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Puerto Rico *Sicydium* goby diversity: species-specific insights on population structures and distributions

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ABSTRACT

Sicydiine gobies are major contributors to Caribbean stream fish biodiversity, and ecosystem functions and services. In the Caribbean, Sicydiine gobies are represented by a single genus, *Sicydium*, but species-level *Sicydium* taxonomy and distributions remain unresolved in this region. A previous study posited that four species of *Sicydium* are present in Puerto Rico, including the recently described *Sicydium gilberti*; however, that study relied solely on morphological characters, and samples from a limited number of locations. Identification of *Sicydium* to species in studies with more comprehensive sampling has not occurred due to the impracticality of field identification. In our study, we employed morphological data and Mitochondrial Cytochrome C Oxidase I (mtCOI) genetic sequence data, collected from over 500 *Sicydium* spp. individuals at 16 broadly distributed sites, to provide an inventory of the species of *Sicydium* that occur in Puerto Rico. Additionally, we reveal new information on the spatial patterns of distribution, relative abundance, population structure, and observed molecular diversity among *Sicydium* taxa in Puerto Rico's river systems. This information is essential to advance ecological and evolutionary knowledge of Caribbean fish assemblages, guide stream fish conservation, and to effectively manage fisheries.

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Introduction

Amphidromous *Sicydium* gobies are a major component of Caribbean stream biodiversity. They yield ecosystem functions and services, such as nutrient biotransport, forage for predatory fishes, and culturally significant artisanal fisheries, and they face a variety of anthropogenic threats [1–4]. Despite cultural and ecological significance and the need for conservation, there is no clear understanding of their systematics and distributions. For example, early studies in Puerto Rico (e.g. [5–8]) mistakenly treated all *Sicydium* populations as a single species, the widespread *S. plumieri* (Bloch, 1786), but up to four *Sicydium* species have been purported to occur on the island [9]. Contemporary studies of riverine fishes of Puerto Rico are relatively abundant. The habitats, populations, anthropogenic threats, and life histories of the entire native freshwater fish assemblage of this island have been detailed (e.g. [10–16]). However, due to the impracticalities of field identification and the lack of definitive information on species distributions, *Sicydium* are routinely identified only to genus.

Watson [9] presented the only study of *Sicydium* species composition in Puerto Rico and the Dominican Republic and was the first to indicate that multiple species of *Sicydium* occur in Puerto Rico. Watson evaluated

the diagnostic ability of a suite of morphological characters by comparing specimens collected from the Dominican Republic and Puerto Rico, with topotypic representatives of nominal species described from throughout the Caribbean region. Characters evaluated included morphometrics, meristic and other standard counts, sensory pore distributions, and dentition. Upper jaw dentition, which requires a microscope to examine, was the most useful character for distinguishing the four *Sicydium* forms that Watson found to occur in Puerto Rico. Three of the forms were allocated to the following nominal species: *Sicydium buscki* (Evermann and Clark 1906), described from the Dominican Republic and known from Puerto Rico and Cuba; *S. plumieri* (Bloch 1786) and *S. punctatum* (Perugia 1896), both described from Martinique and held to be widely distributed in the Caribbean. The fourth group was described as a new species, *S. gilberti* Watson 2000, possibly limited to the Dominican Republic and Puerto Rico.

Although Watson's [9] work advanced the understanding of Caribbean *Sicydium* species compositions, his morphological species identifications have not been corroborated with genetic evidence. Moreover, Watson provided little information on species distributions and population structures in Puerto Rico. A study by Cook et al. [17] addressed questions of genetic population structure in Puerto Rico but was

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ambiguous about species identifications and did not include all four *Sicydium* species. Those authors used ATPase 6 and 8 mitochondrial DNA gene sequence data to infer intra-taxon gene flow in *Sicydium* collected from five Puerto Rico river systems that flow into three marine regions: the Caribbean, Atlantic, and Mona Passage. *Sicydium* were identified as *S. buscki*, *S. punctatum*, and *Sicydium* spp., but no information was provided about how species were identified. Presumably, identification was based solely on genetic sequences.

A species-specific and definitive inventory of the *Sicydium* commonly found in Puerto Rico, their distributions, relative abundances, and population structures, is needed to guide stream fish conservation. Dams and other anthropogenic instream barriers are a major threat to native freshwater fishes in Puerto Rico because all native stream fish are diadromous and there are at least 335 such barriers on the island, which has an area of only about 9,000 km² [11]. Cooney and Kwak [11] constructed species-specific logistic regression models that predict the minimum height of a barrier that will interrupt migration for all native fish species except *Sicydium*. The barriers for *Sicydium* were modelled at the genus level because it was not possible for Cooney and Kwak [11] to identify specimens to the species level in the field. A species-specific inventory of *Sicydium* is also needed for the effective management of an amphidromous postlarvae fishery. In Puerto Rico, the postlarvae of *Sicydium* spp. – locally known as “cetí” – are harvested in certain rivers during the last-quarter moon phase from June through January, which are peak *Sicydium* postlarval recruitment periods [16]. Although the harvest from this fishery is small and temporally limited in its availability, cetí is a regional delicacy, with strong cultural significance [8,18]. Basic ecological information, including the species composition of harvested and un-harvested rivers, is critically needed for conservation managers to begin active management to ensure the sustainability of this traditional fishery. Our study is the first to utilize both morphological and genetic data to reliably identify Puerto Rico *Sicydium* gobies collected. Additionally, we provide new information on the spatial patterns of distribution, relative abundances, and observed molecular diversity among *Sicydium* taxa in Puerto Rico’s river systems.

Methods

Sicydium were collected with a backpack electrofisher (pulsed direct current) and dip nets from 16 sites spanning 13 Puerto Rico river systems in July of 2009. Three of the 13 river systems sampled – the Río Grande de Añasco (two sites), Río Grande de Arecibo (one site), and Río Grande de Manatí (three sites) – support active cetí fisheries (pers. observation).

Collected fish were anaesthetized with tricaine methanesulfonate (MS 222), fin-clipped for tissue samples, tagged, and then fixed in buffered 10% formalin to serve as vouchers. Fin clips were preserved in 95% ethanol, and corresponding numbers were assigned to the voucher specimens and tissue vials. In July 2009, 505 voucher specimens and corresponding tissue samples were collected. An additional 65 tissue samples, collected in 2008 from one of the same sites sampled in 2009 (denoted with a star in Figure 1), were included for genetic analyses (Figure 1). Vouchers were transferred to 70% ethanol and deposited in the collection of the North Carolina Museum of Natural Sciences (NCSM). Tissues were also deposited in the NCSM collection and are maintained in ultracold (–80° C) storage (Appendix 1).

We tentatively accepted Watson’s findings and used them to assign each voucher specimen to a working taxon group based on rigorous microscopic examinations of dentition configuration and tooth morphology. In assigning the morphological working taxon group, both the configurations and placement of the teeth, as well as the cusp morphology of individual teeth, were noted and carefully compared to the illustrations provided by Watson [9]. The morphological identifications were then compared with haplotype data derived from molecular methods (described below) to determine agreement of genetic data with morphological taxon group assignments. Each voucher was measured (total length, mm) and length-frequency histograms (LFHs) for each *Sicydium* species’ island-wide population were constructed with R [19]. Finally, the relative abundance of each species at each site sampled was calculated as the quotient of the catch of the individual species to the total *Sicydium* catch at the site.

Total DNA was extracted from tissue samples using the Macherey-Nagel NucleoSpin 96 Tissue kit and processed on an Eppendorf Robotic liquid handler (epMotion 5075) or with the Macherey-Nagel 8 Tissue kit and processed with the Macherey-Nagel vacuum manifold. Extracted DNA was quantified with a Nanodrop spectrophotometer and standardized to a working concentration of approximately 40 ng/μL. PCR amplification, sequencing of the mitochondrial COI gene, and primer cocktails followed protocols standardized in Ivanova et al. [20] and Ratnasingham and Hebert [21] with no modifications. Additional data were downloaded either from the BOLD website (www.barcodinglife.org) or GenBank (www.ncbi.nlm.nih.gov/genbank) to provide reference samples for systematic comparisons. All of the Mitochondrial Cytochrome C Oxidase I (mtCOI) sequence data obtained in this study were deposited with GenBank under accession numbers MK655481–MK656029. Sequence data from *S. buscki*, *S. plumieri*, and *S. punctatum* sampled from the 16 sites during both collection years (2008 and 2009) were converted to FASTA format and examined for

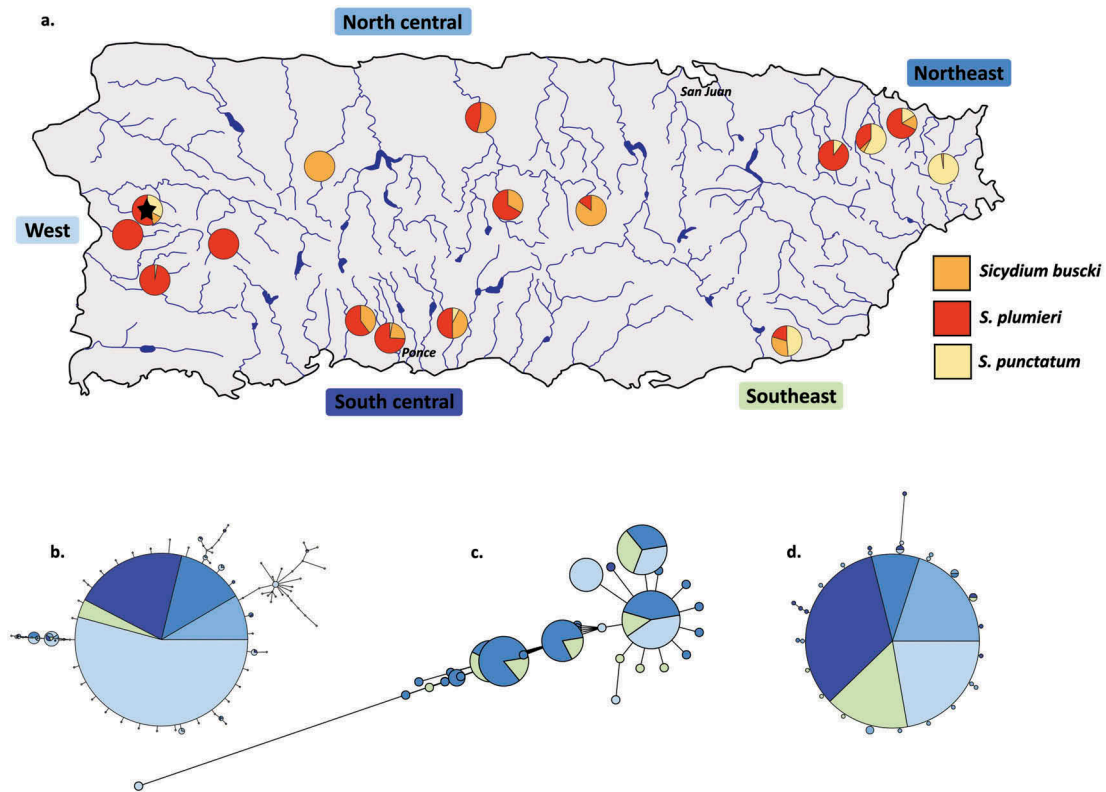


Figure 1. The distributions and Mitochondrial Cytochrome C Oxidase I haplotype networks of all three species of *Sicydium* gobies that occur in Puerto Rico. (a.) Pie charts illustrating relative abundance of each species caught (percent of area of pie with corresponding species colour is equal to the percent of catch) at 16 sampling sites on a map of the island of Puerto Rico; the site marked with a star was sampled in 2008 and 2009, and all other sites were sampled in 2009 only. Also displayed are the locations of the cities of San Juan and Ponce, major drainage basins, and the five regions of the island sampled. (b.) *Sicydium plumieri* haplotype network where the size of each haplotype's pie is proportional to its frequency and the distances between connected haplotypes are proportional to the number of pairwise differences between the two, colours correspond to the regions in (a.) and the percent of area of each colour is equal to the percent of individuals captured from that region. (c.) *Sicydium punctatum* haplotype network. (d.) *Sicydium buscki* haplotype network.

missing data. All sequences with missing data were eliminated from the dataset, and each sequence was identified with the region of Puerto Rico where it was collected. Sequences were aligned using Clustal Omega [22].

The PEGAS software package in program R was used to construct a haplotype network, generate descriptive statistics of population genetics, and analyse geographic patterns of genetic structure for *S. buscki*, *S. plumieri*, and *S. punctatum* [19, 23]. The haplotype network was visualized with a figure, in which the size of each haplotype's circle is proportional to its frequency and the distances between connected haplotypes are proportional to the number of pairwise differences (Figure 1). The descriptive statistics of population genetics that were calculated included the number of haplotypes, haplotype diversity (H_d), and nucleotide diversity (π). A hierarchical Analysis of Molecular Variance (AMOVA) was used to test for within-species genetic population differentiation at the regional and sampling-site scales. A Euclidean pairwise genetic distance matrix was used as the input for the AMOVA, and the statistical significance of the variance components was determined with 1,000 random permutations.

Results

Our findings verify that three genetically distinct species of *Sicydium*: *S. buscki*, *S. plumieri*, and *S. punctatum* are present in Puerto Rico. Average divergence among the three clades was as follows: between *S. buscki* and *S. punctatum*, 2.5%; between *S. buscki* and *S. plumieri*, 8.5%; between *S. punctatum* and *S. plumieri*, 8%. They are reliably distinguished with microscopic examination of dentition because our morphological and genetic classifications agreed at very high rates (Table 1). Specifically, when we excluded failed sequencing attempts, mismatch between morphological and genetic identification of these three species was less than 3%. We found no evidence that *S. gilberti* is a genetically-distinct species of *Sicydium*, and this morphotype was very rare in our samples. 13 of the 505 individuals that we examined had tooth morphology consistent with what Watson [9] described as *S. gilberti*, but none were identified as *S. gilberti* with DNA barcoding. Of the 13 *S. gilberti* morphotype specimens, based on mtCOI sequences, 11 were identified as *S. plumieri* and the other two were identified as *S. buscki*.

Table 1. Agreement between morphological and genetic identifications of 505 *Sicydium* individuals collected from streams throughout Puerto Rico.

Morphological identification	Genetic identification	Number of individuals	Percent of all morphologically identified individuals
<i>S. buscki</i>	<i>S. buscki</i>	108	94.7
	<i>S. punctatum</i>	3	2.6
	Failed	3	2.6
<i>S. gilberti</i>	<i>S. buscki</i>	2	15.4
	<i>S. plumieri</i>	11	84.6
<i>S. plumieri</i>	<i>S. plumieri</i>	268	98.9
	Failed	3	1.1
	<i>S. buscki</i>	1	0.9
<i>S. punctatum</i>	<i>S. plumieri</i>	3	2.8
	<i>S. punctatum</i>	101	94.4
	Failed	2	1.9

We examined broad patterns of distribution and ubiquity of the three *Sicydium* species and the *S. gilberti* morphotype in a subset of 490 of the 505 individuals collected. In this examination, we excluded *S. buscki*, *S. plumieri*, and *S. punctatum* that were genetically and morphologically mismatched, or where sequencing failed, and relied on morphological identifications of *S. gilberti* (Table 1). *Sicydium plumieri* was the most ubiquitous and abundant taxon in our samples, 268 individuals (55% of total catch) were captured at 14 of 16 sites (88%) sampled. *Sicydium buscki* and *S. punctatum* were intermediate in terms of abundance and ubiquity, we captured 108 *S. buscki* individuals (22% of total catch) at 12 sites (75%), and 101 *S. punctatum* (21% of total catch) individuals at 9 sites (57%). The *S. gilberti* morphotype had very low abundance but occurred at several sites; the 13 individuals that we captured (3% of the total catch) were found at 7 of 16 sites (44%).

There were apparent spatial patterns in the distribution and abundance of three *Sicydium* species in Puerto Rico at both regional, and individual site-level scales. *Sicydium plumieri* made up the majority of the catch at all sites, but the relative abundance of this species was especially high in West region watersheds of Puerto Rico, i.e., those that drain into the Mona passage (Figure 1). In contrast, *S. buscki* was rare and in low abundance in West region watersheds. The relative abundance and occurrence of *S. buscki* were also low in the four watersheds located in the Northeast region of Puerto Rico that drain into the Atlantic Ocean. *Sicydium buscki* was relatively

abundant in watersheds of the North Central, South Central and Southeast regions of Puerto Rico, which drain into the Atlantic and Caribbean, respectively. *Sicydium punctatum* was relatively abundant and occurred in all samples from watersheds in the Northeast and Southeast regions of Puerto Rico. *Sicydium punctatum* was either absent or in low abundance at all other sampling locations, with the exception of a single site in a West region watershed, the Río Grande de Añasco, where it made up over 30% of the catch.

Length-frequency histograms (LFHs) revealed inter-taxa similarities and differences in size distributions (Figure 2). *Sicydium buscki* and *S. gilberti* size distributions were generally similar; with a mode around 50 and 60 mm, respectively, and did not include individuals larger than 100 mm. The LFH of *S. plumieri* illustrates that it is generally the largest *Sicydium* taxon in Puerto Rico with a mode around 70 mm, and individuals at over 170 mm. The LFH of *S. punctatum* is indicative of two distinct size classes, because it appears to be bimodal around 50 and 100 mm.

Our haplotype network results revealed distinct levels of mtCOI molecular diversity in the three species of *Sicydium* in Puerto Rico, but did not provide evidence of geographic patterns in genetic structure in any of the populations (Figure 1, Table 2). The *S. buscki* (28 haplotypes from 76 sequences) and *S. plumieri* (98 haplotypes from 266 sequences) haplotype networks were similar in overall shape and structure, and evidenced low genetic diversity in both species. For both species, there was

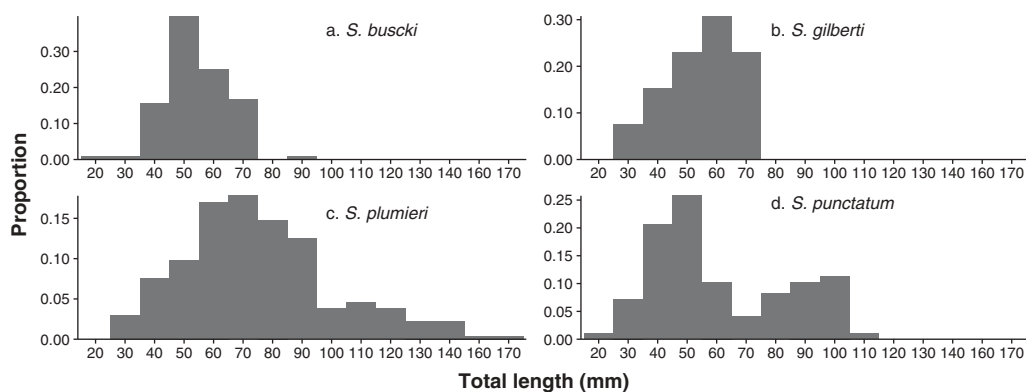
**Figure 2.** Length frequency distribution of (a.) *Sicydium buscki*, (b.) *Sicydium gilberti* (morphotype), (c.) *Sicydium plumieri*, and (d.) *Sicydium punctatum* collected from 16 locations throughout Puerto Rico.

Table 2. Genetic diversity summary statistics for all three species of *Sicydium* in Puerto Rico. H is the number of unique haplotypes/the total number of haplotypes used in the analysis, π is nucleotide diversity, and Hd is haplotype diversity.

Species	H	π	Hd
<i>S. buscki</i>	28/76	0.0040	0.6512
<i>S. punctatum</i>	41/69	0.0059	0.9668
<i>S. plumieri</i>	98/266	0.0043	0.8004

a single, dominant (i.e., most frequent) haplotype that occurred in all regions sampled, and peripheral, low-frequency haplotypes, arranged in a star-shaped pattern around the dominant haplotype (Figure 1). For *S. buscki*, the dominant haplotype frequency was 45 (59%), the dominant haplotype frequency in the *S. plumieri* network was 118 (44%). The *S. punctatum* network indicates the highest molecular diversity of the three species studied, but does not provide clear evidence for regional geographic structure within this diversity. This species' haplotype network consisted of 41 unique haplotypes from 69 sequences with the highest frequency haplotype in six individuals (9%). The *S. punctatum* network had 5 nodes with frequencies of 5 or more and each of these was represented by individuals from multiple regions. One of the nodes is noticeably distinct from all others, this haplotype was exhibited in a single individual, which we sampled from the same site as all other *S. punctatum* individuals in the West region.

Our descriptive genetic diversity statistics and AMOVA analyses support the haplotype network results, indicating variable genetic diversity in species of *Sicydium* in Puerto Rico and a general lack of geographic pattern in genetic structure at the regional scale. Both measures of genetic diversity, Hd and π , were relatively low for *S. buscki* and *S. plumieri*, and greatest for *S. punctatum* (Table 2). The AMOVA analysis revealed that nearly all of the genetic variation of the *S. buscki* and *S. plumieri* populations (96.90 and 99.21%, respectively) occurred within the sample-sites and provided no evidence of genetic structure at either spatial scale for either species (Table 3). Similar to the other two species, the majority of genetic variation (72.99%) in the *S. punctatum* population in Puerto Rico occurred within sample-sites, and there was almost no genetic variation between regions. However, there was significant variation between

sample-sites within regions (30.86%), indicating that some population structure seems to occur at a small spatial-scale in this species; additional time-series studies are needed to support this result.

Discussion

We found no evidence that *S. gilberti*, which was previously reported as a new Caribbean species of *Sicydium* by Watson [9], is genetically distinct from other *Sicydium* species in Puerto Rico; this result calls its validity as a species into question. More generally, our study and recent published and unpublished research highlight the confused nature of Caribbean sicydiine taxonomy [24,25]. Chabarría's [24] results directly support those of our study; a concatenated phylogeny, assembled with data from three genetic markers, nested individuals that were morphologically identified as *S. gilberti* within a *S. plumieri* clade. Our dataset was not specifically designed to examine more than species-specific DNA barcoding, but because of the lack of genetic divergence at this marker (which typically recovers 2–2.5% divergence between valid fish species [26,27]), we posit that individuals with morphology consistent with *S. gilberti* should be considered a phenotypic variant or morphotype rather than a distinct taxon. Furthermore, because *S. gilberti* individuals were genetically identified as *S. buscki* and *S. plumieri*, and the *S. gilberti* size distribution is intermediate of the two species, we hypothesize that the *S. gilberti* morphotype may result from occasional hybridization of these two species. Additional work is warranted to test this hypothesis in a laboratory setting. If this hypothesis is correct, hybridization appears to occur infrequently in Puerto Rico, because the *S. gilberti* morphotype was very rare in our samples despite a large sample size, and extensive spatial coverage of the island. We found that

Table 3. Hierarchical analysis of molecular variance (AMOVA) on COI sequences of all three species of *Sicydium* in Puerto Rico. For each species, the associated percentages of regional variance, variance between sample-sites within regions, error variance (i.e., between individuals within sample-sites), and the associated probabilities of non-differentiation are listed (significant values in bold).

Species	Source of variation	df	Sum of squares	Variance component	Percentage variation	P-value
<i>S. buscki</i>	Region	4	15.39	0.10	3.95	0.2388
	Sample-sites within region	6	14.75	-0.02	-0.85	0.6783
	Error	65	166.22	2.56	96.90	
	Total	75	196.37			
<i>S. plumieri</i>	Region	4	11.41	-0.01	-0.38	0.3506
	Sample-sites within region	9	29.49	0.03	1.17	0.2478
	Error	252	681.22	2.70	99.21	
	Total	265	722.12			
<i>S. punctatum</i>	Region	3	36.87	-0.16	-3.85	0.6324
	Sample-sites within region	4	46.44	1.26	30.86	0.033
	Error	61	182.19	2.99	72.99	
	Total	68	265.51			

the *S. gilberti* morphotype is more common in streams on the western half of the island, but given the very low number of individuals captured at any location, this pattern could be influenced by sampling error.

The occurrence of three sympatric species of *Sicydium* in Puerto Rico with uncommon or non-existent hybridization implies that reproductive isolating mechanisms and niche partitioning occur among these species. Although not previously documented in Puerto Rico, nuptial display behaviour, which is observed in sicydiine males of other species, is a potential reproductive isolating mechanism [28]. More research is needed to determine if this type of behaviour or other cues are used by Puerto Rico *Sicydium* to identify conspecifics during mating. The distinct tooth morphology of these species is a trait that may be related to niche partitioning. Watson [9] postulated that tooth morphology may be an adaptation for the consumption of specific forms of algae or diatoms. The fact that dentition is a reliable character for distinguishing genetically distinct, sympatric species supports the hypothesis that dentition is an adaptation related to interspecies niche partitioning. Further research could confirm if these or other hypotheses can explain the co-occurrence of multiple *Sicydium* species in Caribbean streams.

When synthesized, our results on the spatial distributions, relative abundances, structure, and genetic diversity of *Sicydium* populations of Puerto Rico can be used to inform conservation and management. Our results address a very basic, albeit crucial, question in Caribbean fisheries management and conservation – what is the total native, freshwater fish species richness of Puerto Rico's streams? By demonstrating that there are three, genetically distinct species of *Sicydium* that occur throughout Puerto Rico, we can conclude with confidence that there are nine native freshwater fish species that commonly occur in the streams and rivers of the island. Of the three *Sicydium* species, *S. plumieri* appears to be the most ubiquitous and abundant, and to have the largest size structure, while *S. punctatum* seems to be relatively rare in central and western watersheds. All three species of *Sicydium* are almost certainly harvested in the cetí fishery because we captured all three in the watersheds (Río Grande de Añasco, Río Grande de Arecibo, and Río Grande de Manatí) where this fishery commonly occurs, and all *Sicydium* recruitment occurs in a limited time-window that overlaps with the fishery [16, pers. observations].

Our results and those of other researchers provide little indication that homing, or watershed endemism occurs in Puerto Rico *Sicydium*, at least at the regional scale. Our finding of low MtCOI genetic diversity (i.e., only a few, commonly occurring haplotypes) and the lack of phylogeographic patterns in haplotype networks suggest that there is population connectivity among rivers and regions of Puerto Rico via dispersal during the marine larval phase. *S. punctatum* had the greatest genetic diversity and was the only species with significant population

structure at any scale. However, because this structuring only occurred at the spatial scale of sites within a region, but not at the larger regional scale, we attribute these results to non-equilibrium dynamics in population structure, which can occur due to the effects of kin-structured founders or extinction and colonization dynamics [17]. This conclusion supports the findings of Cook et al. [17] who found evidence of population connectivity using ATPase 6 and 8 MtDNA genetic markers. With this in mind, fisheries management and research may be informed and enhanced with the understanding that the population dynamics of *Sicydium* in any given stream in Puerto Rico could be affected by populations in other streams on the island, or even throughout the Caribbean.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Appendix 1. North Carolina Museum of Natural Sciences (NCSM) catalogue numbers for collections of *Sicydium* voucher specimens and tissue samples (where available) by species, and region of collection.

Region	<i>Sicydium buscki</i>	<i>Sicydium plumieri</i>	<i>Sicydium punctatum</i>	<i>Sicydium</i> sp.
West	NCSM 53616, NCSM 64059, NCSM 64066	NCSM 53614, NCSM 64058, NCSM 64061, NCSM 64063, NCSM 64076	NCSM 53615, NCSM 64070, NCSM 64084	NCSM 53617, NCSM 64060, NCSM 64062, NCSM 64064, NCSM 64083
North Central	NCSM 64068, NCSM 64082, NCSM 64086, NCSM 64087	NCSM 53617, NCSM 64060, NCSM 64062, NCSM 64064, NCSM 64083		NCSM 64069, NCSM 64080, NCSM 64081
South Central	NCSM 64057, NCSM 64077, NCSM 64085	NCSM 64055, NCSM 64075, NCSM 64088	NCSM 64079, NCSM 64056	NCSM 64078
Northeast	NCSM 64047, NCSM 64050, NCSM 64052	NCSM 64045, NCSM 64046, NCSM 64053	NCSM 64043, NCSM 64048, NCSM 64049, NCSM 64051	NCSM 64044, NCSM 64054
Southeast	NCSM 64074	NCSM 64073	NCSM 64072	NCSM 64071