* | *merluccii* | KT208610 |
| BNSCP09914 | Chondracanthidae | *Chondracanthus* | *merluccii* | KT208757 |
| BNSCP09814 | Chondracanthidae | *Chondracanthus* | *merluccii* | KT209334 |
| KR049021 | Chondracanthidae | *Acanthochondria* | *tchangi* | LEGOPOE004 |
| KR049020 | Chondracanthidae | *Acanthochondria* | *spirigera* | LEGOPOE003 |

**Identity of the parasitic copepod**

DNA barcoding revealed just one genetic lineage within our copepod samples, and the extremely low within-group sequence divergence (< 0.5 %) suggests they are a single species (Figure 3). Our sample provides reasonable confidence that additional species are absent (estimated proportion of copepods that are other species = 0, 95% CI = 0 to 0.051, n = 69). We thus found no evidence for cryptic copepod species specialized on one or more of these goby hosts, nor any segregation of copepods by host habitat.

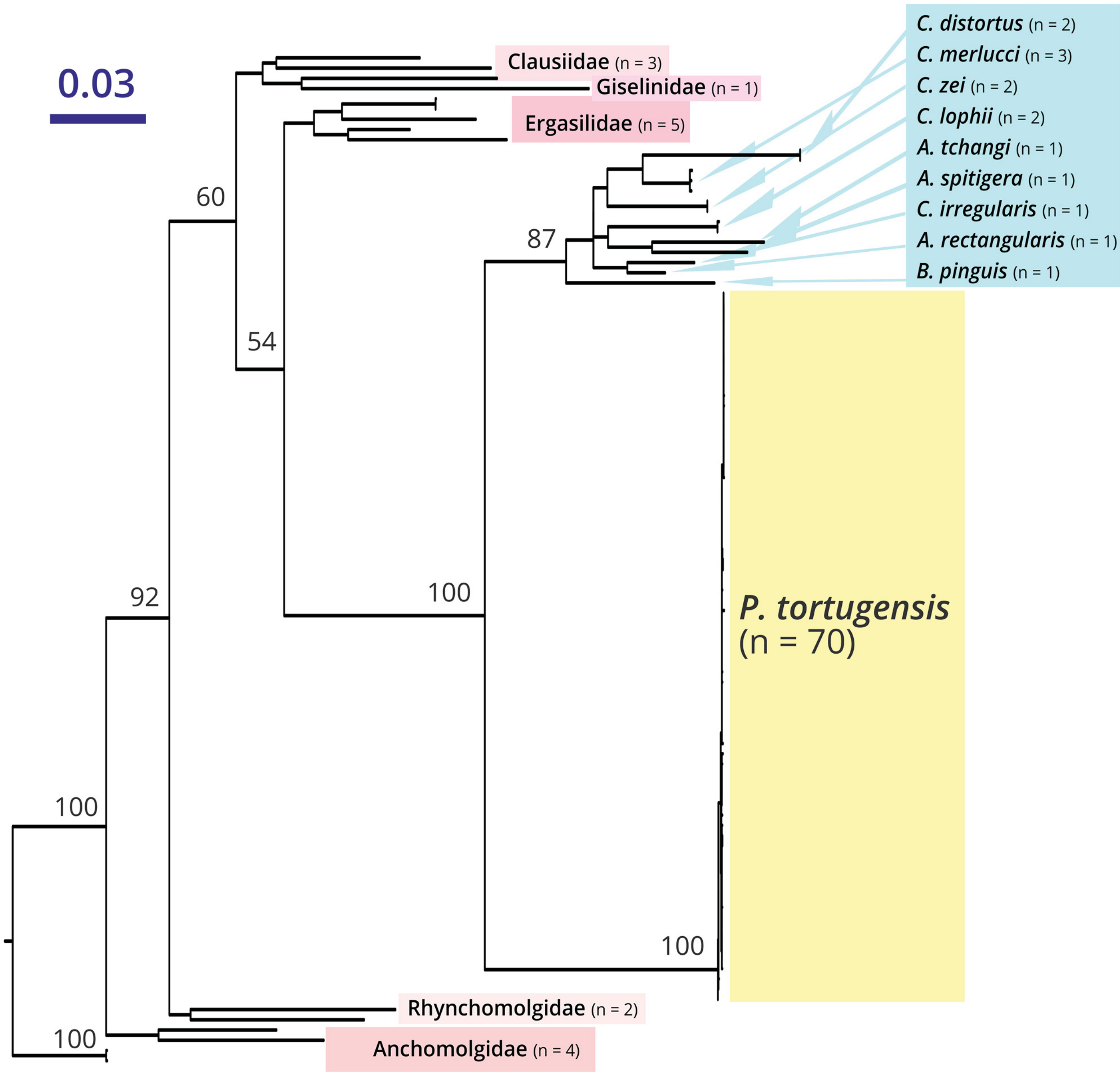


**FIGURE 3.** Maximum likelihood tree derived from COI sequences of our copepod samples (labeled as *P. tortugensis*) plus voucher sequences from related copepods in the suborder Ergasilida (see Table 5 for a list). Sequences of copepods confamilial to *P. tortugenis* (Chondracanthidae) are labelled to species (and shaded blue in the online colour version), and members other taxa are labeled to family (and shaded pink in the colour online version). Support values for bipartitions are indicated, and divergence represented by dark blue scale bar = 3 %.

## Discussion

### Clarified pharodes-coryphopterus interactions in the BVI

The three coryphopterus gobies previously grouped as *C. glaucofraenum* have each been identified using COI sequences at several locations across the tropical western Atlantic (Baldwin *et al.* 2009; Baldwin & Robertson 2015; Victor 2008; Volk *et al.* 2020). Our results extend the confirmed ranges of *C. glaucofraenum* and *C. venezuelae* to the BVI and, because these species are widespread in the region, their presence was not surprising. The apparent absence of *C. tortugae* from our sites was, in contrast, unexpected because this species has been reported at sites in the U.S. Virgin Islands and Puerto Rico less than 100 miles from the BVI (Victor 2008).

Goby habitat associations were consistent with previous reports for *C. glaucofraenum*, but not for *C. venezu-*

*elae*, and provide another reason why the absence of *C. tortugae* was surprising. We expected to find *C. tortugae* in Harris Ghut because it provides the type of shallow, clear-water patch reef habitat it reportedly prefers (Greenfield & Johnson 1999; Victor 2008, 2015). Finding *C. glaucofraenum* only at White Bay West was in agreement with accounts of it preferring areas with fine silty sand and more turbid water (Garzón-Ferreira & Arturo Acero 1990; Greenfield & Johnson 1999). The fact that we found *C. venezuelae* at both of our protected inshore sites expands the reported habitat range for the species, which heretofore was documented to be primarily a species of deeper offshore reefs in buttress-canyon habitats and rocky points with strong currents (Victor 2015). Because we sampled just two sites, however, our data are preliminary and defining habitat associations will require additional sampling. Defining habitat preferences will also be facilitated by quantitative measurements of variables that gobies use to select habitat, such as sediment grain size, water-clarity, and substratum composition, so that cross-study comparisons can be more explicit (Baldwin & Robertson 2015; Victor 2015; Volk *et al.* 2020) .

We conclude that the parasitic copepods infecting *C. venezuelae*, *C. glaucofraenum* and *C. dicrus* in the BVI are all *P. tortugensis*. COI sequence data are sparse for parasitic copepods (Boxshall & Hayes 2019). We could find no published sequences of putative conspecifics (*P. tortugensis*) or congeners against which to compare our samples, and sequences from confamilial taxa (family Chondracanthidae) are few. Our samples, nonetheless, cluster more closely with sequences from confamilial copepods (*Chondracanthus* and *Acanthochondria*) than with various other cyclopoid copepods (Figure 3), which is consistent with the classification of our samples within the Chondracanthidae (Østergaard *et al.* 2003). All copepods previously identified using morphological characters from the same hosts at the same sites were classified as *P. tortugensis*. Because this past classification was based on a fairly large sample (88 copepods in 2001-4 (Petrik-Finley 2005) plus 10 copepods in 2018 (G. Forrester, unpublished data)), we consider it unlikely that any other copepod species are present but not sampled.

**Ecological significance of pharodes-coryphopterus interactions in the BVI**

Our findings allow us to clarify an ecologically significant host-parasite interaction involving *P. tortugensis* and three abundant shared hosts in the BVI (*C. venezuelae*, *C. glaucofraenum* and *C. dicrus)*. Surveys from 2001–2004 showed that *P. tortugensis* was widespread in the BVI and the neighbouring island of St. John in the USVI (detected at 27 of 39 sites) and that infections of these gobies were prevalent (mean = 6%, range = 1–25%) (Petrik-Finley 2005). Field and lab experiments using *C. venezuelae* and *C. dicrus* confirmed that *P. tortugensis* is transmitted directly among these hosts (Petrik-Finley 2005). Because these gobies overlap in habitat use, understanding relative rates of transmission among these newly confirmed hosts will thus be critical to define basic features of *P. tortugensis* dynamics, such as net reproductive rate of the parasite and the host density required for its persistence (Dobson 2004; Holt *et al.* 2003).

Past research also revealed strong impacts of *P. tortugensis* on host population dynamics in Harris Ghut (Finley & Forrester 2003; Forrester *et al.* 2019; Forrester & Finley 2006). These hosts, previously identified as *C. glaucofraenum*, can now be confirmed as *C. venezuelae.* Although *P. tortugensis* is sufficiently debilitating to kill some hosts directly (Finley & Forrester 2003; Petrik-Finley 2005), its primary impact occurs by mediating the effects of predation on host gobies. All coryphopterus host species are consumed by several larger species of reef fish, and predation is the proximate cause of most goby deaths (Forrester & Steele 2000). When threatened by predators, these gobies temporarily flee to shelter within reef crevices. For *C. venezuelae*, the scramble for access to crevices resembles the childhood game of musical chairs (Forrester & Steele 2004; Samhouri *et al.* 2009; Vance *et al.* 2010). Infection with *P. tortugensis* compromises their ability to compete for refuges and so makes them far more vulnerable to predators than uninfected individuals (Forrester *et al.* 2019; Forrester & Finley 2006). Our discovery that *P. tortugensis* also frequently infects *C. dicrus* and *C. glaucofraenum* broadens the scope of this interaction and makes it important to discover whether *P. tortugensis* similarly mediates vulnerability to predation for these gobies. Of particular interest is whether the three gobies compete inter-specifically, as well as intra-specifically for refuges, and whether the effects of *P. tortugensis* on competitive ability are equivalent among goby hosts.

**Host range of P. tortugensis and its relationship with other Pharodes**

Although we clarified the identities of common hosts and parasites in the BVI, considerable uncertainty remains about the host range (the number of host species infected) of *P. tortugensis* in the BVI and elsewhere and its relationship with the four other known species in the genus (Appendix Table 2). Copepods tend to have broader host ranges than other macroparasites of fishes (Poulin 1992) and *P. tortugensis* is reported from 15 host species (Table

1). *Pharodes tortugensis*, *P. banyulensis* (Delamare Deboutteville and Nunes-Ruivo) and *P. clinii* (Vaney and Conte) are morphologically very similar, and both Ho (1971) and Walters (1953) speculated that variations in morphology could represent intraspecific differences. A priority for future testing with COI sequence data is, therefore, the hypothesis that *P. tortugensis*, *P. banyulensis* and *P. clinii* are actually one broadly distributed generalist species.

On the other hand, it is also possible that *Pharodes* includes cryptic species that lack obvious phenotypic differences. Most known hosts of *P. tortugensis* and its congeners are gobies and blennies (Appendix Table 2), which suggests they show significant co-evolution (association by descent) within these families (Paterson & Poulin 1999). Some hosts, however, come from other fish families, such as wrasses, porgies, and scorpionfish (Table 1, Appendix Table 2). We thus hypothesize that the *Pharodes* most likely to be cryptic species are those occupying phylogenetically distant hosts, especially those differing in habitat use, ecology and physiology from gobies and blennies (Noble 1989). Our inability to locate additional hosts in the BVI was consistent with more thorough previous searches (Petrik-Finley 2005), suggesting that *P. tortugensis* rarely or never infects other hosts in the area. We suggest two testable hypotheses to explain this observation. First, the copepod we identified as *P. tortugensis* may be a locally abundant BVI endemic and *P. tortugensis* elsewhere in the tropical Atlantic are actually one or more different species that never reach high prevalence on any one host. Alternatively, *P. tortugensis* may be a widespread generalist parasite that, for unknown reasons, has become locally prevalent on these three coryphopterus hosts in the BVI.

## Acknowledgements

We thank Lianna Jarecki, Dive BVI and the Guana Island staff for logistical support during the field work, and Elaine Shen for assistance during DNA barcoding. Funding for the project was provided by the Falconwood Foundation and the URI Division of Research & Economic Development through a Proposal Development Grant Award.

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**APPENDIX TABLE 1.** Genbank Accession IDs for gobies and copepods sequenced during this study. Host and parasite ID numbers allow matching of each copepod to its respective goby host.

| Species | Host ID | Parasite ID | GenBank Accession ID |
|---|---|---|---|
| *C. venezuelae* | 6 | - | MW412102 |
| *C. venezuelae* | 7 | - | MW412101 |
| *C. venezuelae* | 8 | - | MW412100 |
| *C. venezuelae* | 9 | - | MW412099 |
| *C. venezuelae* | 10 | - | MW412098 |
| *C. venezuelae* | 11 | - | MW412097 |
| *C. venezuelae* | 12 | - | MW412096 |
| *C. venezuelae* | 13 | - | MW412095 |
| *C. venezuelae* | 15 | - | MW412094 |
| *C. venezuelae* | 16 | - | MW412093 |
| *C. venezuelae* | 17 | - | MW412092 |
| *C. venezuelae* | 19 | - | MW412091 |
| *C. venezuelae* | 20 | - | MW412090 |
| *C. venezuelae* | 21 | - | MW412089 |
| *C. venezuelae* | 22 | - | MW412088 |
| *C. venezuelae* | 23 | - | MW412087 |
| *C. venezuelae* | 25 | - | MW412086 |
| *C. venezuelae* | 28 | - | MW412085 |
| *C. venezuelae* | 30 | - | MW412084 |
| *C. venezuelae* | 32 | - | MW412083 |
| *C. venezuelae* | 37 | - | MW412082 |
| *C. venezuelae* | 38 | - | MW412081 |
| *C. venezuelae* | 39 | - | MW412080 |
| *C. venezuelae* | 40 | - | MW412079 |
| *C. venezuelae* | 41 | - | MW412078 |
| *C. venezuelae* | 44 | - | MW412077 |
| *C. venezuelae* | 45 | - | MW412076 |
| *C. venezuelae* | 46 | - | MW412075 |
| *C. venezuelae* | 48 | - | MW412074 |
| *C. venezuelae* | 49 | - | MW412073 |
| *C. venezuelae* | 50 | - | MW412072 |
| *C. venezuelae* | 51 | - | MW412071 |
| *C. venezuelae* | 52 | - | MW412070 |
| *C. venezuelae* | 53 | - | MW412069 |
| *C. venezuelae* | 54 | - | MW412068 |
| *C. venezuelae* | 55 | - | MW412067 |
| *C. venezuelae* | 56 | - | MW412066 |
| *C. venezuelae* | 72 | - | MW412065 |
| *C. venezuelae* | 82 | - | MW412064 |
| *C. venezuelae* | 99 | - | MW412063 |
| *C. venezuelae* | 107 | - | MW412062 |
| *C. venezuelae* | 112 | - | MW412061 |
| *C. venezuelae* | 114 | - | MW412060 |

*......continued on the next page*

**APPENDIX TABLE 1. (Continued)**

| Species | Host ID | Parasite ID | GenBank Accession ID |
|---|---|---|---|
| *C. venezuelae* | 139 | - | MW412047 |
| *C. venezuelae* | 143 | - | MW412043 |
| *C. glaucofraenum* | 122 | - | MW412059 |
| *C. glaucofraenum* | 124 | - | MW412058 |
| *C. glaucofraenum* | 125 | - | MW412057 |
| *C. glaucofraenum* | 127 | - | MW412056 |
| *C. glaucofraenum* | 128 | - | MW412055 |
| *C. glaucofraenum* | 129 | - | MW412054 |
| *C. glaucofraenum* | 130 | - | MW412053 |
| *C. glaucofraenum* | 134 | - | MW412052 |
| *C. glaucofraenum* | 135 | - | MW412051 |
| *C. glaucofraenum* | 136 | - | MW412050 |
| *C. glaucofraenum* | 137 | - | MW412049 |
| *C. glaucofraenum* | 138 | - | MW412048 |
| *C. glaucofraenum* | 140 | - | MW412046 |
| *C. glaucofraenum* | 141 | - | MW412045 |
| *C. glaucofraenum* | 142 | - | MW412044 |
| *C. dicrus* | 159 | - | MW412042 |
| *C. dicrus* | 160 | - | MW412041 |
| *C. dicrus* | 161 | - | MW412040 |
| *C. dicrus* | 162 | - | MW412039 |
| *C. dicrus* | 163 | - | MW412038 |
| *C. dicrus* | 164 | - | MW412037 |
| *C. dicrus* | 166 | - | MW412036 |
| *P. tortugensis* | 6 | 1 | MW412035 |
| *P. tortugensis* | 6 | 2 | MW412034 |
| *P. tortugensis* | 8 | 1 | MW412033 |
| *P. tortugensis* | 8 | 2 | MW412032 |
| *P. tortugensis* | 10 | 1 | MW412031 |
| *P. tortugensis* | 10 | 2 | MW412030 |
| *P. tortugensis* | 12 | 1 | MW412029 |
| *P. tortugensis* | 12 | 2 | MW412028 |
| *P. tortugensis* | 15 | 1 | MW412027 |
| *P. tortugensis* | 15 | 2 | MW412026 |
| *P. tortugensis* | 17 | 2 | MW412025 |
| *P. tortugensis* | 19 | 1 | MW412024 |
| *P. tortugensis* | 19 | 2 | MW412023 |
| *P. tortugensis* | 21 | 1 | MW412022 |
| *P. tortugensis* | 21 | 2 | MW412021 |
| *P. tortugensis* | 23 | 1 | MW412020 |
| *P. tortugensis* | 23 | 2 | MW412019 |
| *P. tortugensis* | 25 | 1 | MW412018 |
| *P. tortugensis* | 25 | 2 | MW412017 |

*......continued on the next page*

**APPENDIX TABLE 1. (Continued)**

| Species | Host ID | Parasite ID | GenBank Accession ID |
|---|---|---|---|
| *P. tortugensis* | 30 | 1 | MW412016 |
| *P. tortugensis* | 30 | 2 | MW412015 |
| *P. tortugensis* | 37 | 1 | MW412014 |
| *P. tortugensis* | 37 | 2 | MW412013 |
| *P. tortugensis* | 38 | 2 | MW412012 |
| *P. tortugensis* | 39 | 1 | MW412011 |
| *P. tortugensis* | 40 | 1 | MW412010 |
| *P. tortugensis* | 41 | 1 | MW412009 |
| *P. tortugensis* | 44 | 1 | MW412008 |
| *P. tortugensis* | 45 | 1 | MW412007 |
| *P. tortugensis* | 45 | 2 | MW412006 |
| *P. tortugensis* | 46 | 1 | MW412005 |
| *P. tortugensis* | 46 | 2 | MW412004 |
| *P. tortugensis* | 48 | 1 | MW412003 |
| *P. tortugensis* | 48 | 2 | MW412002 |
| *P. tortugensis* | 49 | 1 | MW412001 |
| *P. tortugensis* | 49 | 2 | MW412000 |
| *P. tortugensis* | 50 | 1 | MW411999 |
| *P. tortugensis* | 50 | 2 | MW411998 |
| *P. tortugensis* | 51 | 1 | MW411997 |
| *P. tortugensis* | 52 | 1 | MW411996 |
| *P. tortugensis* | 52 | 2 | MW411995 |
| *P. tortugensis* | 53 | 1 | MW411994 |
| *P. tortugensis* | 53 | 2 | MW411993 |
| *P. tortugensis* | 54 | 1 | MW411992 |
| *P. tortugensis* | 54 | 2 | MW411991 |
| *P. tortugensis* | 55 | 1 | MW411990 |
| *P. tortugensis* | 55 | 2 | MW411989 |
| *P. tortugensis* | 56 | 1 | MW411988 |
| *P. tortugensis* | 56 | 2 | MW411987 |
| *P. tortugensis* | 72 | 1 | MW411986 |
| *P. tortugensis* | 72 | 2 | MW411985 |
| *P. tortugensis* | 122 | 1 | MW411984 |
| *P. tortugensis* | 122 | 2 | MW411983 |
| *P. tortugensis* | 134 | 1 | MW411982 |
| *P. tortugensis* | 134 | 2 | MW411981 |
| *P. tortugensis* | 137 | 1 | MW411980 |
| *P. tortugensis* | 139 | 1 | MW411979 |
| *P. tortugensis* | 139 | 2 | MW411978 |
| *P. tortugensis* | 142 | 1 | MW411977 |
| *P. tortugensis* | 142 | 2 | MW411976 |
| *P. tortugensis* | 143 | 1 | MW411975 |
| *P. tortugensis* | 143 | 2 | MW411974 |

*......continued on the next page*

**APPENDIX TABLE 1. (Continued)**

| Species | Host ID | Parasite ID | GenBank Accession ID |
|---|---|---|---|
| *P. tortugensis* | 159 | 1 | MW411973 |
| *P. tortugensis* | 159 | 2 | MW411972 |
| *P. tortugensis* | 160 | 1 | MW411971 |
| *P. tortugensis* | 160 | 2 | MW411970 |
| *P. tortugensis* | 161 | 1 | MW411969 |
| *P. tortugensis* | 161 | 2 | MW411968 |
| *P. tortugensis* | 166 | 1 | MW411967 |
| *P. tortugensis* | 166 | 2 | MW411966 |

**APPENDIX TABLE 2.** Known hosts of other *Pharodes* species (Horton *et al.* 2020).

| Parasite species | Host Family | Host species | Host common name |
|---|---|---|---|
| *Pharodes biakensis* | Scorpaenidae | *Caracanthus unipinna* | coral croucher |
| *Pharodes banyulensis.* | Blenniidae | *Salaria pavo* | peacock blenny |
| *Pharodes banyulensis.* | Gobiidae | *Deltentosteus quadrimaculatus* | four-spotted goby |
| *Pharodes clini* | Clinidae | *Clinitrachus argentatus* | cline |
| *Pharodes clini* | Labridae | *Symphodus ocellatus* | ocellated wrasse |
| *Pharodes ninnii* | Gobiidae | *Gobius auratus* | golden goby |
| *Pharodes ninnii* | Gobiidae | *Knipowitschia panizzae* | Adriatic dwarf goby |