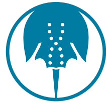

Identity and Diversity of Polydorids (Annelida: Spionidae) on Coralline Algae Encrustations of *Conus* Shells at French Polynesia

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Abstract

Polydorids (family Spionidae) are marine annelids that mostly bore into and occupy a variety of calcareous substrates, including mollusc shells and coralline algae. Here we evaluated the identity of polydorids inhabiting coralline algae encrustations of shells of Conus *species (family Conidae) from French Polynesia through analyses of sequences of a region of the mitochondrial 16S gene. We observed polydorids in samples of 18 specimens of six* Conus *species from three locations in French Polynesia. We obtained sequences of 138 polydorid specimens, which represented seven distinct haplotypes. Although it is unclear whether the distinct sequences represent intraspecific variation, they are most similar to a GenBank sequence of* Dipolydora armata*. For 12 of the 14* Conus *specimens for which multiple polydorid individuals were sequenced, we recovered identical haplotype sequences; two haplotype sequences were obtained from the other three* Conus *specimens, but one of the haplotypes predominated the samples. Moreover, no particular polydorid haplotype showed any obvious association with any of the* Conus *species. These results suggest that the polydorids inhabiting the encrustations on cone snails of French Polynesia are* D. armata *or represent close relatives of this species and that communities of polydorids on individual snails are genetically homogenous and possibly established from settlement of few larvae and asexual reproduction of recruits.*

Introduction

Members of several genera of the annelid family Spionidae bore into and occupy a variety of substrates, including mollusc shells, corals, coralline algae, other calcareous structures, and sponges (Blake, 1996; Radashevsky *et al.*, 2023; Sato-Okoshi *et al.*, 2023). These genera are *Polydora*, *Dipolydora*, *Boccardia*, *Pseudopolydora*, *Boccardiella*, *Carazziella*, *Amphipolydora*, and *Tripolydora*, which together comprise the *Polydora* complex and are commonly referred to as polydorids (Blake, 1996). Some polydorids are generalist borers (Radashevsky and Nogueira, 2003; Simon, 2011; Martin and Britayev, 2018; Radashevsky *et al.*, 2023), while others appear to be specialists for particular hosts or substrates (Martin and Britayev, 1998, 2018; Sato-Okoshi and Takatsuka, 2001). For example, as summarized by Martin and Britayev (1998), *Polydora glycymerica* primarily inhabits the shell of the bivalve *Glycymeris yessoensis* (Radashevsky, 1989), while *P. wobberi* is exclusively associated with a gorgonian coral (*Lophogorgia* sp.) (Light, 1970) and *P. woodwicki* bores into shells of the blacklip abalone *Haliotis ruber* (Blake and Kudenov, 1978).

Cone snails (family Conidae) are a diverse family of venomous marine gastropods that are often encrusted with coralline algae that, together with their shells, provide a habitat for polydorids. Indeed, various gastropods at Guam, including *Conus* species, with heavily encrusted shells, host a number of boring taxa, including polydorids that were observed to also penetrate shell material (Smyth, 1989, 1990). However, the identity of these polydorids and their

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potential associations with cone snail species have not been previously investigated.

While performing field studies of cone snails at locations in French Polynesia, we observed many *Conus* species with thick coralline algae encrustations that supported large numbers of polydorids (Fig. 1). Here we sought to determine the identity and genetic diversity of these polydorids. To accomplish this, we obtained sequences of a region of the mitochondrial *16S* gene from polydorids that were collected along with feces of cone snails at four locations in French Polynesia. We compared recovered sequences to GenBank sequences of polydorids and used phylogenetic approaches to assess identity, evaluate potential associations among different *Conus* species, and determine the structure of communities of polydorids on coralline algae of individual snails.

Materials and Methods

Polydorid specimens

While performing studies of the diets of cone snails at four locations in French Polynesia (Rangiroa and Makemo [Tuamotu Archipelago], Huahine [Society Islands], and Rurutu [Austral Islands]) in 2022 and 2023 (Fig. 2), we observed polydorids occupying the coralline algal encrustations of shells of *Conus* species (Fig. 1), especially for snails from the lagoon at Rangiroa, which were heavily encrusted. To obtain fecal samples from snails to characterize their prey, we placed individuals in small cups with seawater immediately after they were collected and then recovered any feces that were produced. When individuals with heavily encrusted shells were placed in cups, polydorids tended to gradually emerge from the coralline algae and so were often collected along with fecal materials. We preserved fecal samples (and accompanying polydorids) in 95% ethanol. Later, while viewing samples under a microscope, we separated polydorids from feces and placed individual specimens, including individuals from the same fecal sample, into separate centrifuge tubes for DNA extraction.

DNA analyses

We extracted DNA from individual polydorids using the Omega Biotek E.Z.N.A. mollusc DNA kit (Omega Biotek, Norcross, GA). In brief, we incubated specimens in centrifuge tubes containing 350 μL of ML1 buffer with 25 μL of proteinase K (20 mg mL^{-1}) at 60 °C for at least 30 min or until the specimen was fully dissolved. After incubation, we added 350 μL of chloroform to each sample and gently mixed the solution by inverting tubes five to 10 times. We centrifuged tubes at 10,000 *g* for 2 min to separate organic and aqueous phases and then carefully transferred 200 μL of the upper aqueous layer to new centrifuge tubes. We then added 200 μL of MBL buffer and 200 μL of absolute ethanol to each sample and gently inverted tubes 10 times to facilitate DNA precipitation. We transferred the mixture to a HiBind DNA column (Omega Bio-tek), added 500 μL of HBC buffer, and then used a vacuum manifold to remove the liquid. We performed two washes with 700 μL of DNA wash buffer with a vacuum manifold and then centrifuged columns at 15,000 *g* for 2 min after the final wash. We eluted DNA by adding 50 μL of preheated elution buffer (60–70 °C) to each HiBind column. After a 2-min incubation at room temperature, we centrifuged columns at 10,000 *g* for 1 min to collect eluted DNA, which was then stored at −20 °C.

We amplified an ~314-base pair (bp) region of the mitochondrial *16S* gene using primers (16SANNf2 and 16Spr1) (Duda *et al.*, 2009) that were appended with M13 and M13R vector primers for sequencing. Each amplification reaction had a total volume of 10 μL, with 1 μL of template DNA, 5 μL of 2X GoTaq Green Master Mix (Promega, Fitchburg, WI), 0.25 μL of each 10 μmol L^{-1} primer, and 3.5 μL of nuclease-free water. We used the following thermocycling conditions: 40 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. We included positive and negative controls to verify amplification specificity and detect contamination. For reactions that initially failed, we used diluted genomic DNA and attempted new amplifications. We visualized amplification products using gel electrophoresis on a 1.5% agarose gel run at 90 V for 15 min. We confirmed successful amplification based on the expected size of ~400 bp, using a DNA ladder as a size reference. We diluted successful amplification products 1∶5 with nuclease-free water and submitted them to Eurofins Genomics (Louisville, KY) for sequencing in both directions using M13 and M13R primers.

We imported chromatogram files into Geneious Prime v2024.0.7 (Dotmatics, Woburn, MA), where we removed primers, trimmed sequences for quality, assembled consensus sequences for each template, and inspected consensus sequences and alignments. We used the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST) for nucleotide sequences to identify sequences of polydorids from GenBank that are most similar to the sequences we obtained. We also inspected GenBank sequences to identify polydorid species for which sequences of 10 or more individuals have been reported and downloaded these sequences as well as sequences from related species (based on the phylogeny of Abe and Sato-Okoshi, 2021). We obtained these latter sets of sequences to use as a proxy for observed levels of intra- and interspecific variation of *16S* sequences of polydorids.

We aligned downloaded sequences with those we generated using Muscle in Seqotron v1.01 (Fourment and Holmes, 2016), edited the alignment manually, trimmed all sequences to the length of the longest sequences we obtained, and exported the alignment for subsequent analyses. Given the small lengths of some of the recovered and GenBank sequences, we examined sequences from the same sample (for our data) or same reported species (for

Figure 1. *Conus ebraeus* individual from the lagoon at Rangiroa heavily encrusted with coralline algae and numerous polydorids emerging from algae and settling on the bottom of collection cup.

GenBank data) to determine whether the sequences are identical for the lengths compared and then utilized the longest sequence to represent these sequences in phylogenetic reconstructions. We used Bayesian approaches with MrBayes v3.2.7a (Ronquist *et al.*, 2012) to construct the tree. We also used MEGA11 (Stecher *et al.*, 2020; Tamura *et al.*, 2021) to evaluate different substitution models for our data and to estimate genetic distances among sequences with the best model determined.

We compared levels of sequence divergence of the sequences we recovered with levels of intra- and interspecific variation of sequences of polydorid sequences from GenBank. In particular, we calculated maximum genetic distances among members of species for which 10 or more sequences were available from GenBank and minimum distances between sequences of these species and their inferred sister species.

Morphology

We examined polydorid specimens under a microscope to evaluate morphological characteristics of specimens. We also compared their morphologies to features of polydorid species with sequences that are the best match to the sequences we recovered to infer whether they possess features that distinguish this species from other polydorids.

Results

Samples and microscopic examinations

We observed polydorid specimens within 17 fecal samples of five *Conus* species from Rangiroa, *C. chaldaeus* ($n = 4$), *C. ebraeus* ($n = 5$), *C. lividus* ($n = 2$), *C. rattus* ($n = 3$), and *C. sanguinolentus* ($n = 3$), although two of the three fecal samples from *C. sanguinolentus* are from the same individual (Table 1). We also observed polydorids in fecal samples of a specimen of *C. miliaris* from Makemo and one of *C. lividus* from Rurutu (Table 1). While the shells of *Conus* specimens from Rangiroa and Makemo (for which polydorids were obtained) were heavily encrusted with coralline algae, the specimen from Rurutu had only a small encrustation on its shell.

DNA analyses

We obtained sequences of a region of the mitochondrial *16S* gene from 138 polydorid individuals that were present in fecal samples (Table 1; GenBank accession nos. PX281698–PX281835). We obtained only short fragments from a few of the individuals, owing to difficulties with sequencing. Overall, sequences ranged in size from 185 to 314 bp (average size: 303.7 bp), including 20 sequences with lengths less than 300 bp.

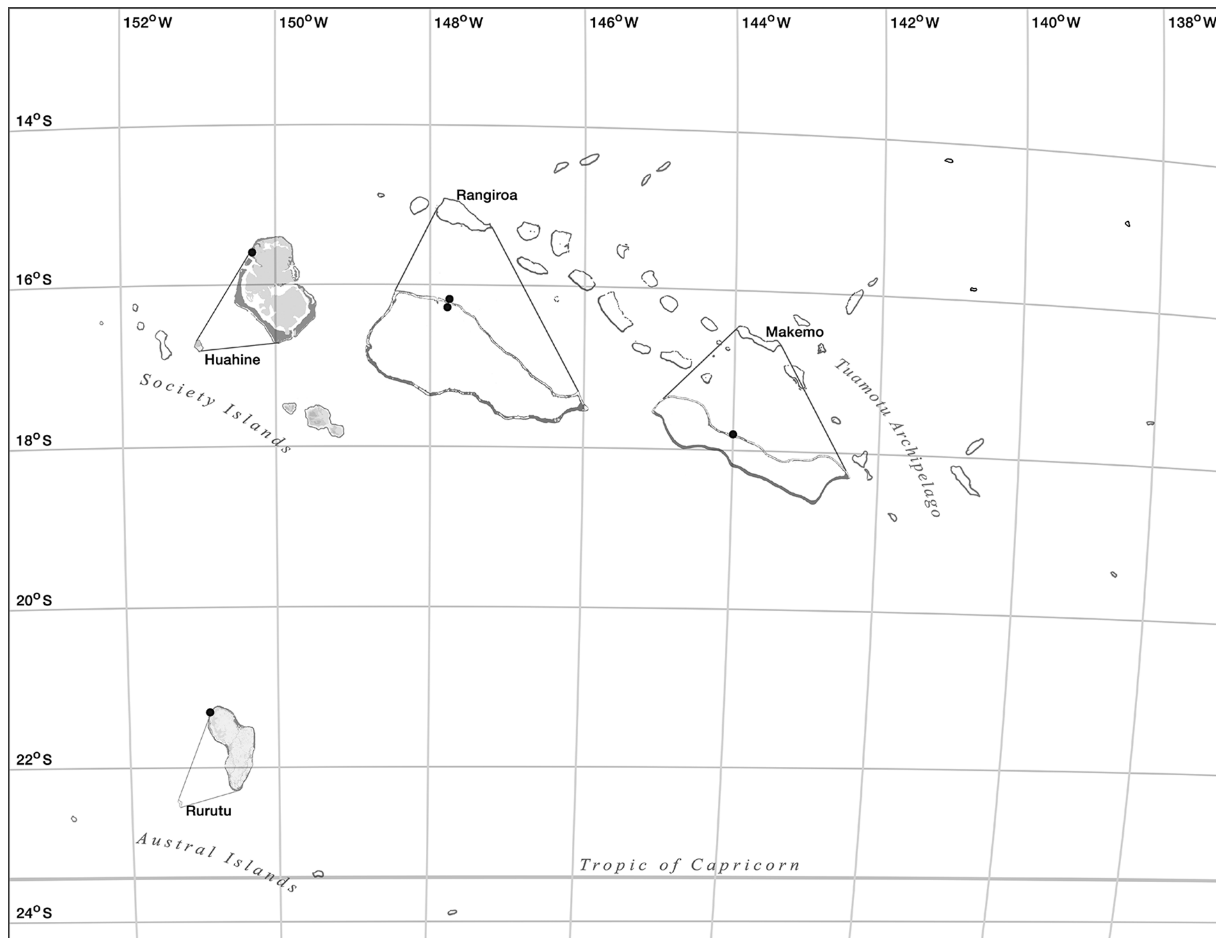

Figure 2. Map of collecting sites at French Polynesia. Dots indicate approximate locations of collecting sites in inset maps of islands and atolls. Coordinates of locations: 14°56′54.5″ S, 147°40′28.3″ W (Rangiroa, ocean side); 14°57′13.9″ S, 147°40′18.4″ W (Rangiroa, lagoon side); 16°33′12.0″ S, 143°44′30.0″ W (Makemo); 16°42′27.0″ S, 151°02′31.0″ W (Huahine); 22°26′28.1″ S, 151°22′36.1″ W (Rurutu). Illustration by John Megahan.

A GenBank sequence of *Dipolydora armata* (GenBank accession no. LC595729) shows greatest identity to our sequences (>95%) based on BLAST searches; other GenBank sequences exhibited less than 84% identity to our sequences. We also downloaded sequences of six polydorid species for which 10 or more sequences are reported in GenBank, including *Boccardia proboscidea* ($N = 36$), *Dipolydora bidentata* ($N = 15$), *Dipolydora giardi* ($N = 31$), *Polydora hoplura* ($N = 31$), *Polydora websteri* ($N = 17$), and *Pseudopolydora paucibranchiata* ($N = 16$). In addition, we downloaded GenBank sequences of additional species of polydorids that show close affinity to these six species based on BLAST searches.

We aligned the downloaded GenBank sequences with our recovered sequences, trimmed the GenBank sequences to be the same length as the sequences we obtained, and determined the best substitution model (Tamura three-parameter model [Tamura, 1992], with gamma distribution and gamma shape parameter of 0.22). We constructed a tree with our recovered sequences, the GenBank sequence of *D. armata*, and a GenBank sequence of *B. proboscidea* (LC595721) using Bayesian methods with 1,000,000 generations and the GTR substitution model (mean standard deviation of split frequencies after run: 0.003125) (Fig. 3). We utilized the *B. proboscidea* sequence to root the tree, as sequences of this species are among those of various *Boccardia* species that occur close to the sequence of *D. armata* in a recent spionid phylogeny (Abe and Sato-Okoshi, 2021).

Most of the sequences we obtained occur in three distinct clades in the tree (Fig. 3, clades A, B, D); among these clades, sequences differ at a minimum of 13 sites and Tamura three-parameter distance of 0.0521 and up to 14 sites and Tamura three-parameter distance of 0.0583. For the most part, sequences occurring in each clade are identical, but two sets of sequences show one base difference from other sequences in their clades (*i.e.*, rattus TR280 in clade A and lividus TR297 in clade B; Fig. 3). A single sequence from *C. rattus* (rattus TR538) and *C. lividus* (lividus AR038) did not occur in any of these clades and differ from other sequences at a minimum of five and nine sites and Tamura three-parameter distance of 0.0174 and 0.0329, respectively, and a maximum of 15 sites and Tamura three-parameter distance of 0.0650. Although they are represented by only a single sequence in

Table 1

Fecal samples from *Conus* species containing polydorid

Sample	Species	Location	UMMZ	*n*	Clade(s)
TR281	*C. chaldaeus*	Rangiroa, lagoon	311513	11	D
TR283	*C. chaldaeus*	Rangiroa, lagoon	311512	1	D
TR513	*C. chaldaeus*	Rangiroa, lagoon	311643	12	D
TR543	*C. chaldaeus*	Rangiroa, lagoon	311726	9	B
TR027	*C. ebraeus*	Rangiroa, lagoon	311279	9	A
TR029	*C. ebraeus*	Rangiroa, lagoon	311281	12	D
TR030	*C. ebraeus*	Rangiroa, lagoon	311284	8	B, D
TR047	*C. ebraeus*	Rangiroa, lagoon	311262	1	B
TR502	*C. ebraeus*	Rangiroa, lagoon	311627	11	A
AR038	*C. lividus*	Rurutu	310491	2	E
TR297	*C. lividus*	Rangiroa, lagoon	311497	8	B, D
TR369	*C. lividus*	Rangiroa, ocean	311606	10	B
TM197	*C. miliaris*	Makemo, ocean	310970	1	B
TR280	*C. rattus*	Rangiroa, lagoon	311496	11	A, B
TR538	*C. rattus*	Rangiroa, lagoon	311718	1	C
TR539	*C. rattus*	Rangiroa, lagoon	311725	2	A
TR398	*C. sanguinolentus*	Rangiroa, lagoon	311636	10	B
TR399	*C. sanguinolentus*	Rangiroa, lagoon	311638	9	A
TR519	*C. sanguinolentus*	Rangiroa, lagoon	311636	10	B

University of Michigan Museum of Zoology (UMMZ) catalog numbers of specimens for which fecal samples were taken, location of specimen, number of polydorid sequences (*n*) obtained, and clade membership of sequences.

the tree, we refer to these sequences as clades C and E in the following text.

The GenBank sequence of *D. armata* clusters outside of clades A–D in the tree, albeit with low support (Fig. 3). This sequence differs from sequences of clade A at a minimum of 12 sites (Tamura three-parameter distance; 0.0486), clade B at a minimum of 12 sites (Tamura three-parameter distance: 0.0462), clade C at 10 sites (Tamura three-parameter distance: 0.0372), clade D at 10 sites (Tamura three-parameter distance: 0.0381), and clade E at eight sites (Tamura three-parameter distance: 0.0303).

We recovered sequences of multiple polydorids from 14 cone snail specimens (Table 1). For all but three of these individuals, the sequences from the same specimen were identical, including all 20 sequences obtained from two fecal samples of an individual of *C. sanguinolentus* (Table 1; Fig. 3). The exceptions include single specimens of *C. ebraeus* (ebraeus_TR030), *C. lividus* (lividus_297), and *C. rattus* (rattus_TR280), in which one sequence was distinct and identical to sequences of polydorids obtained from other individuals (Fig. 3).

For the five *Conus* species for which we recovered polydorid sequences from multiple specimens, the sequences from different individuals occur in more than one clade (Table 1; Fig. 3). In addition, aside from the two clades that are represented by polydorids obtained from single specimens (*i.e.*, C and E), the other three clades (A, B, and D) include polydorids from the coralline algae encrustations of different *Conus* species, although not all clades have polydorids from all species examined.

Most of the sequences that we recovered are from fecal samples from a single site in the lagoon at Rangiroa (*i.e.*, the only lagoon site we sampled); we observed polydorids only in three fecal samples from other locations (Table 1). Polydorid sequences obtained from a specimen of *C. lividus* from the ocean side of the atoll of Rangiroa (lividus_TR369) and *C. miliaris* from the ocean side of the atoll of Makemo (miliaris TM197) are identical to sequences occurring in clade E (Fig. 3). Nonetheless, the two polydorid sequences obtained from a specimen of *C. lividus* from Rurutu are distinct from all other polydorid sequences recovered (Fig. 3) and differ at a minimum of seven sites and Tamura three-parameter distance of 0.0248 from these other sequences.

We evaluated levels of intra- and interspecific divergence among *16S* sequences of *B. proboscidea* ($n = 36$), *D. bidentata* ($n = 15$), *D. giardi* ($n = 31$), *P. hoplura* ($n = 31$), *P. websteri* ($n = 17$), and *P. paucibranchiata* ($n = 16$) and top BLAST matches of these sequences (all recovered from GenBank). Sequences of each of these species differed at between five (for *D. bidentata*) and 14 (for *D. giardi*) sites and exhibited the largest Tamura three-parameter distances of between 0.0210 (for *D. bidentata*) and 0.0627 (for *D. giardi*). We observed the lowest level of interspecific divergence among sequences of *P. websteri* and *P. haswelli*, which differed at as few as 15 sites with a Tamura three-parameter distance of 0.0600. As reported above, some of the polydorid clades we recovered differ at up to 15 sites and a Tamura three-parameter distance of 0.0650.

Morphology

We did not observe any clear morphological differences among the polydorid specimens we examined. These specimens possess several of the diagnostic morphological features of *D. armata* (Blake, 1996; Radashevsky and Nogueira,

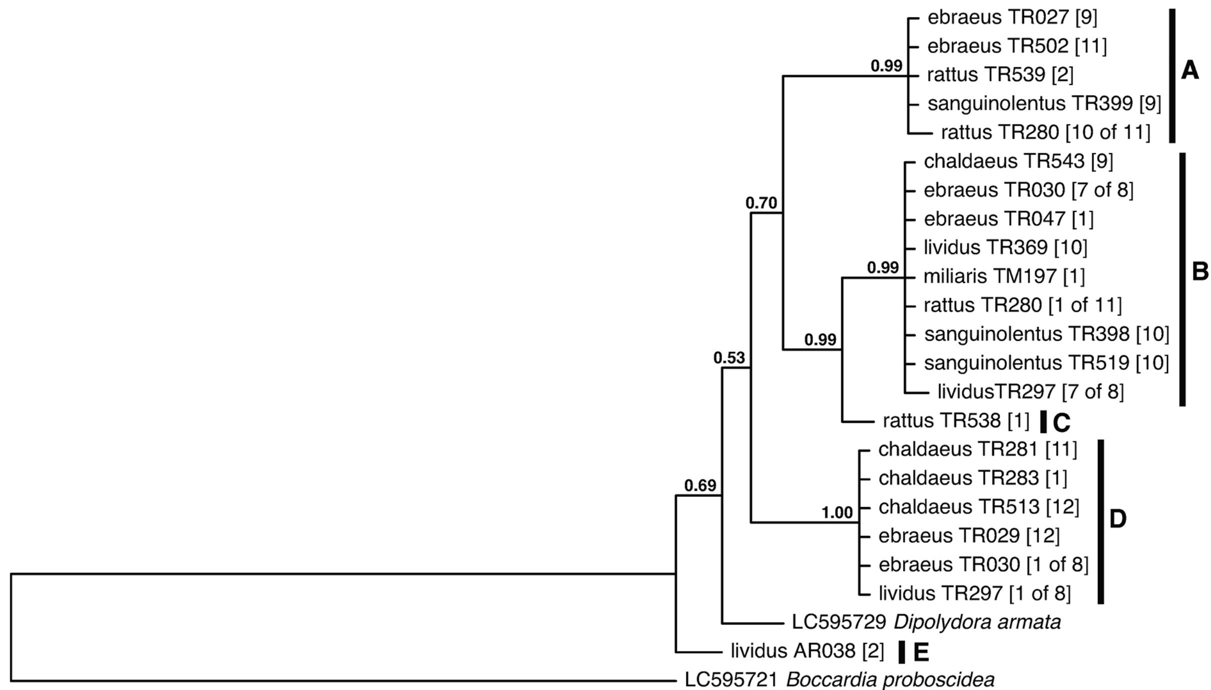

Figure 3. Phylogeny produced using Bayesian approaches based on analyses of sequences of a region of the mitochondrial *16S* gene of polydorids. Letter codes and bars are used to identify unique tip clades or sequences. Names of sequences include the specific epithet of the *Conus* species from which the sample of polydorid was obtained and the code of the fecal sample; names are followed by numbers in brackets with the number of identical sequences observed from that sample; samples for which all sequences were not identical are indicated by the fraction of sequences that were represented by the particular sequence (*e.g.*, 7 of 8). GenBank sequences of *Dipolydora armata* and *Boccardia proboscidea* are also included, and sequence names include their GenBank accession numbers; the latter sequence was used to root the tree. Posterior probabilities are reported at internal nodes.

2003) (Fig. 4), including bidentate spines on setiger five (Fig. 4B, C) and a cup or cuff-shaped pygidium (Fig. 4D).

Discussion

Our results reveal that the polydorids inhabiting the coralline algal encrustations of *Conus* species from French Polynesia represent a genetically diverse set of individuals. The sequences we recovered show closest affinity with a GenBank sequence of *Dipolydora armata* (Fig. 3); other GenBank sequences of polydorids are quite divergent from these sequences. The specimens also possess diagnostic morphological features of *D. armata* (Fig. 4). In addition, the identity of sequences of multiple polydorids from the same cone snail specimen implies that polydorid communities in coralline algae present on *Conus* shells are genetically homogeneous. Finally, the different clades of polydorids that we detected do not exhibit any association with particular species of cone snails.

Clades—distinct species or intraspecific variation?

Overall, we observed seven unique haplotypes among the 138 polydorid sequences we obtained, including one that was uniquely observed at Rurutu (*i.e.*, lividus_AR038; Fig. 3). Given that two of these unique haplotypes show only one base difference from other sequences, we assigned these seven unique haplotypes to five clades that represent either separate, though closely related, cryptic species or unique haplotypes of a single species. Other annelids show high levels of genetic diversity at mitochondrial gene regions and little morphological differences, so it is often not clear whether distinct clades of sequences or unique haplotypes represent different species (*e.g.*, *Palola*) (Schulze, 2006, 2015). In some cases, other polydorid species show levels of intra- and interspecific diversity at *16S* that are lower than levels of divergence among some of these haplotypes. Other polydorids, however, including *Polydora hoplura*, exhibit high levels of diversity at mitochondrial sequences (Williams *et al.*, 2016). Given that six of the seven unique haplotypes were all detected from the same lagoon site at Rangiroa, we suspect that these haplotypes represent intraspecific variation of a single species.

Dipolydora armata *(Langerhans, 1880)*

The close affinity of our sequences to a GenBank sequence of *D. armata* and the similar morphologies of the specimens we examined and *D. armata* suggest that the polydorids recovered from French Polynesia are this species or a close, morphologically similar relative. The GenBank sequence of *D. armata* was originally determined from a specimen from Izu-Oshima Island, Japan (Abe and Sato-Okoshi, 2021), that, as reported in the GenBank accession record, occurred on a shell of the disk abalone, *Haliotis discus*. In a previous molecular phylogenetic analysis of spionids that utilized this sequence, *Dipolydora* is not monophyletic—while

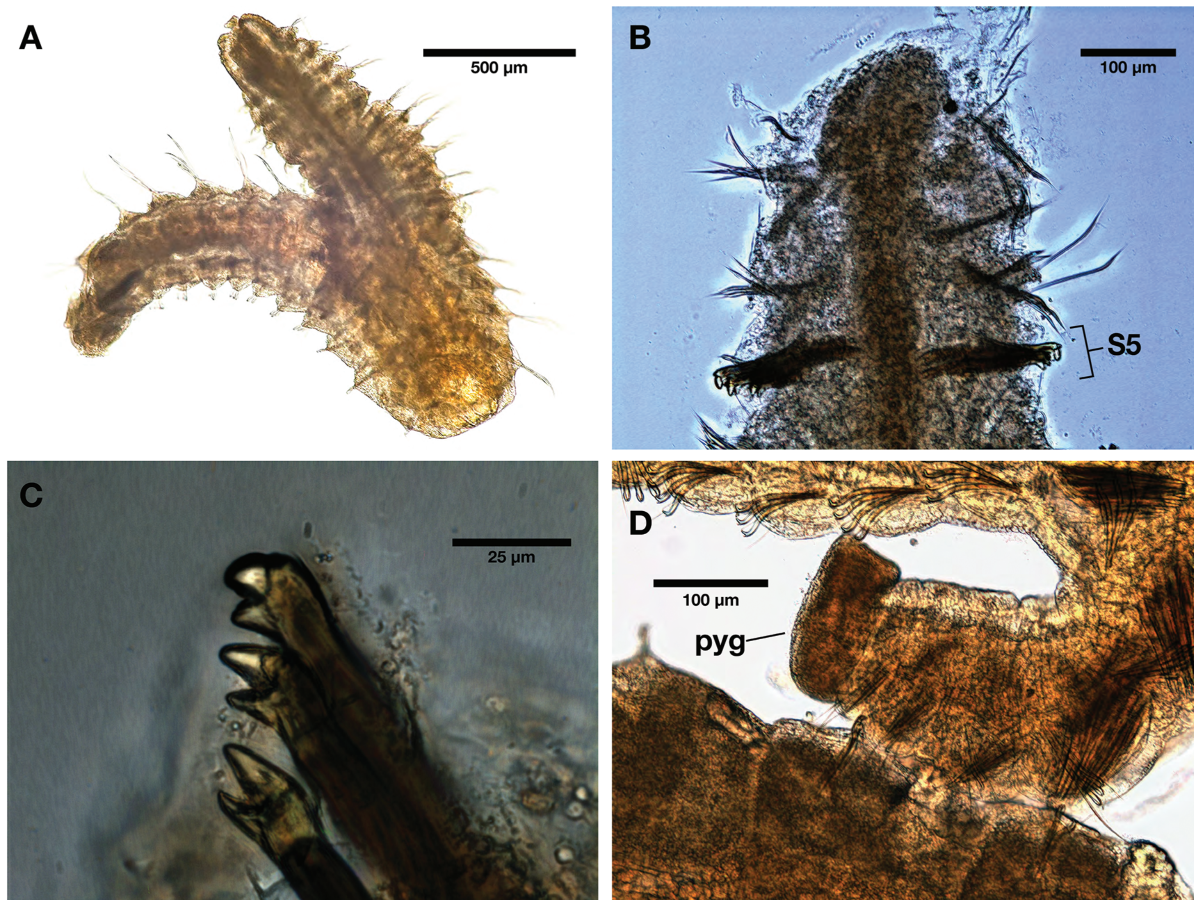

Figure 4. Images of polydorid specimens recovered from fecal samples of cone snails. (A) Entire polydorid. (B) Anterior region. (C) Bidentate modified spines of setiger five. (D) Posterior regions illustrating the cup/cuff-like pygidium. pyg, pygidium; S5, setiger five.

sequences of several *Dipolydora* species occur together in a single clade, the sequence of *D. armata* clusters quite far from this clade in a clade with sequences of various *Boccardia* species (Abe and Sato-Okoshi, 2021). *Dipolydora armata* has a worldwide distribution in tropical and subtropical oceans (Radashevsky and Nogueira, 2003), including Hawaii (Ward, 1981) and the Marshall Islands (Hartman, 1954; Woodwick, 1964), although Radashevsky and Nogueira (2003) caution that it may be composed of a number of cryptic species. This species bores into a variety of substrates, including coralline algae, stony coral, hydrocorals, coral rubble, bivalve and gastropod shells, barnacle, and the coralline algae encrustations of shells of live gastropods and gastropod shells occupied by hermit crabs (Blake and Kudenov, 1978; Blake, 1996; Sato-Okoshi, 1999; Radashevsky and Nogueira, 2003).

Polydorid communities in encrustations of cone snail shells

The high degree of sequence homogeneity of polydorids recovered from individual cone snail specimens is surprising given the overall diversity of haplotypes that we observed from a single site within the lagoon at Rangiroa. Indeed, the polydorid communities that inhabit the coralline algae encrustations on single snails appear to be composed largely of individuals with the same *16S* haplotype and not a random selection of haplotypes. In addition, two unique haplotypes (*i.e.*, those represented by lividus_TR297 and rattus_TR280) that differed from a more common haplotype (*i.e.*, other members of clade E and A, respectively) at just one site were exclusively observed from single *Conus* specimens. Moreover, for the three *Conus* specimens for which two haplotypes were observed (*i.e.*, ebraeus_TR30, lividus_TR297, and rattus_TR280), the majority of the sequences were of the same haplotype.

These results imply that one or a few larvae settle on an appropriate substrate (*i.e.*, the coralline algal encrustations of a gastropod shell) and then reproduce asexually to establish a local population. Alternatively, once larvae with a particular haplotype settle, larvae with other haplotypes cannot colonize successfully. The former hypothesis seems more likely to explain our observations, especially given that spionids are known to reproduce asexually through

architomy, in which new individuals develop from fragments of an original single individual (Blake, 1996; Gibson and Harvey, 2000), including *D. armata* (Radashevsky and Nogueira, 2003).

Polydorids and cones snail species

Conus species for which multiple individuals were examined hosted polydorids from multiple haplotype clades. Although the cone snails provide a substrate for the coralline algae only where the polydorids reside, presence of multiple haplotypes on particular species suggests a lack of association with the encrustations on particular *Conus* species, which aligns well with previous studies showing that many polydorid species exhibit broad host ranges (Radashevsky and Nogueira, 2003; Simon, 2011; Martin and Britayev, 2018; Radashevsky *et al.*, 2023). Although some clades were not detected from select *Conus* species, we likely had too small of sample sizes of individual species for adequately addressing any specificity between polydorid clades and cone snail species.

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Data Accessibility

Voucher specimens are deposited in the University of Michigan Museum of Zoology Mollusk Division. Sequences are deposited in GenBank (accession nos. PX281698–PX281835).

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