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ORIGINAL PAPER

Phylogeography of the marine interstitial nemertean *Ototyphlonemertes parmula* (Nemertea, Hoplonemertea) reveals cryptic diversity and high dispersal potential

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Abstract We conducted a phylogeographic study of the meiofaunal nemertean *Ototyphlonemertes parmula*, an apparent species complex from the littoral zone of coarse-grained beaches, using a 494-bp fragment of the mitochondrial cytochrome oxidase 3 gene (*cox3*). Six populations from the Gulf and Atlantic coasts of Florida, two from New England, and one from the Caribbean were sampled in March and August 2005. Three major lineages were identified, separated by *cox3* sequence divergence of 16–18%, with partially overlapping ranges. Tests for hybridization using ISSR markers detected nuclear gene exchange within but not between the major mitochondrial lineages, indicating the presence of cryptic species. One lineage dominating the Atlantic coast of Florida shows no evidence of geographic structuring. Another lineage shows a phylogenetic break between the Atlantic and Gulf coasts, suggesting that unsuitable habitat may act as a barrier to dispersal. Long-distance migration is evidenced by shared haplotypes between Florida and the eastern Caribbean. Overall, the widespread distribution of individual haplotypes and lack of structuring within geographic regions contrast with *O. parmula*'s strongly sediment-bound lifestyle. We speculate that dispersal of adults by storms and/or sediment transport may be more important than few and potentially short-lived planktonic larvae to explain geographic diversity in *O. parmula* and may be important for meiofauna in general.

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Introduction

Marine meiofauna, metazoans inhabiting the aqueous interstices of sand grains, represents an enormous diversity of life; however, meiofaunal organisms share certain ecological characteristics, including a general lack of free-swimming larvae (Higgins and Thiel 1988; Giere 2009). Though we might expect meiofauna to show a high degree of population structuring as a result (Bohonak 1999; Collin 2001), many meiofaunal species appear to have widespread distributions (Westheide et al. 2003; Giere 2009). The presence of such wide-ranging distributions despite the lack of a dispersive larval stage has been called the "meiofauna paradox" (Giere 2009), though the same phenomenon occurs among direct-developing macrofauna as well (Johannesson 1988; Rogers et al. 1998; Sponer and Roy 2002). In cases of the latter, several methods of colonization by adult individuals have been inferred (Paulay and Meyer 2002).

Molecular studies of marine meiofauna have shown that wide distributions often consist of multiple cryptic species (e.g., Rocha-Olivares et al. 2001; Casu and Curini-Galletti 2004; Derycke et al. 2008; Casu et al. 2009), a pattern seen

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in many marine organisms, including those with planktonic larvae (Knowlton 2000). In addition, meiofaunal species exhibit phylogeographic breaks—boundaries between intra-specific lineages—where no obvious barriers to dispersal exist, suggesting restricted gene flow (Derycke et al. 2008). Nonetheless, those intraspecific lineages may display a lack of genetic structuring over surprisingly long distances (Schizas et al. 1999; Derycke et al. 2008).

As with macrofaunal marine invertebrates, understanding the consequences of adult dispersal may help explain these patterns. While adults of some meiofaunal taxa actively enter the water column, where they may be dispersed by currents (Palmer 1988; Boeckner et al. 2009), other taxa avoid entering the water column by downward migration and adhesion to sediment (Higgins and Thiel 1988; Giere 2009). Nonetheless, individuals may be eroded from the sediment by wave or current action or dispersed with suspended sediment (Palmer 1988) as well as on macroalgae and other substrata (Thiel and Gutow 2005; Derycke et al. 2008). Relatively few phylogeographic studies of meiofauna exist (see Todaro et al. 1996; von Soosten et al. 1998; Rocha-Olivares et al. 2001; Westheide et al. 2003; Castro-Longoria et al. 2003; Casu and Curini-Galletti 2004; Derycke et al. 2005, 2007, 2008, 2010; Casu et al. 2009, 2011; Andrade et al. 2011), and fewer still across the otherwise well-studied southeastern US phylogeographic boundary (Schizas et al. 1999, 2002). As a result, we lack a good general understanding of population differentiation in the environment occupied by these organisms.

Ototyphlonemertes is a genus of meiofaunal nemerteans found in shallow marine sediment. It occurs in highest numbers and diversity in tropical and subtropical regions, though populations have been found as far north as 60° latitude (Envall and Norenburg 2001). *Ototyphlonemertes* exists preferentially in coarse sediment relatively free of silt or organic particulates. Thus, littoral species most commonly are found on beaches with well-sorted sediments, whereas sublittoral species usually are in areas with significant current activity (Norenburg 1988, pers obs). Females produce as few as ten oocytes to as many as about 200 (Norenburg, pers obs) during a one-year life cycle. A planktonic stage has been observed for some species (Chernyshev 2000; Norenburg and Stricker 2002), though it appears to be short-lived (Mock 1981), and larvae are rare in the plankton even in areas where adults are abundant (Norenburg unpubl obs). Adults avoid entering the water column by active vertical migration in the sediment (Kirsteuer pers comm; Mock and Schmidt 1975) and through the use of adhesive glands (Envall and Norenburg 2001).

Few molecular studies of *Ototyphlonemertes* exist (see Envall and Sundberg 1998; Andrade et al. 2011). A study based on 16S rRNA (Envall and Sundberg 1998) found that morphological species are paraphyletic on a population-level phylogeny, a result consistent with a morphology-based phylogenetic study (Envall and Norenburg 2001) that revealed a high degree of homoplasy in morphological characters. This opens the question of whether *Ototyphlonemertes* populations comprise multiple cryptic species, or whether the extensive morphological convergence indicates wide-ranging species whose traits covary with the environment.

Here, we present a phylogeographic study of *O. parmula* from the littoral zone of the North Atlantic, using a fragment of the mitochondrial cytochrome oxidase 3 gene (*cox3*) and nuclear ISSR markers. Since geographic structuring is often explained by a combination of contemporary (e.g., dispersal) and historical (e.g., vicariance and range expansion) causes (Avise 2004), we subject our mitochondrial sequence data to phylogenetic, population genetic, and demographic analyses. We use ISSR data to test for hybridization between mitochondrial lineages (e.g., Fortune et al. 2008), as well as to provide an independent test of the phylogenetic signal obtained from the mitochondrial marker (Ballard and Whitlock 2004). By examining the extent and causes of structuring in *O. parmula*, we seek to offer additional insight into the processes that shape diversity among marine meiofauna in general.

Materials and methods

Sample collection and *cox3* sequencing

Individuals were collected from the littoral zone at six coastal locations in Florida (by AYT, JLN, JMT; 26°25′18.83″N 82°4′48.50″W–27°51′45.05″N 80°26′50.44″W), two in New England (by JLN; 41°4′41.31″N 71°56′5.79″W and 41°28′0.60″N 70°34′34.24″W), and one in the Caribbean (by JLN; 17°54′25.70″N 62°49′25.07″W) in March and August 2005 (Fig. 1, Supplementary Materials Table 1). The sites were selected to sample multiple populations within separate geographic regions, though the exact locations were chosen opportunistically. Florida was sampled more densely than New England, since *Ototyphlonemertes* occur with greater diversity in Florida (Envall and Norenburg 2001). *O. parmula* has not previously been reported from regions north of Florida and has not been found in New Jersey, Delaware, Virginia, and North Carolina despite active efforts (Norenburg unpubl obs).

Samples were stored in 95% ethanol or in RNALater (Ambion, Inc). DNA was extracted from the whole individual using the QIAamp DNA Mini Kit (Qiagen, Inc). The mitochondrial *cox3* gene fragment was amplified using one of the following primer sets: *cox3*-forward-1 TGATGG CGGGATGTGGTTCGTGAAGG and *cox3*-reverse ACAAA ATGCCAATATCAAGCAGC, or *cox3*-forward-2 CAGTG

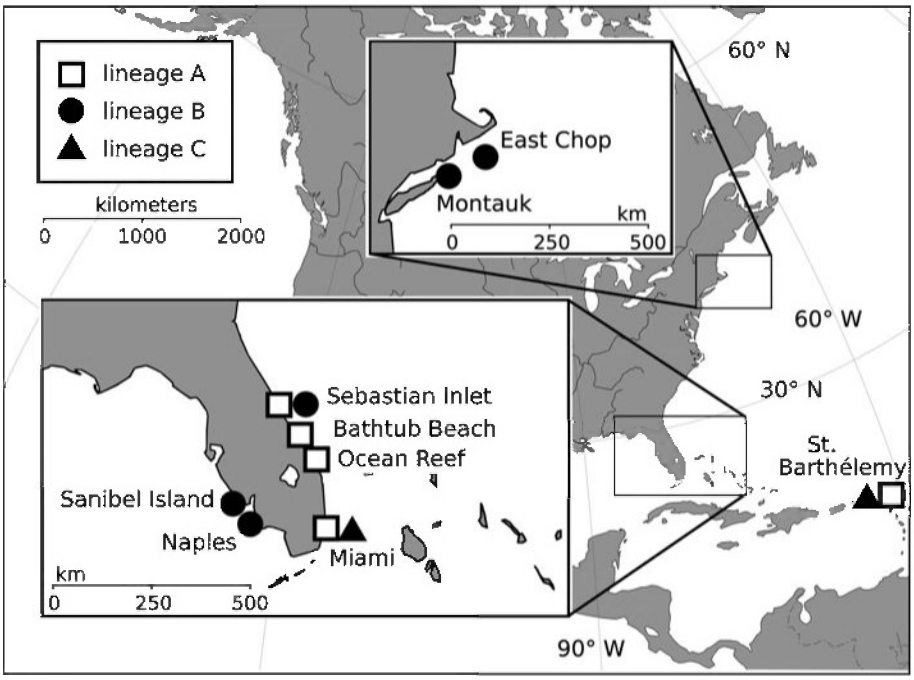


Fig. 1 Sampling locations and distributions of the three major *cox3* mitochondrial lineages of *Ototyphlonemertes parmula* identified in this study. *Open Square* lineage A, found on the east coast of Florida and in smaller numbers at St. Barthélemy; *Filled circle* lineage B, showing a distribution from the Gulf Coast to New England; *Filled triangle* lineage C, found at St. Barthélemy with isolated individuals occurring at Miami Beach. Detailed structuring within each lineage is shown in Fig. 2

ATGGCGGGATGTGGTTCGTGA and *cox3*-reverse (as above). We designed these primers based on an alignment of *cox3* sequences from related organisms and verified them using the OligoAnalyzer software v. 3.0 (Integrated DNA Technologies). Following amplification, PCR products were purified using ExoSAP-IT (USB, Inc.) and sequenced using the DYEnamic ET Dye Terminator Cycle-Sequencing Kit (Amersham Biosciences) on an Amersham Biosciences Megabace 1,000 automated DNA sequencer.

Cox3 data analysis

Nucleotide sequences were aligned using MAFFT v. 5.667 (Katoh et al. 2002). No internal gaps were present in the alignment. *Cox3* nucleotide sequences and translated amino acid sequences were entered into NCBI BLAST (Altschul et al. 1990), and both returned strong matches with *cox3* from other protostomes. *Cox3* had not previously been sequenced for nemerteans, so the BLAST results were taken as confirmation that the correct product had been sequenced.

MrModeltest v. 2.2 (Posada and Crandall 1998; Nylander 2004) was used to select a model of nucleotide substitution for the sequenced *cox3* fragment. The optimal model, HKY + G, was used to construct a maximum likelihood phylogeny of *cox3* haplotypes with the software PhyML v. 3.0 (Guindon and Gascuel 2003), using *Ototyphlonemertes lactea* as an outgroup. Tree search was performed using the SPR algorithm from a NJ starting tree. The transition/transversion ratio and gamma shape parameter (using four rate categories) were estimated as model parameters, whereas nucleotide frequencies were measured from the data. Tests for substitution saturation were performed with the software DAMBE v. 4.2 (Xia and Xie 2001) using the F84 model of nucleotide substitution (closely related to HKY). Support for the highest-scoring ML tree was determined using 100 bootstrap replicates and approximate likelihood ratio tests (Anisimova and Gascuel 2006). Additionally, a 95% statistical parsimony network (Templeton et al. 1992) was constructed using the software TCS v. 1.21 (Clement et al. 2000) to visualize relationships among *cox3* haplotypes.

Population differentiation based on mitochondrial haplotypes was measured in DnaSP using Hudson's (2000) S_{nn}, as well as Hudson et al.'s (1992) method of estimating F_{st} from sequence data. Population differentiation was analyzed using each site as a whole, as well as split by major mitochondrial lineage (since it is not known whether divergent lineages occurring at a single site are truly sympatric). One lineage (lineage C, Fig. 2) that was found almost exclusively at a single site was excluded from the latter analysis.

Tajima's (1989) and Fu and Li's F* (1993) were calculated for each mitochondrial lineage in order to detect demographic changes or episodes of selection. The McDonald and Kreitman (1991) test was conducted to detect evidence of positive selection on the *cox3* sequence. For the latter test, the major mitochondrial lineages were treated as separate species for the purpose of comparing synonymous and nonsynonymous substitutions, and pairwise comparisons were made between groups that showed violation of neutrality by other tests and those that did not.

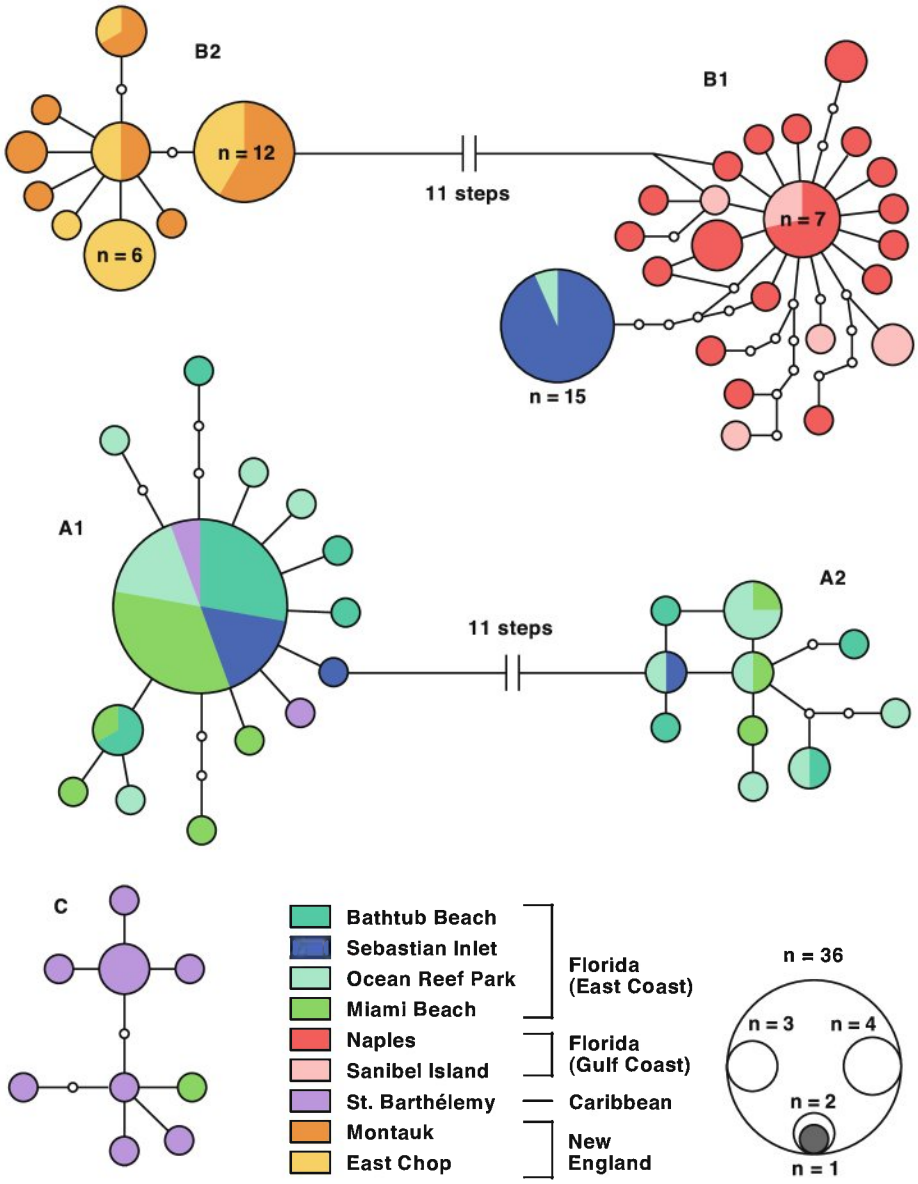


Fig. 2 Statistical parsimony network of *cox3* mitochondrial haplotypes for *O. parmula*, shaded by geographic distribution. Lineage names are indicated next to each haplotype group. All connections between nodes in the network are within 95% confidence limits of statistical parsimony, except for the 11-step connections between A1 and A2 and between B1 and B2, which are within 90% confidence limits

Neutrality tests were performed with the software DnaSP v. 4.10.8 (Rozas et al. 2003).

To examine the influence of demographic history on the pattern of diversity, mismatch analysis was performed using the software Arlequin v. 3.11 (Excoffier et al. 2005). For divergent mitochondrial lineages present in sympatry (e.g., lineages A1 and A2, Fig. 2), mismatch analysis was performed on both the separate and combined data sets. For the demographic expansion model, the parameters τ, θ_0, and θ_1 were estimated, where τ is the time in generations since the expansion, θ_0 is the initial population size, and θ_1 is the final population size, all scaled by the per-locus mutation rate (which is not known in this case). For the spatial expansion model, parameters were estimated as above, except that separate estimates are not made for θ_1 and θ_0 (population size is assumed to be constant). In addition, the number of migrants per generation during the expansion, M, was estimated. The fit of the model to the observed distribution was assessed using the sum of squared deviations (SSD). The significance of SSD, as well as the 95% confidence intervals for parameters and expected mismatch distributions, was calculated using 1,000 bootstrap replicates. In several cases, multiple populations were tested as a single deme because the question of interest (e.g., range expansion) concerned the combined populations, and because the populations were undifferentiated by S_{nn} and F_{st}.

ISSR amplification and data analysis

ISSRs, inter-simple sequence repeats, provide nuclear genotype data using single-primer PCR's (Zietkiewicz et al. 1994). ISSRs were amplified from total DNA using

the primers $(GA)_8T$ and $(CA)_8G$ at an annealing temperature of 46° and 50°C, respectively. These sequences are not expected to be present in the *Ototyphlonemertes* mitochondrial genome. The PCR profile was as follows: denaturing at 94°C for 90 s, then 37 cycles of denaturing at 94°C for 25 s, annealing for 40 s, and extension at 68°C for 80 s, followed by a final extension of 10 min at 68°C. The products were visualized on 1.5% agarose gel run at 1–2 V cm^{-1} for 6 to 12 h. Approximately 25% of the amplifications were repeated to test the reproducibility of bands.

Individuals were scored for the presence or absence of each gel band by visual examination on a computer screen. Bands appearing as the same size in different individuals were assumed to be homologous markers. If two bands of different size were similar enough that an individual could not be scored unambiguously, both bands were dropped from the analysis for all individuals. Individuals that could not be scored unambiguously for the remaining bands were likewise dropped. ISSR data were analyzed using *structure* v. 2.3 (Pritchard et al. 2000; Falush et al. 2007), distance-based tree methods, and AMOVA (Excoffier et al. 1992).

Data were input into *structure* as dominant markers (with band absence as the recessive allele). Models were tested with number of populations (K) ranging from 2 to 7, each using both independent and correlated allele frequencies. All runs used the admixture model to allow for hybrid genotypes. From the above combinations, the best model was identified based on the estimated probability of the data. This model was then used in an explicit test for hybridization using *structure*'s USEPOPINFO model, with a predefined population for each individual based on its best estimated membership. Runs to infer K and assign group memberships were done using 60,000 repeats with 10,000 "burn-in" repeats, and each run was repeated 3 times to check for convergence on the best solution. Runs to test for hybridization were done using 120,000 repeats with 20,000 "burn-in" repeats, with each run performed twice to check for repeatability.

A distance tree of ISSR genotypes was constructed in PAUP* v. 4.0b2 under the minimum-evolution criterion using a heuristic search with 100 random addition replicates and TBR branch swapping. The distance measure used was that of Dice-Sørensen, calculated from band presence–absence data using the program FAMD v. 1.10 (Schlüter and Harris 2006), and input into PAUP* as a user-specified distance matrix.

AMOVA was used to calculate Φ_{st}, an analog of F_{st}, by partitioning the variance in the ISSR data into within-deme and among-deme components. Because we are using ISSR to measure gene exchange between mitochondrial lineages, the analysis was conducted using mitochondrial lineages as demes. AMOVA was performed with the software GenAlEx v. 6 (Peakall and Smouse 2006), using 1,000 permutations to test the significance of pairwise Φ_{st} values. The Dice-Sørensen distance was used in place of the Euclidian metric to avoid counting shared band absences as homologies (Wolfe et al. 1998).

Results

Cox3 diversity and population differentiation

Sequence data were obtained for 494 bp of *cox3* from a total of 156 individuals of *O. parmula* representing nine geographical locations (Fig. 1, Supplementary Materials Table 1). A total of 65 unique haplotypes were identified (GenBank Accession nos GU306064-GU306128). These haplotypes grouped into three major lineages, both by statistical parsimony (Fig. 2) and maximum likelihood (Supplementary Materials Fig. 1) analysis. Haplotype frequency by population is illustrated in Fig. 2 and listed in Supplementary Materials Table 2.

The three major lineages are separated by an uncorrected sequence divergence of approximately 16–18%. One of these, lineage A, clustered into two distinct lineages, A1 and A2 (Fig. 2), separated by about 3% uncorrected sequence divergence. These occurred in sympatry at all four locations on the east coast of Florida, although a few A1-lineage individuals were found at St. Barthélemy approximately 2,000 km away. Another of the major lineages, lineage B, clustered into lineages B1 and B2, also separated by about 3% uncorrected sequence divergence. Lineage B1 occurred on the Gulf coast of Florida, with smaller numbers represented by a single haplotype on the east coast of Florida (Fig. 2). Lineage B2 was found only at New England locations. The third major lineage, lineage C, was found primarily at St. Barthélemy, although a single individual was found on the east coast of Florida. The sympatric A1 and A2 lineages show approximately the same genetic divergence as lineages B1 and B2, which are separated by a distance of at least 2,400 km. The Gulf and Atlantic coasts of Florida are mostly dominated by highly divergent lineages (lineage A on the Atlantic and lineage B on the Gulf).

Phylogenetic analysis found reciprocal monophyly among the three major lineages (with good support), as well as between lineages A1 and A2 (though with poor support). Lineage B2 was found to be nested within B1. Although B1 and B2 are not distinct phylogenetic lineages, we treat them here as separate evolutionary lineages based on the geographic and genetic distances between them. The branching order of the three major lineages was not well resolved, due in part to substitution saturation at the genetic distances separating them (data not shown). No significant saturation was detected at the low to moderate genetic distances within each lineage.

Table 1 Population differentiation for *Ototyphlonemertes parmula* based on *cox3* sequences for individuals of lineage A (above diagonal: F_{st} based on Hudson, Slatkin, and Maddison; below diagonal: Hudson's S_{nn})

	Btb	Orp	Mia	Seb
Btb	–	0.054	0.042	0.053
Orp	0.514	–	0.092	0.134
Mia	0.457	0.473	–	0.064
Seb	0.454	0.567	0.576	–

Differentiation is not significant ($P > 0.3$) for all population pairs

Table 2 Population differentiation for *O. parmula* based on *cox3* sequences for individuals of lineage B

	Seb	Mon	Ech	Nap	San
Seb	–	0.934	0.944	0.761	0.743
Mon	1.00**	–	0.044	0.820	0.796
Ech	1.00**	0.580‡	–	0.835	0.812
Nap	1.00**	1.00**	1.00**	–	0.008
San	1.00**	1.00**	1.00**	0.622	–

Above diagonal: F_{st} based on Hudson, Slatkin, and Maddison; below diagonal: Hudson's S_{nn} and significance

** Highly significant ($P < 0.001$)

‡ Borderline significance ($P = 0.068$)

Hudson's (2000) S_{nn}, as well as Hudson, Slatkin and Maddison's (1992) F_{st}, detected no differentiation between the two Gulf Coast populations at Naples and Sanibel Island, between the two New England populations at Montauk and East Chop, and among the three east coast Florida populations at Bathtub Beach, Ocean Reef, and Miami Beach (Supplementary Materials Table 3). The population at Sebastian Inlet, consisting of approximately equal frequencies of lineage A and lineage B individuals, was significantly differentiated from other Florida and Gulf populations. When lineages A and B were analyzed separately, lineage A showed no evidence of population differentiation in its range (Table 1) while lineage B individuals at Sebastian Inlet were significantly differentiated from those elsewhere (Table 2).

Haplotype network

The 95% statistical parsimony network of *cox3* haplotypes is shown in Fig. 2. Lineages A1 and B1 each show a star-like radiation from a central haplotype of high frequency. The 11-step connections between lineages A1 and A2 and between B1 and B2 were not homoplasy-free within the 95% confidence limit but were correct within 90% confidence and are displayed for clarity.

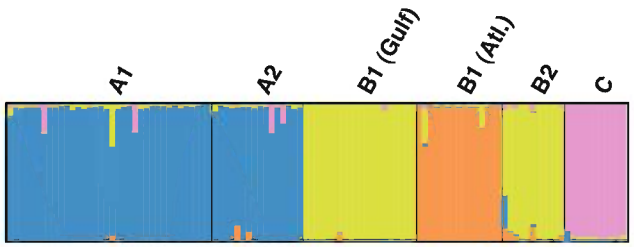


Fig. 3 Analysis of ISSR markers using *structure*. Labels above each section correspond to mitochondrial lineage (Fig. 2). B1 (Atl.) refers to the B1 haplotype on the Atlantic coast of Florida. Each of the four shades represents an ISSR cluster. Within a section, each column is an individual whose composition shows estimated membership in each cluster, under an admixture model with four clusters ($K = 4$). The image was generated from *structure* output using distruct1.1 (Rosenberg 2004)

ISSR diversity

ISSR banding patterns were obtained for 114 of the 156 individuals in the study. After discarding bands that could not be scored reliably, 15 unique, reproducible markers remained. A total of 37 genotypes were identified, where "genotype" refers to an individual's presence/absence states over all 15 markers. Images of the ISSR gels are available in Supplementary Materials Fig. 2. Gel annotation and scoring of individuals are shown in Table 4 and Table 5 of the Supplementary Materials, respectively.

Analysis with *structure* identified four clusters ($K = 4$, with correlated allele frequencies) as the best-fitting model. Individuals clustered as follows (Fig. 3): all individuals from mitochondrial lineage A grouped into ISSR cluster 1. All individuals from mitochondrial lineage B, except those found on the Atlantic coast of Florida, grouped into ISSR cluster 2. All individuals of mitochondrial lineage B from the Atlantic coast of Florida grouped into ISSR cluster 3. All individuals from mitochondrial lineage C grouped into ISSR cluster 4. Individual group identity under the admixture model was highly unambiguous, with greater than 0.95 proportion membership of the above mitochondrial lineages in each of the respective clusters. An explicit test of hybridization found only four individuals with less than 90% membership in their assigned clusters (Table 3). Examination of the ISSR banding patterns reveals that each inference of hybrid ancestry is based on a single band that is not found in other individuals of the same lineage but is common in the inferred source of introgression.

ISSR genotypes grouped into three major clusters according to a minimum-evolution tree (Supplementary Materials Fig. 3). At this level, the nuclear genealogy shows complete concordance with the high-level mitochondrial lineages (rather than with geography). Individuals from the sympatric A1 and A2 mitochondrial lineages did

Table 3 Possible hybrids from explicit hybridization test between *O. parmula* lineages using *structure* (sample IDs correspond to population names and to sample numbers from the Supplementary Materials; admixture "zero generations back" means that the individual is a migrant; admixture one or two generations back means that the individual has a parent or grandparent from the other cluster, respectively)

Sample ID	Assumed ISSR cluster/ *cox3* lineage	P(assumed cluster)	Source of admixture	P(admixture) by # generations back		
				0	1	2
Mia-9	1/A	0.523	2/B	0.003	0.115	0.267
Orp-19	1/A	0.793	3/B1-Atlantic	0.001	0.000	0.195
Mia-20	1/A	0.780	4/C	0.001	0.008	0.206
Ech-7	2/B	0.842	1/A	0.003	0.007	0.138

not form separate clusters based on the nuclear data. Although B1 and B2-lineage individuals also did not form separate clusters, they were differentiated by AMOVA, share relatively few bands (Supplementary Materials Table 5), and differ in terms of genotype composition (Supplementary Materials Fig. 3). One shared ISSR genotype was found between lineages B1 and B2; however, this genotype consists of only a single band presence among the 15 scorable loci.

AMOVA

ISSR data were used to obtain pairwise Φ_{st} values between mitochondrial lineages to test for gene flow between them (Table 4). Because the B1-lineage individuals on the east coast formed a distinct ISSR cluster, these were treated as a separate deme to examine their relationship to each mitochondrial lineage. Overall pairwise Φ_{st} values were high between lineages, indicating high differentiation. The sympatric A1 and A2 lineages were the exception, showing no differentiation based on ISSR data ($\Phi_{st} = 0$, $P = 0.38$). Combined with the ISSR phylogeny and *structure* results, this suggests that these lineages are undifferentiated at the nuclear level, and they were combined for subsequent AMOVA. In contrast, the B1-lineage individuals on the east coast showed a high pairwise Φ_{st} (0.755, $P < 0.001$; Table 4) with the combined A1 and A2 lineages that share their range. This value is higher than that between the A1 + A2 lineage and the geographically distant B2 ($\Phi_{st} = 0.537$) and C ($\Phi_{st} = 0.697$) lineages.

Table 4 AMOVA of ISSR data for *O. parmula* using mitochondrial lineages as demes (below diagonal: pairwise Φ_{st} values; above diagonal: *P* value for $\Phi_{st} = 0$)

	C	B2	A1 + A2	B1 (Atl.)	B1
C	–	0.001	0.001	0.001	0.001
B2	0.521	–	0.001	0.001	0.001
A1 + A2	0.697	0.537	–	0.001	0.001
B1 (Atlantic)	0.887	0.452	0.755	–	0.001
B1	0.826	0.333	0.733	0.828	–

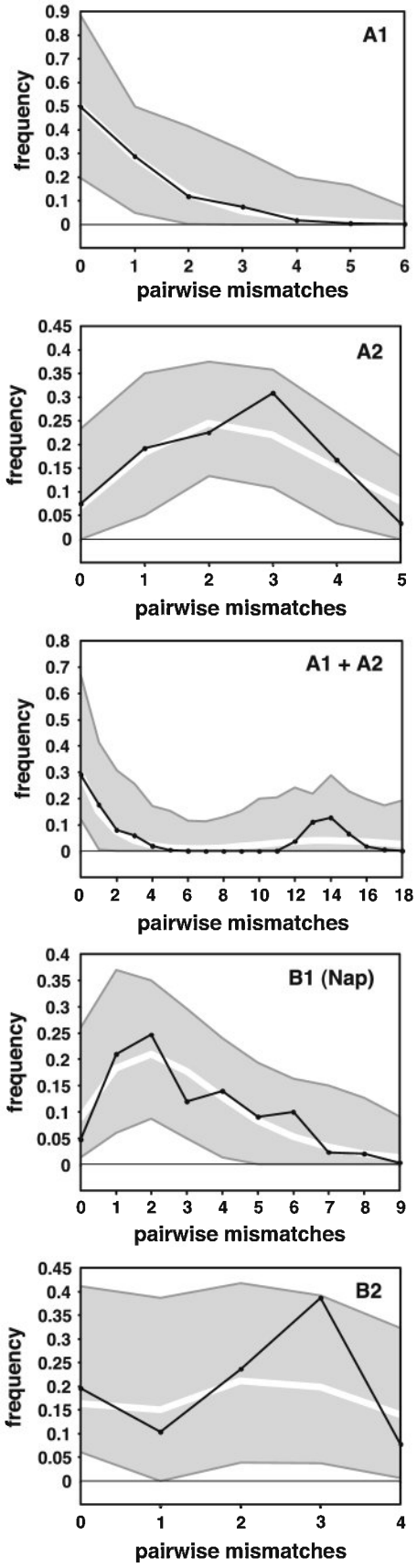
Table 5 Tajima's D and Fu and Li's F* tests for neutral evolution of *cox3* in *O. parmula* by mitochondrial lineage

	Tajima's D	*P* value	Fu and Li's F*	*P* value
All *O. parmula*	2.996	(<0.01)	1.193	(>0.10)
B1	−1.497	(>0.10)	−2.631	(<0.05)
B1 (Nap only)	−2.169	(<0.05)	−3.018	(<0.05)
B2	−0.5726	(>0.10)	−0.8282	(>0.10)
C	−1.083	(>0.10)	−1.687	(>0.10)
A1 + A2	−0.5871	(>0.10)	−1.977	(>0.05)
A1	−2.440	(<0.01)	−4.893	(<0.02)
A2	−1.043	(>0.10)	−1.167	(>0.10)

Selective neutrality and mismatch distributions

Results of neutrality tests based on *cox3* sequence data are shown in Table 5. A significant positive value of Tajima's D was obtained for the combined set of all *O. parmula* haplotypes. This often results when a subdivided population is pooled for analysis (Simonsen et al. 1995). Significant negative values of Tajima's D and Fu and Li's F* were obtained for the Naples population and for lineage A1. Negative values typically result from population growth following a bottleneck, or a selective sweep (Tajima 1989; Simonsen et al. 1995). The McDonald–Kreitman test found no evidence of selection in the *cox3* sequence itself (data not shown).

Mismatch distributions plotted against expected distributions under a model of spatial expansion are shown in Fig. 4. Parameter estimates and measures of deviation from the model are shown in Supplementary Materials Table 6. The population at Naples (B1 lineage) fit a spatial expansion model only slightly better (SSD = 0.0096) than a demographic expansion model (SSD = 0.0154). Combined with Tajima's D, the mismatch distribution is consistent with growth following a population bottleneck. The New England populations (B2 lineage) fit a spatial expansion



◀ **Fig. 4** Mismatch distributions for *cox3* nucleotide sequences from *O. parmula*. *Black lines* correspond to observed distributions from the indicated lineages; *white lines* and *shaded* areas correspond to expected distributions (with 95% confidence intervals) under a model of spatial expansion. Demographic expansion models were similar except that lineage B2 and combined A1 + A2 lineages fell outside the 95% confidence intervals

model better (SSD = 0.0434) than a demographic expansion model (SSD = 0.0521), and the observed distribution was outside the 95% confidence interval for the latter. However, both models were comparatively poor fits in this case.

Distributions for lineages A1 and A2 were explained approximately as well by a demographic expansion model as by a spatial expansion model with very high migration among demes. Along with the neutrality statistics above, the mismatch distributions are consistent with expansion from a bottleneck in lineage A1, as well as with independent expansion events in the presently admixed A1 and A2 lineages. Lineages A1 and A2, when combined, showed a bimodal mismatch distribution that fell within the 95% confidence interval for a spatial expansion model. However, bimodal distributions such as this also occur when subdivided demes are pooled for analysis (Marjoram AND Donnelly 1994), and a model of independent range expansions for A1 and A2 is a much better fit to the data (Fig. 4).

Estimates of τ suggest similar time since putative expansion events for lineage C ($\tau = 2.85$), A2 ($\tau = 2.69$), and B2 ($\tau = 2.82$), and a much more recent time for lineage A1 ($\tau = 0.14$ for spatial expansion or 0.28 for demographic expansion). In each of these cases, high gene flow was inferred under the spatial expansion model. Range expansion for the combined A1 and A2 lineages would be a much older event ($\tau = 12.92$) with relatively low gene flow ($M = 0.68$). However, we should note that the 95% confidence intervals on τ and M are rather wide (Supplementary Materials Table 6).

Discussion

Phylogenetic structure and cryptic species

We observe a relatively high (16–18%) genetic distance between major mitochondrial lineages, compared to approximately 3% within lineages. Other studies of benthic meiofaunal taxa have delineated cryptic species based on a similar pattern of high sequence divergence between species compared to divergence within species (see Rocha-Olivares et al. 2001; Derycke et al. 2008, 2010). In addition, we find agreement between mitochondrial and nuclear clusters based on *structure* analysis and phylogenetic

comparison, high nuclear differentiation between mitochondrial lineages, and an absence of hybrid genotypes in sympatry. These results suggest that the three major mitochondrial lineages are distinct biological species. The evidence of reproductive isolation is strongest between lineages A and B, which are genetically distinct despite overlapping ranges. Lineage C shows limited co-occurrence with the other lineages at our study sites; thus, we cannot be as confident that it is reproductively isolated.

Unlike the major mitochondrial lineages, which show a lack of shared nuclear markers in sympatry, lineages A1 and A2 share nearly all of their markers (Table 4 and Supplementary Materials Table 5). Due to our relatively small number of markers, we may have missed differences between them. However, lineages B1 and B2, which are divergent to approximately the same degree as A1 and A2 in terms of *cox3* sequence, show little or no sharing of ISSR genotypes (Supplementary Materials Fig. 3) or alleles (Table 4). This suggests that the lack of ISSR differentiation between A1 and A2 is the result of interbreeding, rather than shared ancestry or poor resolution.

Four individuals of potential hybrid ancestry were identified by analysis in *structure*, three of which are possible hybridizations given the distribution of each lineage: one between lineages A and B at the Mia site; one between lineages A and B at the Orp site; and one between lineages A and C at the Mia site (Table 3). No hybrid genotypes were detected at the Seb site where co-occurrence of A and B lineages is greatest, and no likely F1 hybrids were detected at any site. In each of the above cases, the inference of hybridization is based on the apparent introgression of a single ISSR marker. Since unrelated markers of similar size may be indistinguishable on an agarose gel, an inference based only on a single marker has a high probability of error. Thus, we do not believe that the above individuals represent compelling evidence of hybridization; however, we cannot rule out a low level of gene flow between species.

No qualitative difference in morphology is evident between lineages A and B (Tulchinsky 2006), suggesting that these lineages have experienced morphological stasis since the time of their divergence. A slight difference is seen in the shape (length to width ratio) of the stylet between the two lineages, although there is no difference in how the length increases with an increase in width (Tulchinsky 2006). In addition, the middle chamber of the proboscis is approximately 17% longer in lineage A compared to lineage B (Tulchinsky 2006). Both the stylet and the middle chamber are components of the feeding apparatus and thus may have diverged under ecological pressure; however, both differences may be also explained by a correlation with body size (Gould 1966), which by itself is not a useful character for distinguishing species within *Ototyphlonemertes* (Envall and Sundberg 1998). Nonetheless, either a difference in morphometry of feeding structures or in body size could contribute to habitat partitioning, which would isolate the lineages to some degree in terms of competition and mating. Given the genetic divergence between the lineages, however, intrinsic pre- or post-mating isolation is likely as well.

Dispersal and range expansion

Two mitochondrial lineages (A1 and A2) show a continuous distribution along the east coast of Florida, with no evidence of structuring within either, despite likely poor larval dispersal. This may indicate high gene flow due to adult dispersal, which has been inferred for other direct-developing marine invertebrates (e.g., Rogers et al. 1998; Sponer and Roy 2002). The two New England populations do not show significant structuring in terms of F_{st} and S_{nn} based on *cox3* sequences; however, they do appear to show more structuring than the Atlantic Florida populations (Tables 1, 2; Fig. 2). This may be because the Florida sites experience a higher frequency of hurricanes and other powerful storms, which cause large quantities of sediment to be suspended in the water column and transported by oceanic currents (Wren and Leonard 2005). In addition, the Florida sites, unlike the New England sites, are joined by mostly continuous habitat, where sediment suspended by normal wave activity can be transported by long shore or tidal currents. Because *Ototyphlonemertes* is behaviorally and morphologically adapted to remain attached to sediment (Kirsteuer 1977; Envall and Norenburg 2001), either form of sediment transport may play a dominant role in its dispersal, though the degree to which local mixing translates into long-distance gene flow is unclear (McNair et al. 1997).

Sediment transport alone seems unlikely to explain the distribution of the B1 lineage, which is present on both coasts of Florida, but on the Atlantic coast appears almost entirely restricted to the Sebastian Inlet population. Lineage A shows no evidence of structuring along the east coast of Florida, so the restricted distribution of lineage B1 is somewhat puzzling. If populations are mixed by ongoing dispersal, we would expect to find individuals of lineages A and B1 in similar proportions at all east coast Florida sites (see also Andrade et al. 2011). However, our sampling may not be representative of the actual proportions, especially if habitat partitioning is present.

Recent (i.e., post-pleistocene) range expansion may explain both the structuring within lineage B1 and apparent lack of population differentiation within lineage A. If lineages have occupied their current range long enough for an equilibrium to have been reached between gene flow and genetic drift, then lack of differentiation implies high levels of gene flow. However, if populations are not at equilibrium,

then their pattern of diversity may be dominated by historical events. Because of the large population sizes of meiofaunal species, populations take longer to become differentiated by genetic drift, so structuring may not be apparent for a relatively long time following the cessation of gene flow.

Recent range expansion of lineages A1 and A2 into the Atlantic coast of Florida is consistent with the observed signature of population growth (Table 5; Fig. 4; Slatkin and Hudson 1991) and would explain the apparent lack of genetic structure if the expansion was sufficiently rapid and population sizes sufficiently large that differentiation had not yet had time to occur (Slatkin 1993). Such a pattern has been reported from post-pleistocene expansions of macrofauna in Europe and North America (Hewitt 2000). Among meiofauna, rapid range expansion may be facilitated by adult dispersal, which increases the probability that rare long-distance transport will result in successful colonization (Johannesson 1988).

Mismatch analysis of lineages A1 and A2 under a model of independent range expansions estimates a high inter-deme migration rate during each expansion (Supplementary Materials Table 6), suggesting that present-day gene flow would be high as well. However, a high migration rate would be inferred even if dispersal was rare, provided the expansions occurred recently and were rapid relative to the substitution rate of the marker (Ray et al. 2003; Excoffier 2004). A follow-up study using faster-evolving markers (e.g., microsatellites) may be able to detect structuring that the present study missed and thus distinguish between ongoing gene flow and the signature of past range expansion.

Demographic analyses also are consistent with a dynamic of recurrent population extinction and recolonization. Metapopulation dynamics may especially be important on subtropical coasts, where hurricanes can scour bottom sediment to substantial depths (Teague et al. 2006), and local populations of *Ototyphlonemertes* have been observed to undergo extinction or near-extinction following hurricanes (Norenburg, pers obs). However, the conditions under which recurrent extinction and recolonization could result in reduced (rather than increased) structuring between populations are rather limited based on theoretical considerations (Wade and McCauley 1988; Barton and Whitlock 1997) and are unlikely to apply in our case.

In addition, a selective sweep of the mitochondrion may leave a genetic signature similar to population growth (Tajima 1989). The McDonald–Kreitman test does not indicate selection on the *cox3* fragment sequenced, though selection elsewhere in the mitochondrial genome can affect linked markers through hitchhiking (Maynard Smith and Haigh 1974; Bazin et al. 2006). However, ISSR genotypes show a concordant phylogeographic pattern, suggesting that our results have not been systematically biased by selection.

Phylogeographic divisions

The discontinuity between lineages B1 (Florida) and B2 (New England) is consistent with known biogeographic boundaries in the region, including the well-studied Cape Canaveral boundary which is believed to be the product of hydrogeographic conditions (Reeb and Avise 1990; Collin 2001). However, in the case of *O. parmula,* a long expanse of unsuitable habitat north of Florida likely acts as an additional barrier to gene flow. A similar region of unsuitable habitat exists around the southern tip of Florida (Norenburg, pers obs) and may help explain why the Gulf and Atlantic coasts of Florida are dominated by different lineages despite currents connecting them, as well as why the B1 lineage on the Atlantic appears to be genetically isolated from the Gulf. However, some studies of coastal organisms have found phylogeographic disjunctions even in the absence of such barriers, reflecting maintenance of historical structuring (e.g., Waters et al. 2005).

The phylogenetic nesting of lineage B2 within the diverse subtropical B1 lineage suggests that B2 may have originated in the latter. The habitat presently occupied by lineage B2 was glaciated during the last glacial maximum (LGM), and post-glacial colonization from populations in Florida is inconsistent with the sequence divergence between the groups; thus, lineage B2 likely spread to New England from past Atlantic populations. Despite extensive searching along the mid-Atlantic coast, only one site in North Carolina (Norenburg unpubl obs) is known to have sand that is sufficiently sorted and coarse; it has yielded one species of *Ototyphlonemertes*, of a morphotype different from *O. parmula* (Kirsteuer 1977). The existence of a northern refugium is unlikely given the current range limitation of *Ototyphlonemertes* to below 60°N (Envall and Norenburg 2001). This suggests that coarse-grained beaches supporting littoral *Ototyphlonemertes* may have existed on the mid-Atlantic coast of North America during the LGM. Comparative studies of coastal meiofauna may support this idea.

Vicariance due to Pleistocene sea level changes has been invoked to explain the initial divergence of lineages split by the northeast Florida boundary and other coastal biogeographic boundaries (Reeb and Avise 1990; Lavery et al. 1996). While no molecular clock has been calibrated for meiofaunal nemerteans, the substitution rate of *cox3* is likely higher than that of the relatively well-conserved *cox1*, whose estimates range from 2.8 to 6.0% my^{-1} in other marine invertebrates (Young et al. 2002; Lessios et al. 2003). Thus, *cox3* sequence divergence of approximately 3% between Florida and New England lineages (B1 and B2) is roughly consistent with Pleistocene vicariance, when the Gulf was separated from the Atlantic by low sea levels.

Lineages A1 and A2, also divergent by approximately 3%, are present in sympatry on the east coast of Florida, suggesting secondary contact following allopatric isolation (Avise et al. 1987). The degree of divergence suggests that, like B1 and B2, these lineages may have been isolated by Pleistocene sea level changes. If that is the case, secondary contact between A1 and A2 may have occurred when rising sea levels at the end of the Pleistocene removed the barriers to expansion by current transport (Reeb and Avise 1990). Several unsampled areas are connected by currents to the Atlantic coast of Florida (Gyory et al. 2005; Rowe et al. 2005) and may provide more direct evidence for this hypothesis.

These currents, including the east-to-west currents of the Caribbean (Gyory et al. 2005) and Antilles (Rowe et al. 2005), may explain the apparent dispersal of a lineage C individual from St. Barthélemy to the east coast of Florida. However, the degree to which gene flow is possible between these two sites is unclear. Several macrofaunal species show phylogeographic breaks in the intervening region (e.g., Baums et al. 2005), but to date no studies of meiofauna exist for the northern Caribbean. The presence of three A1-lineage individuals at St. Barthélemy cannot be explained by the dominant current patterns. Anthropogenic transport is one possibility; however, until the intervening region can be sampled, it is unknown whether any long-distance transport of individuals has occurred, or whether one or both lineages are distributed throughout the Caribbean. Long-distance transport over open ocean, though it may be rare, has been detected in other studies of coastal meiofauna (von Soosten et al. 1998; Westheide et al. 2003).

Taxonomy and species delimitation

Resolving morphologically cryptic species with respect to taxonomy is a problem increasingly faced by all biologists interested in biological diversity (Hart and Sunday 2007). The "Fila" morphotype of Envall and Norenburg (2001) contains two named species, *O. fila* and *O. parmula*. Corrêa (1950, 1953) described both species from Ilhabela (São Sebastião), Brazil, and distinguished them based on morphology of the proboscis and caudal adhesive plate; however, subsequent studies have found only one Fila-type species in Brazil (Norenburg unpubl obs; Andrade et al. 2011).

Corrêa (1961) assigned the Fila morphotype she reported from Florida to *O. fila* rather than *O. parmula*. For that reason, we initially assumed the species in our study to be *O. fila* (Envall and Norenburg 2001; Tulchinsky 2006). However, Andrade et al. (2011) found strong evidence that our North Atlantic specimens comprise a monophyletic group within a Brazilian *O. parmula* clade. Furthermore, the Brazil and Florida specimens share a feature not recognized by Corrêa and, to date, not known for any other *Ototyphlonemertes* morphotype: a vesicular sigmoid region at the anterior of the posterior proboscis (fig 6C Envall and Norenburg 2001).

Therefore, we assign our specimens to *O. parmula* based on nomenclatural priority. The two Brazil *O. parmula* lineages are moderately to highly diverged from the Florida lineages (approximately 6–14% *cox3* divergence; Andrade in litt). Thus, Brazilian and North Atlantic *O. parmula* together comprise a species complex, and there is no basis for upholding Corrêa's original inference of two morphologically distinct species. We consider *O. fila*, as used by Corrêa (1953, 1961) and by subsequent authors (e.g., Envall and Norenburg 2001; Tulchinsky 2006; Norenburg 2009), to be a synonym of *O. parmula*.

Conclusion

Our findings share several features with other meiofaunal phylogeographic studies, suggesting general patterns in meiofaunal diversification. A lack of genetic structuring within relatively wide geographic regions, despite limited dispersal potential, may suggest a high degree of passive adult dispersal (Schizas et al. 1999; Derycke et al. 2008). At the same time, however, we detect instances of unexplained structuring. This apparent contradiction may be explained by infrequent long-distance dispersal combined with a high colonization success rate (Derycke et al. 2008). Such a dispersal mode may promote rapid range expansion while maintaining boundaries that reflect historical events. Whereas rafting on macroalgae or other floating substrates may facilitate this mode of dispersal in some systems (Thiel and Gutow 2005), transport of storm-suspended sediment may play a similar role for *O. parmula*. We see evidence of trans-Caribbean dispersal, supporting other studies that infer long-distance dispersal of coastal meiofauna over open water, including transoceanic transport (von Soosten et al. 1998; Westheide et al. 2003). Such cases of cosmopolitanism are counterbalanced by the frequent detection of cryptic species, often with overlapping ranges (Rocha-Olivares et al. 2001; Derycke et al. 2008, 2010), pointing to a history of allopatric isolation followed by secondary contact.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards All experiments comply with the current laws of the country in which they were performed.

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