Coral-infecting parasites in cold marine ecosystems

Highlights
- Coral parasites are found in cold-water ecosystems
- They infect animals related to corals, such as anemones and corallimorphs
- Biogeography is a major factor in parasite evolution
- Current strategies for sampling microbial diversity overlook important parasites

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In brief
Coralicolids are coral parasites that were thought to be restricted to coral reef ecosystems. Trznadel et al. show that they are widely distributed, infecting related hosts, such as anemones, in cold marine environments. This shows that coralicolids are globally relevant parasites and raises questions about biases in microbial diversity surveys.
Coral reefs are a biodiversity hotspot,1,2 and the association between coral and intracellular dinoflagellates is a model for endosymbiosis.3,4 Recently, corals and related anthozoans have also been found to harbor another kind of endosymbiont, apicomplexans called corallicolids.5 Apicomplexans are a diverse lineage of obligate intracellular parasites6 that include human pathogens such as the malaria parasite, Plasmodium.7 Global environmental sequencing shows corallicolids are tightly associated with tropical and subtropical reef environments,5,8,9 where they infect diverse corals across a range of depths in many reef systems, and correlate with host mortality during bleaching events.10 All of this points to corallicolids being ecologically significant to coral reefs, but it is also possible they are even more widely distributed because most environmental sampling is biased against parasites that maintain a tight association with their hosts throughout their life cycle. We tested the global distribution of corallicolids using a more direct approach, by specifically targeting potential anthozoan host animals from cold/temperate marine waters outside the coral reef context. We found that corallicolids are in fact common in such hosts, in some cases at high frequency, and that they infect the same tissue as parasites from topical coral reefs. Parasite phylogeny suggests corallicolids move between hosts and habitats relatively frequently, but that biogeography is more conserved. Overall, these results greatly expand the range of corallicolids beyond coral reefs, suggesting they are globally distributed parasites of marine anthozoans, which also illustrates significant blind spots that result from strategies commonly used to sample microbial biodiversity.

RESULTS AND DISCUSSION

Corallicolids commonly infect cold-water anthozoan hosts
Analyses of environmental data have consistently shown corallicolids are restricted to coral reef environments.5,9,11 Although this correlation is striking, it may be misleading because the vast majority of marine sequence data currently comes from the water column and, to a lesser extent, benthic substrates. Sampling these environments will successfully measure parasitic diversity only if the parasite broadcasts some life stages into the environment, as do, for instance, marine alveolate (MALV) dinoflagellates.12 However, many parasites, including many apicomplexans, have comparatively closed infection strategies—moving from one host to another with only limited exposure to the outside environment—and these will escape detection without direct sampling of their hosts.13,14 Corals attract a lot more attention than most invertebrates, so it is possible, despite the hundreds of millions of data points suggesting the opposite,9 that the corallicolids have a much wider geographical distribution than presently appreciated. Indeed, the discovery of corallicolids in subtropical Anthozoa sampled from deep water off coral reef ecosystems9 suggests they can survive in cold temperatures. But the only direct information on their occurrence in plausible hosts from ecosystems outside tropical or subtropical coral reef environments is a comprehensive biogeographical examination of the temperate sea anemone, Anthopleura elegansissima, whose bacterial microbiome yielded no indication of corallolid plastids across the Northeast Pacific.15 Though this is consistent with the conclusion that corallicolids are restricted to tropical and subtropical coral reefs, it is also based on a single species.

To more systematically test whether corallicolids are more widespread geographically, we collected 325 samples from nine species of cold-water anthozoans from non-coral reef environments at five locations in coastal British Columbia (BC). Collectively, this covers a diverse range of anthozoans from cold water in temperate marine environments, including stony corals, sea anemones, corallimorphs, zoanthids, octocorals, and tube-dwelling anemones (see Figure 1A for a summary of anthozoan groups). Samples were screened for corallolid infection using PCR and sequencing of nuclear small subunit rRNA (SSU rRNA). This confirmed the lack of corallicolids in Anthopleura elegansissima,15 but showed other cold-water anthozoans are in fact infected, some at high frequency. The anemone Metridium senile appeared to have the highest rates of infection,
with 77.8% of individuals testing positive (n = 36), followed by the corallimorph *Corynactis californica*, in which 68.7% of tested individuals were infected (n = 32). The anemones *Anthopleura artemisia* and *Urticina grebelnyi* were also infected, but at lower rates (Table S1). Corallicolids were not detected or detected at very low frequencies in only a couple of individuals in *Pachycerianthus* sp. and *Urticina clandestinea*, respectively (Table S1). To corroborate these findings, deep sequencing of the eukaryotic microbiome was carried out for representative positive- and negative-testing species. This confirmed the presence of numerous genetically distinct corallicolids in a variety of cold-water anemones (Figure 1B) and suggested that even in species where infection frequency is high (e.g., *M. senile*), the parasite load within a given host is likely low (Figure S1). The bacterial microbiome was also sequenced, but sequences corresponding to corallicolid plastid SSU rRNA in BC samples were not observed, although we could detect them in anemones from tropical coral reefs. This may be because the copy number of plastid SSU is lower, as in other corallicolid plastid SSU rRNA in BC samples were not observed, although we could detect them in anemones from tropical coral reefs. This may be because the copy number of plastid SSU is lower, as in other corallicolids, or because of an amplification bias.

To directly observe the presence and frequency of cold-water corallicolids in their host, we examined the two hosts whether the phylogenetic separation is genuine, and to better understand the possible explanations—providing that the phylogeny based on short sequence reads is even a correct representation of the data. For example, coral reef sampling is strongly biased toward stony corals and locations in the Atlantic, whereas cold-water hosts are biased toward anemones and to the Pacific. To test whether the phylogenetic separation is genuine, and to better distinguish between potential drivers behind this separation, we characterized full-length SSU rRNA genes from communities of corallicolids in *M. senile* and *C. californica*. To better address the possibility that host taxonomy was a factor, we also sampled five species of anemone from the tropical coral reef environment, identified two species of *Edwardsiella* anemones from Curacao (Figures 2C and 2D) with high infection frequencies, and

**What drives the structure of corallicolid diversity?**

Environmental sequences are too short to yield a well-supported phylogeny, but we nevertheless noted an obvious tendency for amplicon sequence variants (ASVs) from temperate anemone parasites to cluster together to the exclusion of parasites from coral reefs in Figure 1 (note the clustering of blue- and red-shaded boxes). Though this may at first glance appear to suggest that the temperature of the habitat is an important factor in corallicolid ecology or evolution, there are actually several possible explanations—providing that the phylogeny based on short sequence reads is even a correct representation of the data. For example, coral reef sampling is strongly biased toward stony corals and locations in the Atlantic, whereas cold-water hosts are biased toward anemones and to the Pacific. To test whether the phylogenetic separation is genuine, and to better distinguish between potential drivers behind this separation, we characterized full-length SSU rRNA genes from communities of corallicolids in *M. senile* and *C. californica*. To better address the possibility that host taxonomy was a factor, we also sampled five species of anemone from the tropical coral reef environment, identified two species of *Edwardsiella* anemones from Curacao (Figures 2C and 2D) with high infection frequencies, and
characterized full-length SSU rRNA genes from their corallicolid communities.

The phylogenetic tree based on these longer sequences was inferred using ML and Bayesian methods, resulting in a tree with greater resolution and statistical support, but still representing parasites sampled from various hosts, climates, depths, and geographical zones and locations (Figure 3). Not every node of the tree is supported, and no factor correlates perfectly with the phylogeny, except at the shallowest level in the tree; at deeper levels they are scattered among several highly supported clades. This is not to say these criteria do not impact corallicolid diversity, but rather that their effects are likely most important over short evolutionary timescales; closely related hosts may have closely related parasites, but there is no “anemone clade”, and host-parasite co-evolution does not appear to be a major factor at a deep phylogenetic level. This is consistent with previous observations from the Gulf of Mexico and Caribbean, which found no strict clustering based on host species but noted associations of some corallicolids with hosts at lower taxonomic levels. Water depth and latitudinal zone tended to cluster somewhat more, but sampling across these criteria is also more biased as there are relatively few samples from greater depth and higher latitudinal zones and they tend to be co-biased with location and host identity, making these promising factors for further study. The factor that currently correlates most strongly with ancient branches in the tree is geographical location. All the deepest-branching and best-supported clades in the phylogeny (e.g., every clade supported over 85%) include only samples either from the Atlantic or the Pacific Oceans. There are more weakly supported groups in both Figures 1 and 3 that include both Atlantic and Pacific samples, so parasites may spread at some low frequency; nevertheless it does seem that geography plays

Figure 2. Cold-water corallicolids and hosts
(A–D) Host species that were chosen for detailed observations as they appear in their natural habitat: (A) the white plumose anemone, Metridium senile; (B) and (C) two undescribed members of the genus Edwardsiella, both small, transparent sand-dwelling anemones from coral reef environments (both pictured at night using blue light to excite their fluorescence, as this is how they were found and sampled in nature); and (D) the corallimorph confusingly known as the strawberry anemone, Corynactis californica.
(E and F) Two specific individuals of C. californica that tested positive for corallicolids and were analyzed by microscopy, shown to the right.
(G–I) Fluorescence in situ hybridization showing corallicolids in mesenterial filaments of C. californica. In each panel on the left is a bright-field (BF) image of fixed tissue with the same fields shown hybridized to probes for corallicolid plastid (center: ARL-V 16S) and nuclear SSU rRNA (right: Type-N 18S). See Figure S2 for controls. Each panel shows infection at different magnifications: (G) low magnification image of a whole mesenterial filament with two infection foci; (H) shows a close up of several foci in a mesenterial filament; and (I) shows a high magnification of two corallicolid cells in a membrane, which we interpret to be intracellular stages released from their host cell during fixation. All samples were pre-incubated with blocking primers to reduce non-specific hybridization to nematocysts (see Figure S2).
Figure 3. Biogeography and corallicolid diversity
Maximum likelihood phylogeny of all available SSU rRNA genes from corallicolids greater than 800 base pairs (bp) in length (with two exceptions being slightly shorter sequences generated in this study—Corallicolid sp. ex Metridium senile no. 69 and Corallicolid sp. ex Metridium senile no. 70; see STAR Methods and (legend continued on next page)
major role in structuring the diversity of corallicolids over long evolutionary timescales.

These observations lead to interesting questions about parasites from other regions. For example, consider the range of *M. senile* (or something very closely related to it, as the species-level taxonomy is in flux), which extends from the North Pacific to the North Atlantic. If corallicolids infect Atlantic *M. senile*, are they more closely related to Pacific corallicolids from the same host, or alternatively more similar to tropical Atlantic corallicolids, as the phylogenetic pattern would currently suggest? Corallicolids from other areas, such as the West Pacific, Mediterranean, Red Sea, or Indian Ocean, might also help reveal larger biogeographical patterns underlying the spread of these parasites around the world’s oceans.

**Implications for microbial biodiversity sampling**

The discovery of corallicolids in coral hosts led to them being called a “third player in the game” of coral symbiosis. Indeed, their tight association with coral tissue, observed directly and also in the striking correlation between the presence of corallicolid sequences and coral reef environments, further reinforced this. However, here we show that, despite the overwhelming signal for this correlation in environmental data, corallicolids are not restricted to tropical reef regions, but are likely globally distributed parasites of diverse anthozoans in all their marine habitats. This example has implications for the way we sample, measure, and interpret environmental diversity, because it is clearly missing an important and potentially massive fraction of that diversity. Parasites are known to be both hugely diverse and very important in structuring natural communities, including both animal and protist hosts. Some of these are already appreciated to be important and major components of marine diversity. For example, marine alveolates (MALVs) are two lineages that typically account for as much as 60% of sequences in surveys of marine eukaryotic diversity, and they are so well represented because they broadcast huge numbers of infectious cells into the water column as a key part of their parasitic strategy. Our direct observations also support this, because MALVs were one of the most prevalent classes in the anemone microbiomes (60.5% of all samples) and showed the highest average relative abundance across all samples (21%).

But we also know that other important parasitic lineages remain in a tight physical association with their host or hosts throughout their life cycle, and indeed it appears that corallicolids likely fall into this camp. The importance of these on land is well documented (e.g., consider malaria, which is abundant in many parts of the world and virtually never found outside a host), but in the ocean these continue to fly under our radar, probably because of sampling bias. Indeed, corallicolids were only discovered because of an unrelated interest in the bacteria associated with coral. To see this kind of diversity more clearly, we need to change the way we define “the environment” to include the bodies of animals and other potential hosts within the larger environment, because they are a reservoir of an interesting and ecologically significant component of biodiversity that is not represented anywhere else. When we do so, we anticipate that a number of new or understudied parasitic and host-associated groups will turn out to be diverse, widespread, and ecologically significant, despite currently being totally invisible.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2024.03.026.

**ACKNOWLEDGMENTS**

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**AUTHOR CONTRIBUTIONS**

The study was conceived by P.J.K. and M.T., collections were done by M.T. and C.C.H., data analysis done by M.T. and S.J.L., project supervision was provided by W.K.K. and P.J.K., and funding acquired by P.J.K.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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Table S3. New lineages characterized in this study are highlighted by gray boxes. Bootstrap and posterior probability support above 80% or 0.8 are indicated at each node. To the right, four variables are indicated by color-coded boxes: host order, latitudinal zone, sampling depth, and geographical location. Clades supported at 85% or greater consistently contain sequences from the same ocean. Hosts acquired from the aquarium trade branched in a position consistent with their expected geographical origin, but because their exposure to other potential hosts since their collection cannot be ruled out, variables other than the host are left blank. Additional trees based on Bayesian analyses or with additional short sequences are available in Figure S3.

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See also Table S3.
REFERENCES


**STAR METHODS**

**KEY RESOURCES TABLE**

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RESOURCES AVAILABILITY

Lead contact
Requests for further information, resources, and reagents can be directed to the lead contact Patrick Keeling (pkeeling@mail.ubc.ca).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- New 18S SSU genes characterized in this study have been deposited to GenBank and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. Microbiome raw reads from anthozoan hosts have been deposited in the NCBI Short Read Archive (SRA) under BioProject number PRJNA1048503, publicly available as of the date of publication.
- This paper does not report any original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Samples were collected at five locations along the coast of British Columbia, Canada, and one site in Curacao (Table S1). The anthozoans were collected by carefully removing them from the substrate to minimize stress and stored in seawater with bubblers while in transit to the laboratory. For a subset of samples, anthozoans were dissected by tissue type (mesenterial filaments, tentacles, dermis, acontia), otherwise the whole polyp was dissected, fixed in 100% ethanol, and stored at room temperature until DNA extraction. DNA extraction was then conducted according to the DNeasy PowerBiofilm Kit protocol, and DNA concentration (ng/μL) was measured using the Qubit 1X dsDNA High Sensitivity (HS) Assay Kit.

METHOD DETAILS

Screening of corallicolid infection
To determine whether sampled anthozoans were positively infected, corallicolid specific primers were designed (18N_F_wk, 18N_R_wk) from cold-water corallicolid amplicon reads that target the V4 region of the 18S rRNA gene obtained from Illumina short-read amplicon sequencing. PCR reactions and thermocycling conditions were conducted using the standard Phusion High-Fidelity PCR Master Mix with HF Buffer protocol (New England BioLabs), modified for a 40 μL reaction: 2 μL of 10 μM Forward Primer, 2 μL Reverse Primer, 20 μL 2X Phusion Master Mix, 12 μL Nuclease-free water, and 12 μL Template DNA. When possible, DNA was diluted to achieve a concentration of 25 ng/μL. UltraPure Distilled Water (Invitrogen) was used as a negative control. PCR products were mixed with 6X GelRed Prestain Loading Buffer with Orange Tracking Dye (Biotium) as per product instruction and visualized (expected band size ~212 bp) using gel electrophoresis with 1% agarose gels.

Microbiome sequencing and phylogenetic analysis
Libraries for eukaryotic microbiome amplicon sequencing were prepared using metazoan-excluding primers which targeted the V4 region of the 18S rRNA gene (UnonMetaF, UnonMetaR). The resulting product was dyed with 6X GelRed Prestain Loading Buffer with Orange Tracking Dye (Biotium) as per product instruction and visualized (expected band size ~212 bp) using gel electrophoresis with 1% agarose gels.
libraries were prepared using prokaryotic-specific “fusion primers” (containing Illumina adaptors and indices) that target the V4 region of the 16S rRNA gene (515FB, 806RB). PCR products from pooled dilutions were then cleaned using the Charm Biotech Just-a-Plate 96-well Normalization Kit before sequencing on an Illumina Miseq using 300bp reads.

PCR primer and sequencing adapter sequences were removed from raw sequencing reads using Cutadapt (v.3.4) and processed in R (v.4.3) following the DADA2 pipeline (v.1.28). In brief, reads were filtered and trimmed using standard filtering parameters (maxN = 0, maxEE = c(2,2), truncQ = 2) before generating run-specific error models for both forward and reverse reads to estimate their respective error rates. The DADA2 sample inference algorithm was run with “pseudo” pooling to increase sensitivity to low frequency sequence variants present in multiple samples. Chimeras were removed from merged reads using the “consensus” method before assigning taxonomy using the naive Bayesian classifier and the PR2 database. The final OTU table was combined with PR2 taxonomic assignments and sample metadata using the phyloseq package (v.1.44.0).

Corallicolid sequences over 0.001 relative abundance (> 0.1%) were identified by aligning ASVs assigned as apicomplexans against full-length reference sequences using the EINS-I MAFFT (v. 7.48) alignment algorithm. A Maximum Likelihood (ML) phylogeny was inferred with IQ-TREE (v.1.6.12) using Model Finder to choose the optimum substitution model. Sequence variants clustering outside of the corallicolid clade were discarded.

To compare anemone ASVs with those from coral, published eukaryotic sequencing surveys of coral microbiomes were identified on GenBank. Data from 12 studies were obtained (Table S2) and processed as above, combining all datasets after independent processing with the DADA2 pipeline. All ASVs above 0.01% in at least one sample and assigned to the family Corallicolidae were extracted, resulting in the inclusion of four studies. Including ASVs assigned to the Order Eimeriida did not reveal additional corallicolid sequences (determined phylogenetically).

A final ML phylogeny with corallicolid ASVs from both anemones and coral was generated as above but using the MAFFT-XINSI algorithm, which uses MXSCARNA to assess secondary structure information. The resulting tree file was imported to R using the phyloseq package and re-rooted using the ape package (v.5.7.1). A cladogram was plotted using the ggTree (v.3.8.2) and ggTreeExtra (v.1.10.0) packages, adding accompanying host information as additional layers.

To visualize entire microbiomes, eukaryotic and prokaryotic ASVs were independently agglomerated to the Class and Order level (respectively), their read counts transformed to relative abundance, and filtered to only include those greater than 0.1% relative abundance in at least one sample. Only samples with more than 1000 reads were included. The resulting dataset was plotted as a heatmap using the ggplot2 package where fill color scales to abundance values.

**Full-length SSU sequencing and phylogenetic analysis**

Primer sets used previously to amplify the SSU of coral-infecting corallicolids resulted in the amplification of Actiniaria and other non-coral Anthozoa hosts, therefore, a series of primer sets were developed and tested: CCH_F, 18N_R_wkexA; 18N_F_wk, FAD4; and More_F1, FAD4 were used to amplify the majority of *M. senile*, and both Edwardsiella sp. The aforementioned primer sets were less successful in obtaining long and high-quality fragments from *C. californicus*. Thus, R-coralFront and F-coralEnd were designed, and used along with 18N-R1 (CCH_F, R-coralFront; F-coralEnd, FAD4; 18N_F_wk, 18N-R1) to fill in gaps of the *C. californicus* SSU sequence. PCR reactions and thermocycling conditions were conducted using the Phusion High-Fidelity PCR Master Mix with HF Buffer following the manufacturer's protocol. UltraPure Distilled water was used as a negative control and samples previously confirmed to contain corallicolids were used as a positive control. PCR products were visualized via gel electrophoresis as above and purified using the Monarch DNA Gel Extraction Kit before quantification. Products with sufficiently high yield were sent to the Sequencing + Bioinformatics Consortium at the University of British Columbia in BC, Canada for Sanger dyeoxy sequencing using the Applied Biosystems 3730S 48-capillary DNA Analyzer and BigDye Terminator v3.1 Sequencing Chemistry.

Sequence chromatograms were inspected, and low-quality ends were trimmed manually using Geneious Prime. The identity of trimmed sequences was confirmed via BLASTn searches of the NCBI megaBLAST nucleotide database. Fragments of the same host individual were then assembled using Geneious Prime de novo assembler. Assembled sequences with a minimum HQ of 85% (as defined by the default setting of Geneious Prime), and publicly available corallicolid sequences (Table S3) were filtered to select only those longer than 800bp, with the exception of two sequences generated in this study (659bp and 707bp), to be used for phylogenetic analysis. An alignment was then constructed with nine additional Apicomplexa sequences as the outgroup with MAFFT v. 7.481 using the MAFFT-XINSI algorithm. The alignment was then visually inspected with Geneious Prime, and miss-alignments were manually corrected. In cases of identical sequences isolated from both the same host species and location, the shorter sequence was removed before being trimmed with trimAl 1.2rev59 using a gap threshold of 0.4 and a similarity threshold of 0.001. The sequence MH304759 was divergent and removed. The trimmed alignment, containing 1582 positions, was then used to conduct a ML analysis using IQ-TREE 1.6.12. The ModelFinder feature was used to find the best-fit model as determined by IQ-TREE (TN + F + R2). To corroborate the resulting tree, PhyloBayes-MPI (v.4.0.3) was used to perform a Bayesian analysis on the same alignment using four parallel chains and the CAT+GTR+G4 model. A consensus tree (Figure S3A) was built after approximately 29,000 trees in each chain and reported a maxdiff value of 0.03.

As supplemental information two additional ML tree were also constructed. Both trees used the same parameters stated above, with the inclusion of all sequences over 659bp, and all identical sequences from the same host species and location were included in the subsequent alignment and ML analysis (Figure S3B); the second also contains the divergent sequence MH304759 (Figure S3C).
The ModelFinder feature was once again used to find the best-fit model as determined by IQ-TREE (HKY+F+R2 for both). For all ML iterations (including Figure 3) 1000 non-parametric bootstrap pseudoreplicates were performed.

**Fluorescence in situ hybridization (FISH)**

*M. senile* and *C. californicus* from Quadra Island, British Columbia were transported to UBC and maintained in a benchtop aquarium for several days (*M. senile*) or several weeks (*C. californicus*). Individuals selected for FISH were co-sampled for 18S identification and screened positive for corallicolids using 18N_F_WK and 18N_R_WK as PCR primers. 13 individuals of *C. californicus* were dissected in the sagittal plane to generate tissue sections that contained tentacles, body wall, and mesenterial filaments. Mesenterial filaments from 20 *M. senile* individuals were isolated with forceps into small tissue clumps (< 1 cm³). Tissues from both species were fixed with 4% paraformaldehyde in 0.6 M sea salts for 1 h at room temperature. Tissue sections were rinsed with sea salts and dehydrated in a graded ascending ethanol series (30%, 50%, 70%, 95%, 100% x3) for 15 min at each step. Tissues were cleared in 6% H₂O₂ and 70% EtOH (v/v) for 72 h on a rotary shaker (80 RPM) at room temperature. Samples were rehydrated in aqueous wash buffer (WB) containing 50 mM Tris-HCl and 900 mM NaCl for 15 min x2. Non-specific binding of fluorescent probes to nematocysts were quenched by incubating tissue sections with 0.1 μM EUB338-Fluorescein bacterial probe in hybridization buffer (WB + 0.1% v/v sodium dodecyl sulfate) for 3 h at 46 °C in a dark humidity chamber. Samples were then washed twice for 10 min in a probe-free hybridization buffer. Tissues were hybridized with 0.1 μM corallicolid-specific probes (WK16 and WK17 5 in hybridization buffer for 12 h at 46 °C in a dark humidity chamber. After washing the tissues with probe-free hybridization buffer and subsequently dH₂O, tissues were counterstained with 1 μM DAPI for 10 min at room temperature. Tissue sections were mounted in citifluor mounting solution with a #1.5 coverslip and sealed with paraffin wax.

Bright field and fluorescence imaging was performed with an Olympus BX53 microscope equipped with an Olympus DP80 digital camera and CellSence Standard software (Olympus). Standard filter sets for DAPI, eGFP (EUB338), TRITC (ARL-V 16S probe), and Cy5 (type-N 18S probe) were used to collect the fluorescence emission from each probe respectively. Image acquisition parameters (i.e., laser intensity and exposure time) were optimized for each objective lens to maximize the signal-to-noise ratio, then kept constant for the remainder of the imaging session.