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# Evidence for spatial niche partitioning in the ectocommensal *Symbion americanus* (Cycliophora) on its lobster host, *Homarus americanus* (Arthropoda, Malacostraca)

**Shoyo Sato** | **Allison Law** | **Gonzalo Giribet**

Museum of Comparative Zoology, Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts, USA

**Correspondence**
Gonzalo Giribet, Museum of Comparative Zoology, Department of Organismic and Evolutionary Biology, Harvard University, 26 Oxford Street, Cambridge, MA 02138, USA.
Email: ggiribet@g.harvard.edu

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## Abstract

*Symbion americanus* is a microscopic marine invertebrate in the phylum Cycliophora that lives as an ectocommensal on the mouthparts of the American lobster, *Homarus americanus*. Previous phylogeographic work on *S. americanus* identified three lineages corresponding to one described and two potential new species, along with evidence of sympatry. But these studies did not explore whether individuals of *S. americanus* from different genetic lineages segregate onto different host mouthparts. The present study examines the population structure and microhabitat of 196 individuals of *S. americanus* from lobsters from five North American localities (from Newfoundland, Canada, to Boston, MA) collected between June and September 2019. Specimens were sequenced at two mitochondrial DNA (mtDNA) markers, a 487 bp fragment of *cytochrome c oxidase subunit I* (*COI*) and a 481 bp fragment of *16S rRNA*. Phylogenetic analyses recover three distinct lineages of *Symbion americanus*, corroborating previous studies. Population genetic analyses of individuals belonging to the C and G lineages show clear population structure at the level of host mouthparts. Microhabitat data suggest the segregation of different genetic lineages in *S. americanus* onto different host mouthparts, perhaps indicating the role of spatial niche partitioning in the incipient speciation of *S. americanus*.

## Resumen

*Symbion americanus* es un invertebrado marino microscópico del filo Cycliophora, ectocomensal en las piezas bucales del bogavante americano, *Homarus gammarus*. Una serie de estudios filogeográficos previos identificaron tres linajes de *S. americanus* simpátricos, uno de los cuales se corresponde con la especie descrita, y los otros dos como potenciales nuevas especies. Pero dichos estudios no alcanzaron a evaluar si los tres linajes genéticos de *S. americanus* ocupaban las diferentes piezas bucales del hospedador. Nuestro estudio examina la estructura poblacional y el microhábitat de 196 individuos de *S. americanus* en bogavantes colectados en cinco localidades en Norteamérica (de Terranova, Canadá, hasta Boston, Massachusetts) centre junio y setiembre de 2019. Los ejemplares fueron secuenciados para dos marcadores mitocondriales, un fragmento de 487 pb de la

Shoyo Sato and Allison Law contributed equally to this work.

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*citocromo oxidasa* c *subunidad I* (*COI*) y un fragmento de 481 pb del gen ribosomal *16S rRNA*. Los análisis filogenéticos corroboran la presencia de tres linajes de *Symbion americanus* bien diferenciados, como se había sugerido en estudios previos. Los análisis de genética poblacional de individuos de los linajes C y G muestran una clara estructura espacial a nivel de las piezas bucales que habitan. Estos datos de microhábitat indican que hay segregación de los distintos linajes genéticos de *S. americanus* en las diferentes piezas bucales, sugiriendo que la división espacial puede constituir un nicho para la incipiente especiación de *S. americanus*.

**KEYWORDS**
biodiversity, phylogeography, population genetics, spatial niche partitioning

## 1 | INTRODUCTION

Cycliophorans are microscopic, obligate ectocommensals that live on the setae of lobster mouthparts (Funch & Kristensen, 1995, 1997; Funch & Neves, 2018; Neves, 2016). The life cycle of cycliophorans is extremely complex, with an alternation between an asexual feeding form attached to the lobster and a sexual form that gives rise to motile chordoid larvae. Cycliophorans have been found mostly on lobsters from the family Nephropidae (Obst et al., 2005), although a few individuals have been observed on copepods, which might be secondary hosts (Neves et al., 2014).

Most recent phylogenetic analyses support a clade composed of Cycliophora and Entoprocta (e.g., Kocot et al., 2017; Laumer et al., 2019), although the exact position of this clade remains unstable (see a discussion in Giribet & Edgecombe, 2020). Two species of Cycliophora have been described to date, *Symbion pandora* Funch & Kristensen 1995, which lives on *Nephrops norvegicus* (Linnaeus 1758), and *Symbion americanus* Obst, Funch & Kristensen 2006, which lives on *Homarus americanus* H. Milne-Edwards 1837. A third known species of Cycliophora commensal on the European *Homarus gammarus* (Linnaeus 1758) has yet to be described. *Symbion americanus* demonstrates greater levels of morphological variation than *S. pandora* (see Obst et al., 2006). Morphological differences that set *S. americanus* apart from *S. pandora* include the thicker cuticle on females, chordoid cysts possessing an extra toe appendage, and greater morphological variation in the chordoid larvae in the former (Obst et al., 2006).

A study of the microhabitat of *S. pandora* by Obst and Funch (2006) described their distribution throughout the mouthparts of individuals of *N. norvegicus*. It was observed that across 65 Norway lobsters, two different life stages of the cycliophoran (feeding individuals and chordoid cysts, which arise from nonfeeding females) settle on different regions of host mouthparts. Feeding individuals of *S. pandora* densely aggregated medially on mouthparts associated with greater contact with suspended food particles in the water column: the mandibular palps, the maxillae, and the first maxillipeds. Contrarily, the chordoid cysts were more evenly distributed over the segments, often aggregating on the lateral parts and in the articulation of the segments of mouthparts. This pattern suggested a trade-off between access to food and damage by lobster mandibles (Obst & Funch, 2006).

Another study showed that the microhabitat of *S. americanus* had certain similarities with the microhabitat of *S. pandora*: Feeding individuals of *S. americanus* tended to attach to the medial rims of the host mouthparts, whereas chordoid cysts tended to attach on lateral areas of the mouthparts (Obst et al., 2006). That study showed that, unlike *S. pandora*, the preference by feeding individuals of *S. americanus* for medial attachment was observed on all host mouthparts, as opposed to only on select mouthparts. But the discovery that multiple lineages of *S. americanus* can coexist on a single American lobster made matters more complicated prior to this study, as it was not possible to discern the specific position of the different lineages on the mouthparts of individual lobsters.

Given the morphological variation in the species, it was not entirely surprising that population genetic studies on *S. americanus* revealed evidence of cryptic speciation (Baker et al., 2007; Obst et al., 2005). Analysis of a 487 bp fragment of the mitochondrial gene *cytochrome* c *oxidase subunit I* (*COI*) supported three cryptic lineages of *S. americanus* living on *H. americanus*. The three cycliophoran lineages were named after the first nucleotide of the *COI* amplicon, C, G, and *T* (Baker et al., 2007). A subsequent multilocus phylogenetic analysis using *COI* and other mitochondrial (*12S rRNA* and *16S rRNA*) and nuclear (*18S rRNA*) genes further supported the existence of the three American lineages (Baker & Giribet, 2007). However, these previous studies focusing on the genetics of cycliophorans (Baker et al., 2007; Baker & Giribet, 2007; Obst et al., 2005) did not explore whether individuals of *S. americanus* from different genetic lineages segregate onto different host mouthparts, because the origin of the sequenced individuals was not reported, and studies focusing on microhabitat did not undertake genetic analyses (Obst et al., 2006; Obst & Funch, 2006). The present study thus investigates precisely whether different genetic lineages of *S. americanus* segregate onto different mouthparts of individuals of *H. americanus*.

## 2 | METHODS

### 2.1 | Collection

Individuals of *H. americanus* were obtained from lobstermen at wholesale markets between June and September 2019. The localities of host specimen are based on lobstermen records, which were only accurate to the province or city level, although this should have little implication for our study, which focuses on the spatial variation within lobsters, and not on geographic variation. Lobster fishermen reported to use trapping and trawling. Lobsters from each region were collected into a communal holding container before being sorted by size at market. The general localities in this study were selected given the observations of multiple genetic lineages of *S. americanus* on the same host by Baker et al. (2007). Lobsters used in this study came from the following localities: Newfoundland, Canada; New Brunswick, Canada; Nova Scotia, Canada; Portland, ME; and Boston, MA. Cyclophorans were observed on all host specimens of *H. americanus*. Specimens of *Symbion* sp. (MCZ IZ-153091) from *H. gammarus* were obtained at a restaurant in Roscoff, France. Given the small number of cyclophorans obtained from lobsters from two populations (Boston and Newfoundland), their data were excluded from further analyses.

### 2.2 | Dissection and DNA extraction

Sterilized forceps were used to dissect mouthparts, which were placed into a dish containing either filtered seawater or 95% ethanol. Following dissection, all mouthparts underwent several washes in the corresponding medium to remove as much lobster tissue and hemolymph as possible. Because cyclophoran density was symmetrical on both left and right mouthparts of the host, individuals of *S. americanus* were dissected from both sides under a dissecting microscope, and setae of the host were combed to detach random cyclophorans (Figure 1). Only individuals of *S. americanus* in feeding stage were collected. Contamination was prevalent in part because of the small size of the cyclophoran, which contain little tissue relative to the large volume of host tissue, which leached into the surrounding medium. In instances of contamination, the extraction protocol was modified to include additional wash steps when separating cyclophorans from the mouthparts.

The DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA) was used to extract genomic DNA from individual cyclophorans (sometimes attached to the host seta) under the manufacturer's protocol, without further modifications. Qubit (Thermo Fisher Scientific, Waltham, MA, USA) fluorometry was unable to quantify DNA in our extractions because of the small amount of DNA present in each cyclophoran specimen.

### 2.3 | Polymerase chain reaction and sequencing

A 487 bp fragment of *cytochrome c oxidase subunit I* and a 481 bp fragment *16S rRNA* were amplified using the polymerase chain reaction (PCR) and sequenced for analysis. *COI* was amplified between primer pairs LCO1490–HCO2198 (Folmer et al., 1994). *16S rRNA*

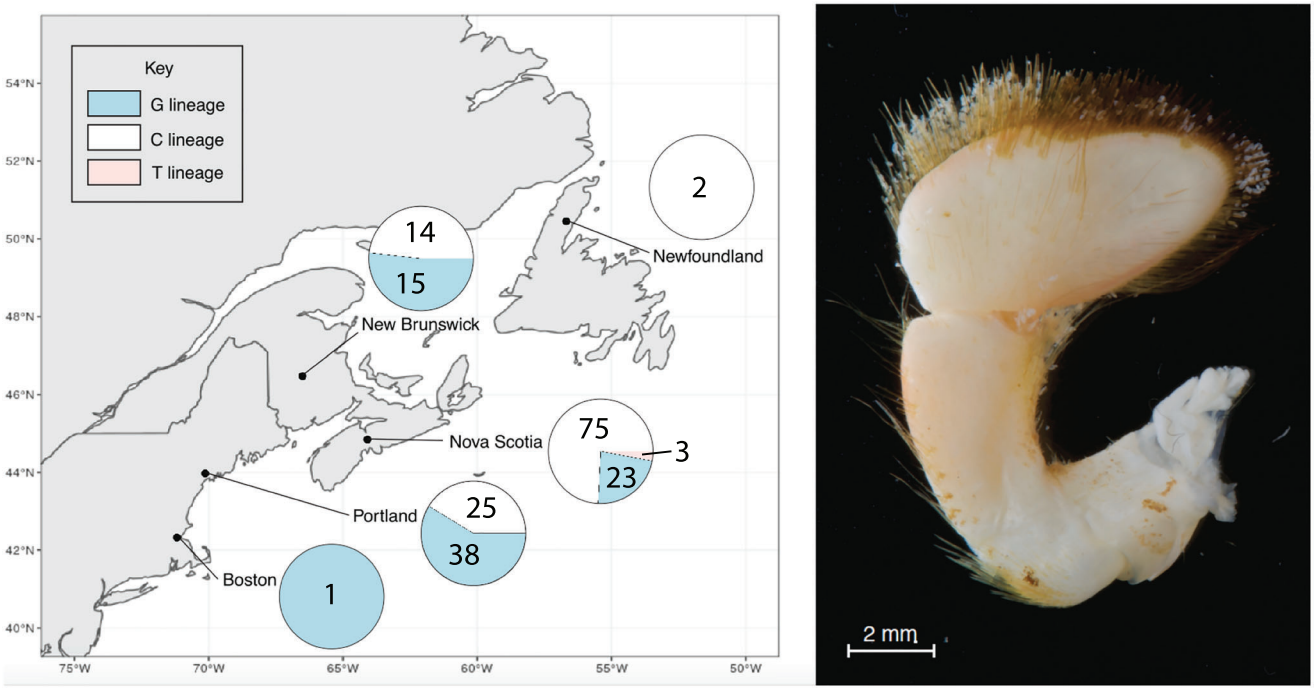


**FIGURE 1** Left: Map of localities and the proportion of genetic lineages of *Symbion americanus* per locality (numerals on pies indicate the number of each haplotype for each population; see also Table S2). Lineages were assigned using *COI* sequences. Right: Prolateral view of the left mandibular palp of specimen MCZ IZ-154720 of the lobster *Homarus americanus*, bearing feeding individuals of *S. americanus*

was amplified using primer pairs 16Sar–16Sb (Edgecombe et al., 2002; Xiong & Kocher, 1991). PCR reactions were done in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) following manufacturer protocols for illustra™ PuReTaq Ready-to-Go PCR Beads (GE Healthcare Life Sciences, Marlborough, MA, USA). All new sequences have been deposited in GenBank (see Table S1).

Gel electrophoresis using 1% agarose was used to check for the presence of the amplified gene fragment. Cyclophoran DNA extractions were first amplified and sequenced for *COI*. One hundred ninety-six individuals of *S. americanus* out of 567 extractions were successfully sequenced, and of these 196 samples, 174 were successfully sequenced for *16S rRNA*. ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to clean up DNA amplifications in 25-µl reactions. Samples were sent to off-site sequencing (Genewiz, Boston, MA, USA) on the same day as DNA cleanup. Geneious 2019.2.1 (Kearse et al., 2012) was used to assemble contiguous DNA fragments.

### 2.4 | Phylogenetic analysis

New sequences of *COI* from this study were aligned with GenBank sequences from Obst et al. (2005), Baker et al. (2007), and Baker and Giribet (2007), with the implementation of MAFFT (Katoh & Standley, 2013) in Geneious (Kearse et al., 2012). A maximum likelihood phylogenetic analysis with integrated model selection (Kalyaanamoorthy et al., 2017) was conducted using IQ-TREE (Nguyen et al., 2015) along with 1000 ultrafast bootstraps (Hoang et al., 2018) to assign individuals to lineages (sensu Baker et al., 2007).

### 2.5 | Population genetic analysis

Once individuals were assigned to lineage, new *COI* and *16S rRNA* sequences from this study (196 individuals in total) were concatenated using SequenceMatrix (Vaidya et al., 2011). Sequences corresponding to the T lineage were removed because of small sample size. A minimum spanning haplotype network (Bandelt et al., 1999) was constructed using POPART (Leigh & Bryant, 2015) to visualize relationships of cyclophoran haplotypes to host identity and mouthpart. All further population genetic analyses were conducted in R (R Core Team, 2021). An analysis of molecular variance (hereon AMOVA) was conducted on the total concatenated dataset using the package *poppr* (Kamvar et al., 2014) to explore whether different host lobsters or different mouthparts contributed more to genetic variation in cyclophorans. Strata were defined as lobster and mouthpart. The data were clone-corrected to account for cyclophorans being sampled during the asexual feeding stage. Significance was determined with 1000 random permutations of the sample matrices (Excoffier et al., 1992) in the package *ade4* (Dray & Dufour, 2007).

### 2.6 | Ecological segregation analysis

Analyses of ecological segregation were conducted in R (R Core Team, 2021). The effects of lobster and mouthpart in determining the lineage of cyclophoran was modeled with an implementation of logistic regression in the package *aod* (Lesnoff & Lancelot, 2012). Mouthparts were grouped to reflect the distribution revealed in the phylogenetic and haplotype analyses by pooling palps and all maxillipeds into one subpopulation and both maxillae into another. Because of the skew in the distribution of lineages on certain lobsters, the presence of multicollinearity was assessed using a variance inflation factor calculation implemented in the package *car* (Fox & Weisberg, 2019). To further verify the minimal effect of individual lobster in determining cyclophoran lineage, host identity was modeled as a random effect in a generalized linear mixed model using the package *lme4* (Bates et al., 2015), again with pooled mouthparts.

## 3 | RESULTS

Maximum likelihood analysis of *COI* data (see Figure S1) recovered the three lineages of *S. americanus* found in previous studies corresponding to a three species complex (Baker et al., 2007; Baker & Giribet, 2007). Both the C and G lineages were recovered with strong support (BS = 95% for both lineages), whereas the T lineage was not well sampled and was recovered with low support (BS = 63%). Once individuals were assigned to lineage, an association could be made between lineage identity and mouthpart origin (see Table S1). There was a qualitative association between lineage and location on mouthparts (Figure 2). The haplotype networks reflect this association. Networks were split into two distinct haplotype groups (Figure 3) corresponding to the C and G lineages. There is extensive overlap between the two haplotypes with regard to host identity (Figure 3A), indicating no clear relationship between lineage and individual lobster. Conversely, the two haplotypes were segregated by mouthpart (pooled for clarity), with little overlap in node color between the two lineages (Figure 3B).

Populations were significantly differentiated among "subpopulations" (= mouthparts) and within "subpopulations" ($p < 0.001$) but not among populations (i.e., individual lobsters; $p = 0.135$) (Table 1). Of the total variance, 47% was associated with differences between different mouthparts, and 44% of the variance was attributed to variation within subpopulations (Table 1). Fixation indices were high among and within subpopulations (Table 1).

The logistic regression analysis found that the odds of finding the C lineage of *S. americanus* were 220% greater than the odds of finding the G lineage of *S. americanus* on palps or maxillipeds (95% CI [221.8, 218.2]). There was no evidence of multicollinearity in lobster or mouthparts (VIF = 1.03, 1.35, respectively). The generalized linear mixed model also recovered similar results, with the odds of finding the C lineage of *S. americanus* being 215.7% greater than the odds of finding G lineage *S. americanus* on palps or maxillipeds (95% CI [217.25, 214.15]).

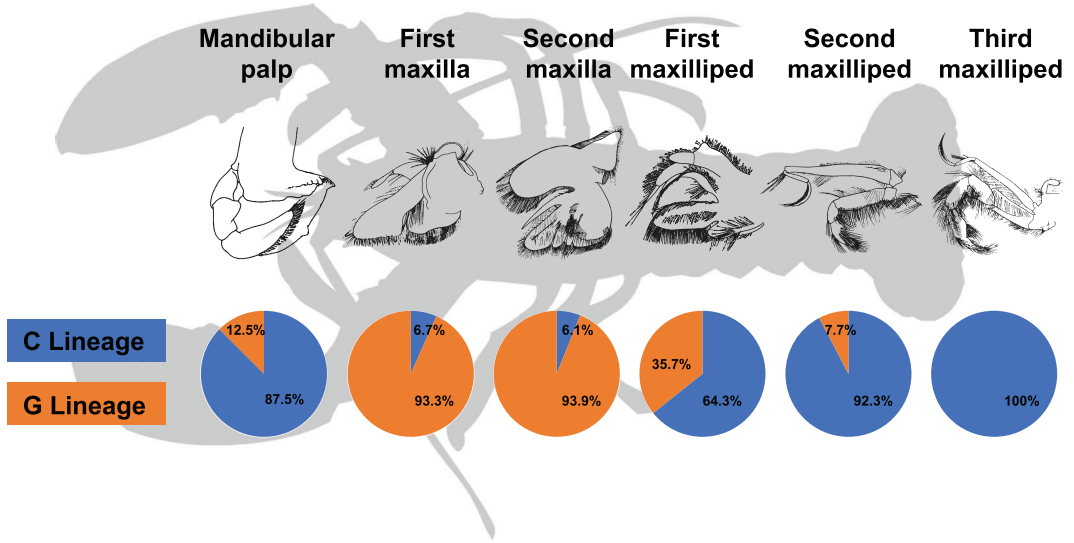


**FIGURE 2** Distribution of C and G lineages of *Symbion americanus* on lobster (*Homarus americanus*) feeding appendages. Lineages were assigned using *COI* sequences

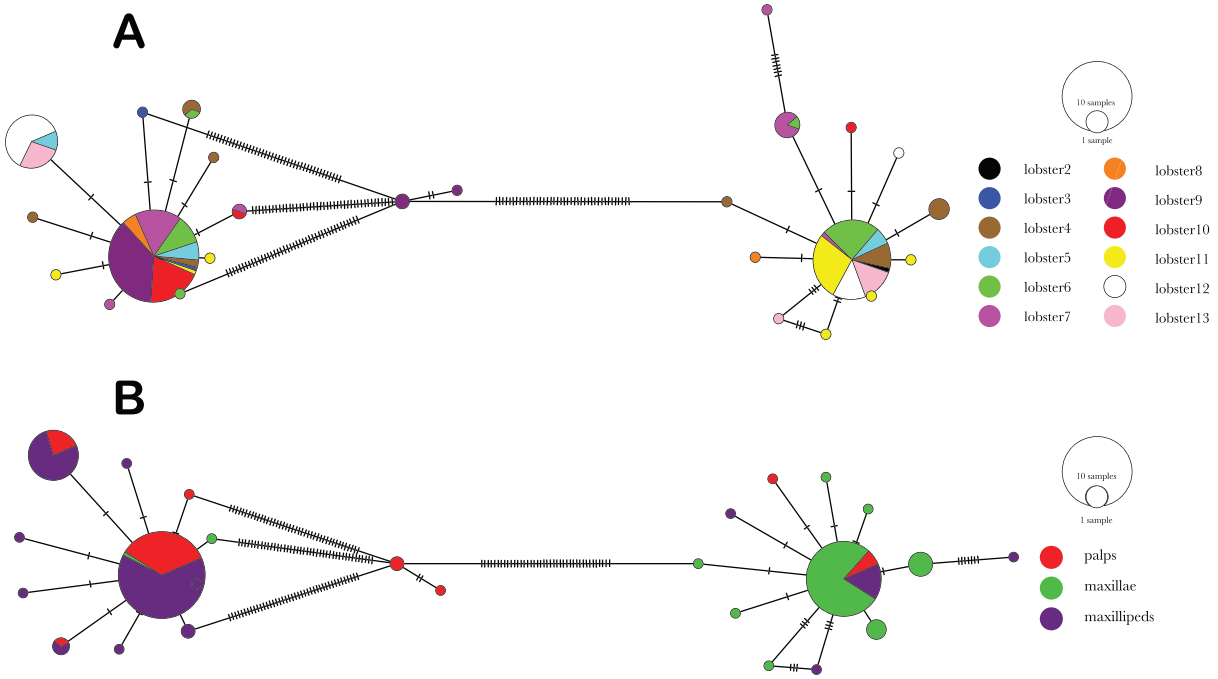


**FIGURE 3** Minimum spanning haplotype networks of individuals of *Symbion americanus*, collected from the lobster *Homarus americanus*, from the concatenated dataset. Nodes in the networks are labeled with host lobster identity (A) and host mouthpart origin (B). Both maxillae and the three maxillipeds are pooled for clarity

**TABLE 1** AMOVA of concatenated dataset

| Source of variation | σ | % of variation | Φ | *p* |
|---|---|---|---|---|
| Between lobsters | 3.69 | 9.45 | 0.09 | 0.135 |
| Between mouthparts | 18.19 | 46.58 | 0.51 | 0.001 |
| Within mouthparts | 17.17 | 43.97 | 0.56 | 0.001 |

*Note*: Strata are defined as lobster identity and mouthpart of origin. The variance (σ) for each hierarchical level is used to calculate the percentage of variation explained by each hierarchy; Φ is the population differentiation statistic, with higher values representing higher amount of differentiation, and *p* values were determined with 1000 random permutations of the sample matrices.

## 4 | DISCUSSION

This study further corroborates a body of work that reveals the existence of three cryptic species within *S. americanus*. However, this is the first work to examine the peculiarity that one host lobster can harbor all three species. Analysis of previously unexplored mouthpart origins of individual cycliophorans revealed a clear preference of different lineages for discrete subsets of lobster feeding appendages. Both logistic regression models and generalized linear mixed models showed that the C lineage was most associated with the palps and maxillipeds and the G lineage with the maxillae, whereas lobster identity had little impact on the distribution of lineages. This result was further substantiated by the molecular data and the underlying demographic structure in populations of *S. americanus*.

The demographic analysis demonstrated high population structure between cycliophorans from different mouthparts, with little to no structure between individuals found on different lobsters. Whether this represents a barrier to gene flow across mouthparts, effectively isolating the different lineages of *S. americanus* even within a single host, or secondary contact, cannot be discerned with the current data. Conversely, there seems to be gene flow in the same mouthparts across lobsters suggesting that cycliophoran larvae are preferentially settling on certain appendages. In the sexual reproductive stage of *Symbion* spp., the strong-swimming chordoid larvae leave their original hosts to find a new host, a phenomenon coinciding with the molting or death of the lobster. This would provide ample opportunity for shuffling and subsequent formation of new subpopulations of cycliophorans, even among those originally from different lobsters.

Lobster mouthpart appendages may be functionally specialized for different feeding processes and the distinct lineages of *S. americanus* may be preferentially using these different microhabitats. It has been found that the different life stages of the European *S. pandora* are associated with different mouthpart regions of its host *N. norvegicus* (see Obst & Funch, 2006). This suggests that there is indeed a potential for different mouthparts to harbor different microhabitat conditions that can be discerned and exploited by individuals of *S. americanus*. This potential niche partitioning may be driving the incipient speciation we find in *S. americanus*, but this would need to be confirmed with nuclear genetic data.

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## ORCID

*Shoyo Sato* https://orcid.org/0000-0002-2970-8702
*Gonzalo Giribet* https://orcid.org/0000-0002-5467-8429

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