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First molecular characterization of *Proctoeces maculatus* (Looss, 1901) (Digenea: Fellodistomidae) infecting blue mussels (*Mytilus edulis*) from the northeastern USA

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Abstract

The digenetic trematode *Proctoeces maculatus* is a cosmopolitan parasite that infects various invertebrates and fish hosts, including the blue mussel, *Mytilus edulis*, along the northeastern U.S. coast. Despite its impact on mussel fitness and the region's aquaculture, little is known about the genetic diversity and connectivity of *P. maculatus* in this region. This study provides the first genetic characterization of *P. maculatus* populations in New England using the D1–D3 region of the 28S ribosomal RNA gene. Bayesian phylogenetic analysis and a haplotype network were used to assess genetic variation and connectivity across six localities in Maine, New York, and southern New England, and to compare these populations to global samples. Our results revealed distinct geographic structuring of *P. maculatus* haplotypes. The ME1 haplotype, unique to Maine, reflects either recent range expansion or isolation driven by environmental and biogeographic factors, such as Cape Cod's role as a phylogeographic barrier. The most common haplotype, US1, was shared by populations in southern New England, New York, and a single specimen from Tunisia, indicating possible historical or anthropogenic connectivity. Two divergent haplotypes from Mississippi and Chile likely represent misidentifications or cryptic species. These findings support the hypothesis that *P. maculatus* is likely a cryptic species complex. Molecular evidence suggests connectivity across distant regions, emphasizing the role of host movement in parasite dispersal. Continued genetic studies, particularly from under-sampled regions, are needed to unravel the diversity and biogeography of *P. maculatus* and its potential impact on declining mussel populations.

Introduction

The digenetic trematode *Proctoeces maculatus* is a widely distributed parasite, recorded from nearly every major oceanic basin, including both sides of the Atlantic, the Mediterranean (its type region), and the Pacific. This species utilizes invertebrates, such as mollusks and polychaetes, as first or second intermediate hosts and reaches sexual maturity as adults in fish. *Proctoeces maculatus* is notably euryxenous, having been reported in 65 species of fish and 26 invertebrates – an unusual trait among marine trematodes (Vermaak *et al.* 2023). A recent morphological and molecular study by Vermaak *et al.* (2023) suggested that *P. maculatus* is likely both cosmopolitan and composed of cryptic species, complicating its identification and the assessment of its connectivity across regions.

On the eastern coast of the United States, *P. maculatus* infects the native blue mussel, *Mytilus edulis*, a keystone species and an economically important bivalve for New England's blue economy (Evensen *et al.* 2023; Fairbanks 2016). Studies indicate that *P. maculatus* imposes significant fitness costs on *M. edulis*, including reduced reproductive output (Valderrama *et al.* 2004) and, in rare cases, mortality (Costau *et al.* 1993). High infestation rates are also associated with reduced mussel growth, diminished filtration capacity, and lower byssal thread production (Lauckner 1983; Thieltges 2006; Stier *et al.* 2015).

Despite its impact as a pathogen of an economically important species, the genetic characterization and connectivity of *P. maculatus* along the east coast of the United States remain unknown. This is particularly relevant as climate change is thought to be driving the range expansion of *P. maculatus* in this region. In 1983, Cape Cod, Massachusetts, a major phylogeographic boundary in the western Atlantic, marked its northernmost distribution (Pondick 1983). More than two decades later, it was reported in Great Bay, New Hampshire, approximately 180 km north of Cape Cod (Markowitz *et al.* 2016). Most recently, *P. maculatus* was documented infecting *M. edulis* in Casco Bay, Maine, approximately 90 km north of Great Bay.

The purpose of this study was to provide the first genetic characterization of *Proctoeces maculatus* from the eastern United States, with a specific focus on its northeastern range (Maine

to New York). Using the 28S ribosomal RNA genetic marker, we assessed genetic variation and connectivity among populations in New England and compared them to other global samples.

Materials and methods

More than 300 specimens of *Mytilus edulis* were collected from six localities in the northeastern United States in 2023 and 2024 (Figure 1, Table 1). At each site, prevalence was determined by observing the presence of the parasite in the mantle tissue of the mussels under a dissecting microscope. Sporocysts, and adult trematodes when present, were isolated from the mantle tissue and frozen at -30°C for 24 hours. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's protocol. DNA concentrations ranged from 12 to 60 ng/μL per sample. The D1–D3 region of the 28S nuclear ribosomal RNA gene was selected for amplification due to its previous success in elucidating the genetic intra- and inter-relationships of *Proctoeces* (Antar and Gargouri 2016; Vermaak *et al.* 2023; Wee *et al.* 2017) and other digenetic trematodes (Choudhury *et al.* 2007; Blasco-Costa *et al.* 2016; Shylla *et al.* 2013). A ~900 bp fragment was amplified using primers Digl2 (5′-AAGCATATCACTAAGCGG-3′) (Tkach *et al.* 2001) and 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′) (Snyder and Tkach 2001), following the PCR protocol described by Tkach *et al.* (2003). Amplicons were visualized on a 2% agarose gel, excised, and purified using the QIAquick Gel Purification Kit (Qiagen). Sequencing of the purified amplicons was performed using both forward and reverse primers with Big Dye Terminator Cycle sequencing chemistry at Azenta LLC (Plainfield, NJ).

Returned sequences were initially identified using the NCBI BLASTn tool. Sequences of *P. maculatus* generated in this study were then combined with 28S sequences published by Vermaak *et al.* (2023) and other 28S sequences of *Proctoeces* from the GenBank database (Table 1). The sequences were aligned and edited using the MUSCLE alignment tool in Biopython (Cock *et al.* 2009). After editing, a 790-bp fragment with 135 polymorphic sites remained for analysis. For phylogenetic analysis, an unrooted Bayesian tree was constructed in MrBayes ver 3.2 (Ronquist *et al.* 2012) using the GTR + G nucleotide substitution model, as determined by AICc best model fit in jModelTest2 (Darriba *et al.* 2012). For tree-building parameters, Markov chain Monte Carlo chains were run for 5 million generations with the burn-in parameter set for 25% of sampled trees. The resulting phylogenetic tree was visualized in FigTree ver 1.4.3 (Rambaut 2009). Intra- and interspecific kimura-2-parameter (K2P) genetic distances were also calculated using MEGA11 (Tamura *et al.* 2021) to determine levels of genetic relatedness. Finally, to determine population admixture and connectivity, a median-joining network was constructed in PoPART ver. 3.5 (Leigh and Bryant 2015) to determine any geographic patterning of haplotypes.

Results and discussion

Overall prevalence of *P. maculatus* within blue mussels ranged from 4.1% to 90.8% (Maine: 4.1%, N = 98; Massachusetts: 49.8%, N = 66; Rhode Island: 65.5%, N=101; Connecticut: 60.4%, N=45; New York: 90.8%, N = 119). Progenesis was also observed in both Rhode Island and Connecticut populations, where both sporocysts and adults co-occurred in the same host.

Sequence data from the northeastern United States showed a 99.6–99.9% identity match to *Proctoeces maculatus* based on BLASTn analysis. A Bayesian phylogenetic tree provided strong posterior support for the monophyly of *P. maculatus*, encompassing sequences from the northeastern U.S., South Africa, and the Mediterranean (Figure 2). Within the northeastern U.S. clade, samples from Maine exhibited genetic divergence from other New England

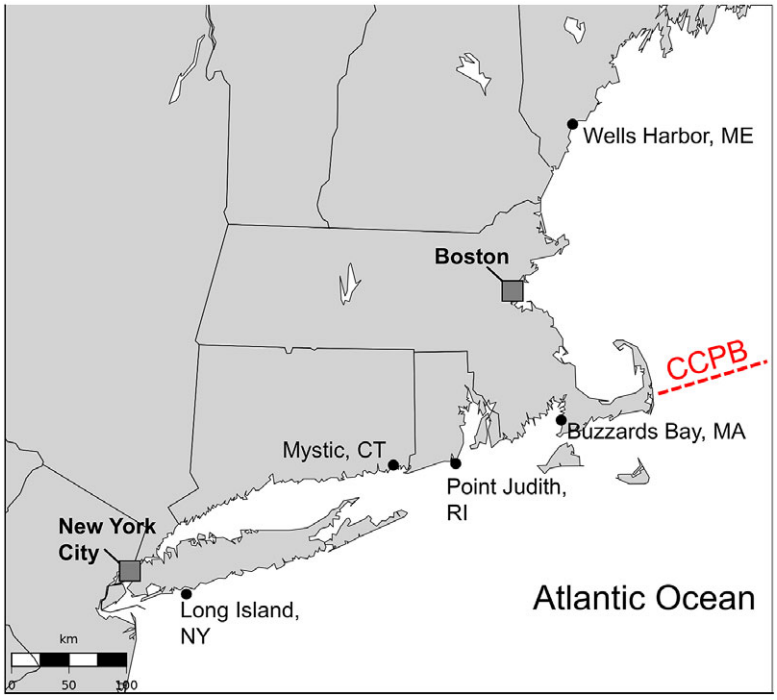

Figure 1. Map showing sampling localities of host mussels (*Mytilus edulis*). State abbreviations: ME – Maine, MA – Massachusetts, RI – Rhode Island, CT – Connecticut, NY – New York. CCBP – Cape Cod Phylogeographic Break.

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Table 1. 28S rRNA sequences data used for phylogenetic and haplotype analysis of *Procteces maculatus*. South African site abbreviations: TNP - Tsitsikamma section of the Garden Route National Park, DHNR – De Hoop Nature Reserve

Species	Host	Locality	GenBank accession number(s)	Sample Size (N)	Reference
Proctoeces maculatus	*Mytilus edulis* (mussel)	Wells Harbor, Maine, USA	PQ640063 – PQ640066	4	Present study
	M. edulis	Buzzards Bay, Massachusetts, USA	PQ640041 – PQ640050	10	Present study
	M. edulis	Point Judith, Rhode Island, USA	PQ640051 – PQ640056	6	Present study
	M. edulis	Mystic, Connecticut, USA	PQ650057 – PQ650062	6	Present study
	M. edulis	Point Lookout, Long Island, New York, USA	PQ640036 – PQ640040	5	Present study
	Sparadon durbanensis (fish)	TNP, South Africa	OR724714	1	Vermaak *et al.* (2023)
	Clinus superciliosus (fish)	TNP, South Africa	OR724715	1	Vermaak *et al.* (2023)
	C. superciliosus	Chintsa East, South Africa	OR724716	1	Vermaak *et al.* (2023)
	Diplodus capensis (fish)	TNP, South Africa	OR724713	1	Vermaak *et al.* (2023)
	D. capensis	DHNR, South Africa	OR724718	1	Vermaak *et al.* (2023)
	D. capensis	Chintsa East, South Africa	OR724717	1	Vermaak *et al.* (2023)
	Archosargus probatocephalus (fish)	Mississippi, USA	AY222284	1	Olson *et al.* (2003)
	Sabella pavonina (polychaete)	Bizerte Lagoon, Tunisia	KX671315	1	Antar and Gargouri (2016)
	Lithognathus mormyrus (fish)	Bizerte Lagoon, Tunisia	KU052937	1	Antar and Gargouri (2016)
	Sicyases sanguineus (fish)	Chile	KT865207	1	Oliva *et al.* (2018)
	Sparus aurata (fish)	Tunisia	KX671302	1	Wee *et al.* (2017)
Proctoeces major	*S. sanguineus*	Chile	KY432618	1	Oliva *et al.* (2018)
	Monodactylus argenteus (fish)	Queensland, Australia	KX671309	1	Wee *et al.* (2017)
Proctoeces insolitus	*Acanthopagrus australis*	Queensland, Australia	KX671300	1	Wee *et al.* (2017)
Proctoeces humboldti	*S. sanguineus*	Chile	KY432601	1	Oliva *et al.* (2018)
Proctoeces choerodoni	*Choerodon cyanodus*	Heron Island, Australia	KX671299	1	Oliva *et al.* (2018)

specimens, with a K2P distance of 0.3%. South African *P. maculatus* formed a distinct clade, separate from U.S. populations, indicating limited connectivity between these regions (K2P distances: 0.4–0.5%). Additionally, two *P. maculatus* specimens from Mississippi (USA) and Chile did not cluster within the primary *P. maculatus* clade. The Mississippi specimen showed K2P divergence distances of 4.7–5% from New England and New York specimens, while the Chilean specimen exhibited K2P distances of 5.8–6.1% when compared to specimens from New England, New York, South Africa, and the Mediterranean. These two specimens likely represent either misidentified trematode species or cryptic species.

The 28S haplotype network revealed a clear geographic pattern of genetic diversity and connectivity among *Proctoeces maculatus* populations, consistent with the phylogenetic tree findings (Figure 3). The most common haplotype, referred to as 'US1', was widely shared among specimens from New York and all New England localities except Maine. Interestingly, US1 was also detected in a single specimen from Tunisia. This could indicate historical gene flow between the northeastern Atlantic and the Mediterranean, facilitated by host species movement. This finding aligns with the biology of the host species, as *P. maculatus* is known to parasitize fish hosts with broad geographic ranges and migratory behavior. Such host mobility could promote long-distance dispersal of the parasite, contributing to the observed genetic connectivity between distant populations. The Tunisian specimen was sampled from Bizerte Lagoon, which harbors *Mytilus galloprovincialis*, a species closely related to *M. edulis* (the two hybridize where their ranges overlap) (Barhoumi *et al.* 2014; Skibinski and Ahmad 1978). Mediterranean populations of *Mytilus galloprovincialis* are also known hosts of *P. maculatus* (Robledo *et al.* 1994). Transoceanic movement of mussels, whether intentional (e.g., through the aquaculture trade) or unintentional (e.g., hull fouling of infected mussels in shipping), likely facilitates occasional connectivity between populations in the western Atlantic and the Mediterranean.

The ME1 haplotype was exclusively shared by *P. maculatus* sampled from Maine mussels. This haplotype's isolation reflects significant differentiation from both U.S. and Mediterranean populations, reinforcing the idea of regional genetic structuring. Maine

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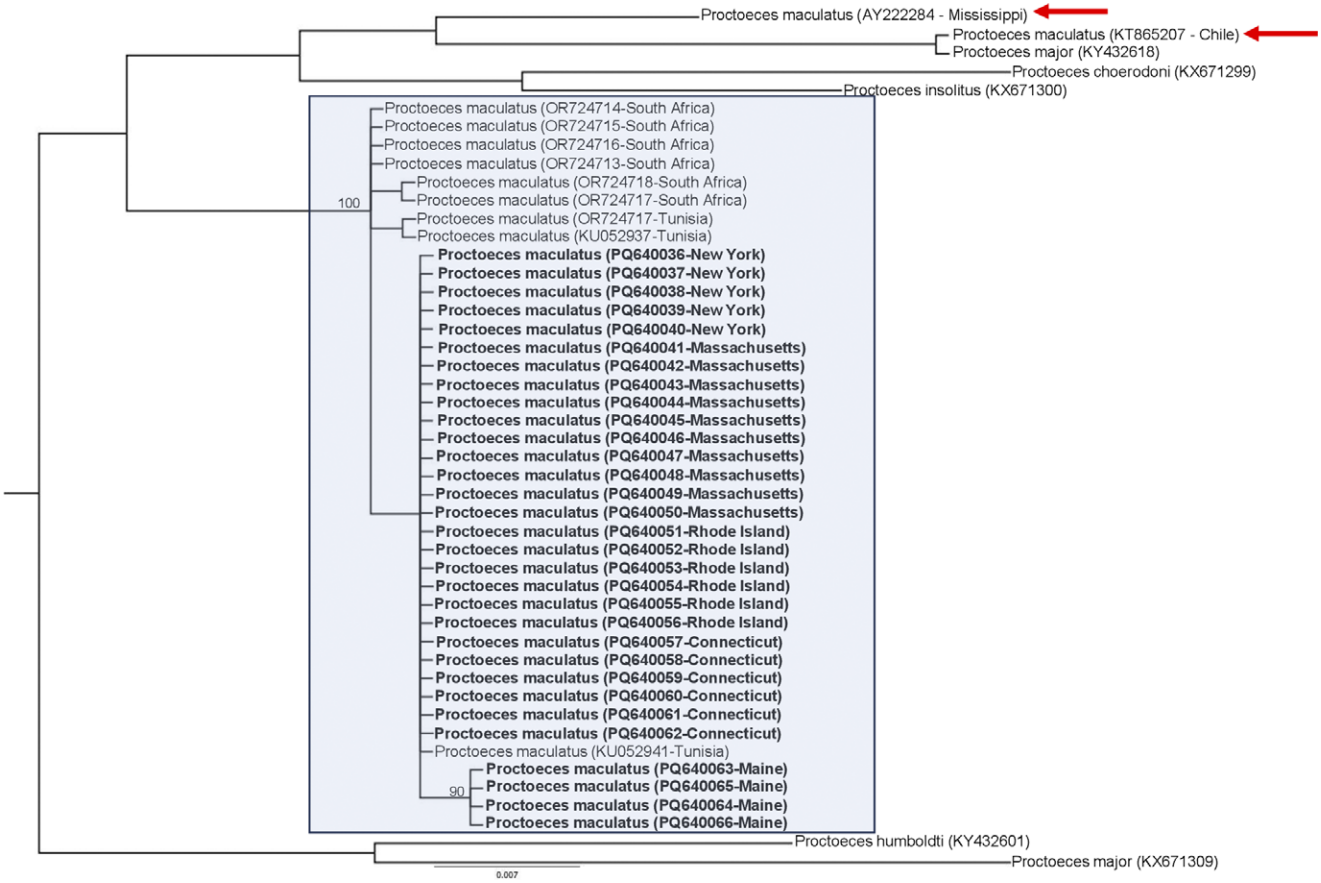

Figure 2. Bayesian phylogenetic tree of *Proctoeces maculatus* and related species obtained from analysis of the 28S rRNA marker. Values above branch nodes represent posterior probability support values derived from Bayesian inference analyses. The *P. maculatus* clade is highlighted, and arrows indicate highly divergent individuals of *P. maculatus* that have likely been misidentified.

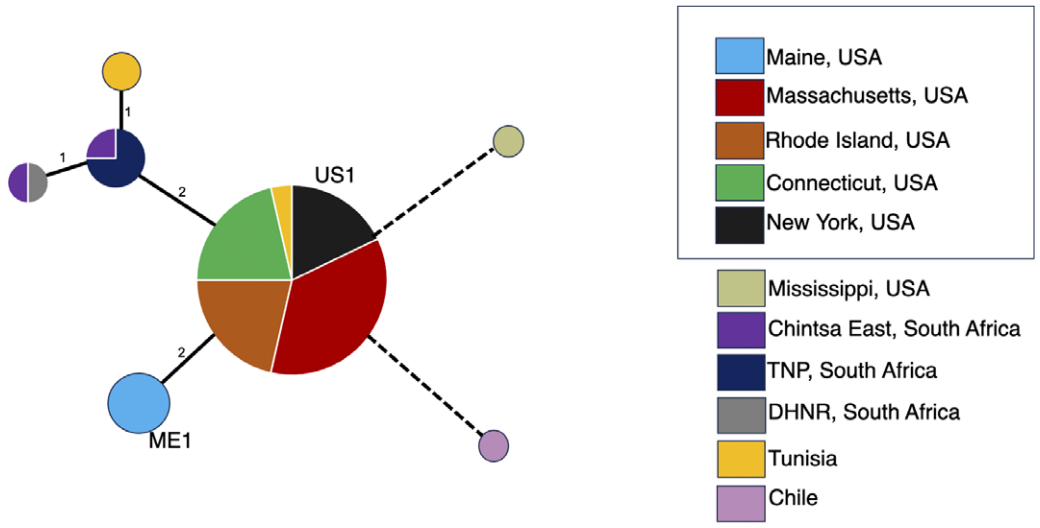

Figure 3. Haplotype network for *Proctoeces maculatus* based on 28S rRNA sequence data. The smallest circles represent a haplotype frequency of one. Each solid connecting line between haplotypes represents one mutational step, while values above lines represent additional mutational steps. Broken connecting lines represent more than 20 mutational steps.

represents the northernmost extent of *P. maculatus*'s range, and mussels in this region are subjected to environmental and oceanographic conditions distinct from those further south, specifically colder waters. In fact, Cape Cod is a well-documented phylogeographic barrier on the U.S. east coast, delineating distinct biogeographic regions and restricting gene flow for numerous marine species, including *M. edulis* (Altman *et al.* 2013; Jennings *et al.* 2009; Riginos and Henzler 2008). Although *P. maculatus*

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resides within the mantle tissue of its mussel host, which provides some insulation from environmental fluctuations, water temperature may still play an indirect role in shaping its distribution. Mussels, as ectothermic hosts, are directly affected by environmental temperature, which influences their health, growth, and reproduction – factors that could affect the parasite's establishment and persistence. Additionally, colder water temperatures in Maine may slow the developmental rates of *P. maculatus* larvae or alter host-parasite interactions, potentially favoring the fixation of a distinct haplotype in this region. For example, studies have found that higher temperatures can enhance transmission rates of trematode parasites in marine snails by increasing the activity and reproduction of both hosts and parasites, whereas colder temperature may reduce transmission efficiency (Friesen *et al.* 2021). One consequence of this is that in cooler climates, such reduced transmissions could lead to isolated parasite populations, promoting genetic divergence and the emergence of unique haplotypes. Alternatively, the isolated ME1 haplotype could reflect *P. maculatus*'s recent arrival to Maine (the species was first reported in Casco Bay, approximately 60 km north of our Maine sample site, in 2021). The northward range expansion of *P. maculatus*, likely driven by warming ocean temperatures or anthropogenic transport via aquaculture or shipping, could have introduced a small founder population with limited genetic diversity. This founder effect, coupled with the Gulf of Maine's distinct environmental conditions at the northern edge of *Mytilus edulis*'s North American range, may have facilitated the establishment of the unique ME1 haplotype. Similar patterns of reduced genetic diversity and haplotype isolation have been observed in other range-expanding parasites (Knapp *et al.* 2009; Wielgoss *et al.* 2008). Unfortunately, despite two years of sampling mussel hosts in the region (Davinack, unpubl. data), we were unable to obtain trematode samples from sites in New Hampshire, which are also located north of the Cape Cod barrier but south of Maine. We recommend that future studies focus on recovering additional samples from New Hampshire to better understand the connectivity of *Proctoeces maculatus* populations north of Cape Cod, which may provide additional genetic diversity information on the species in the northeast United States.

In the haplotype network, the two extreme divergent haplotypes recovered were from single specimens originating from Chile and Mississippi, USA. These required more than 45 mutational steps to connect to the main network, which mirrors their high levels of genetic divergence from other *P. maculatus* specimens and also provides additional evidence that they were either incorrectly identified or represent cryptic species.

Altogether, our results broadly support the hypothesis proposed by Vermaak *et al.* (2023), who argued that *Proctoeces maculatus* is indeed a cosmopolitan species, albeit one composed of unresolved cryptic lineages. Unraveling these lineages remains challenging for two primary reasons: (1) the morphology of isolates within *Proctoeces* is highly conserved, making it difficult to distinguish between taxonomically informative traits and phenotypic variation due to local adaptation, and (2) genetic data from the type locality (Trieste, Italy) and the type host (*Labrus merula*) is currently unavailable. Only with the acquisition of genetic data from topotypic material can definitive conclusions about cryptic species complexes within *P. maculatus* be drawn. Finally, while the identity of the Tunisian *P. maculatus* specimens from the Antar and Gargouri (2016) study was initially questioned due to their geographic separation from the type locality and association with a sparid fish host rather than the labrid host characteristic of *P. maculatus sensu stricto* (Vermaak *et al.* 2023), subsequent analysis concluded that these specimens are best considered *P. maculatus* when all available evidence is taken into account. This conclusion is further supported by the shared haplotype between the Tunisian specimen and the northeastern U.S. populations of *P. maculatus*, providing strong molecular evidence for their conspecificity. Such genetic connectivity is inconsistent with the hypothesis that the Tunisian specimen represents a different species, as interspecific genetic divergence would preclude haplotype sharing.

In conclusion, the genetic diversity and connectivity of *Proctoeces maculatus* populations, including evidence of haplotype sharing between the northeastern U.S. and Tunisia, underscore the complex interplay of biogeographic and anthropogenic factors driving parasite dispersal. The presence of the isolated ME1 haplotype in Maine suggests that environmental factors and recent range expansions both contribute to shaping parasite populations at the edge of their distribution. This is particularly concerning in light of the ongoing decline of blue mussel populations in the northeastern U.S., driven by overharvesting, climate change, and disease. Parasites such as *P. maculatus* may exacerbate these declines by imposing additional fitness costs on mussels, further stressing populations already at their environmental limits. Molecular characterization of parasites remains critical for understanding the broader ecological impacts of environmental change on marine ecosystems. Future population studies using multiple mitochondrial markers and high-resolution SNP datasets (e.g., microsatellites or RAD-Seq) will be needed to provide more fine-scale insights into the connectivity and population dynamics of *P. maculatus*.

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Competing interest. The authors declare none.

Ethical standard. Not applicable. The host is a marine invertebrate species; therefore, ethical approval under the laws of the United States (IACUC) was not required.

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