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Phylogeography of Marine Meiofaunal Nemerteans of the Ototyphlonemertes Fila Species Complex

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PHYLOGEOGRAPHY OF MARINE MEIOFAUNAL NEMERTEANS OF THE *OTOTYPHLONEMERTES FILA* SPECIES COMPLEX

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Abstract

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Morphological conservatism combined with intraspecific variability has obstructed studies of speciation and species boundaries among marine meiofauna. *Ototyphlonemertes* is a genus of meiofaunal nemerteans inhabiting the interstitial spaces of marine sediments. Its members lack pelagic larvae and dispersal potential is believed to be poor. A phylogeographic study of *Ototyphlonemertes fila* is presented using mitochondrial (*cox3*) and nuclear (ISSR) molecular markers. Deep genetic divergence (approximately 18% in *cox3*) was observed between sympatric mitochondrial lineages in Florida. This divergence

was reflected in the nuclear marker as well, suggesting the presence of two cryptic species. The first contains Florida and New England populations separated by 3% *cox3* sequence divergence and showing no evidence of ongoing gene flow. The second contains two co-distributed mitochondrial clades in Florida separated by 3% *cox3* sequence divergence and showing exchange of nuclear alleles. Surprisingly, relatively little fine-scale structuring was found, suggesting that passive dispersal is significant over moderate geographical distances.

Introduction

Marine interstitial meiofauna, also known as mesopsammon, are those organisms that live in the interstitial spaces of marine sediment. Mostly ignored before the early 20th century, this environment has been found to contain a vast diversity of life. Most animal phyla have interstitial representatives, and some phyla, such as Gnathostomulida and Kinorhyncha, have been found only in the interstitial environment (Higgins & Thiel 1988). Although the ecology of meiofauna has been extensively studied, relatively few molecular studies have been conducted on meiofaunal taxa (see Schizas et al. 1999, Castro-Longoria et al. 2003, Derycke et al. 2005, Casu & Curini-Galletti 2006). Consequently, little is known about population structure and speciation in the interstitial environment.

Most interstitial meiofauna lack a pelagic larval stage, and are incapable of existing for prolonged periods of time outside of the sediment. Nonetheless, morphologically indistinguishable populations have been recovered over vast geographical distances, giving the appearance of cosmopolitan or trans-oceanic species (Westheide et al., 2003). Many species of marine macroinvertebrates also appear to have a global distribution, a phenomenon that has been explained by their long-range dispersal capabilities. However, recent molecular evidence has indicated that many species believed to be cosmopolitan are actually a complex of morphologically cryptic sister species. Morphological conservatism,

combined with unseen barriers to gene flow, may mask much of the species diversity in marine environments (Knowlton 2000).

As is the case with macrofaunal species, a few meiofaunal species appear to be truly cosmopolitan based on molecular data (Westheide et al. 2003). In these cases, the mechanism of dispersal remains a mystery. In many cases, however, molecular studies have revealed the presence of cryptic species, often separated by high genetic distances (Derycke et al. 2005, Castro-Longoria et al. 2003). Among meiofauna, many potentially diagnostic morphological characters have been lost due to reduced body size. Thus, great genetic diversity may be hidden behind morphological simplicity. In addition, reduced body size may be accompanied by relaxed developmental constraints (Hanken & Wake 1993), resulting in great intraspecific variation in those few morphological characters that remain. This further obscures species boundaries among meiofaunal organisms.

Ototyphlonemertes is a genus of meiofaunal Nemerteans with a global distribution in intertidal and subtidal coastal areas. They occur in highest numbers and diversity in tropical and subtropical regions, though populations have been found as far north as 60 degrees latitude (Envall & Norenburg 2001). Individuals are generally less than 10mm long and 0.3 mm in diameter. The genus is monophyletic, characterized by lack of adult ocelli, and is one of two genera in the family Ototyphlonemertidae, which is characterized by the presence of a pair of statocysts located on the ventral ganglia (Norenburg 1988). *Ototyphlonemertes* is found only in coarse sediment free of silt or organic particulates, and

so is restricted to relatively high-energy beaches or subtidal areas with significant current activity (Norenburg 1988).

Ototyphlonemertes is a greatly understudied group of organisms. Few molecular studies of the genus exist (see Envall & Sundberg 1998 and Thollesson & Norenburg 2003) and our knowledge of species-level relationships is limited. The organisms possess few reliable morphological characters for phylogenetic analysis; furthermore, existing species descriptions are inconsistent in terms of characters considered, and most describe local varieties on individual beaches. Recently, Envall & Norenburg (2001) have summarized character states for a large number of described species and previously undescribed local varieties, and identified five phylogenetically reliable morphological characters that divide the genus into six taxa termed "morphotypes". These morphotypes co-occur at individual localities on a global scale, as would be expected of distinct species with overlapping distributions. At any one site, individuals belonging to a morphotype form a homogenous group that is distinct from any other morphotypes present. On a larger scale, however, each morphotype is composed of many local varieties. Though nearby varieties differ only slightly, the "endpoints" of this variability would appear to be morphologically distinct species in the absence of any intermediate forms. Because few molecular studies have been conducted on the genus, it is not known whether the local varieties are ecotypes resulting from developmental plasticity, genotypic varieties of a single species, or separate species.

The Fila morphotype includes the recognized species *O. fila* as well as several morphological variants of unknown taxonomic status, and therefore can be considered a species complex. Here, a phylogeographic study of the Fila morphotype is presented using populations from the Atlantic and Gulf coasts of North America and the Caribbean. Its purpose was to gain insight into the pattern of morphological variation present in the genus, to test for the presence of cryptic species, and to test for historical evidence of introgressive hybridization between divergent populations as a possible mechanism for the evolution of diversity within the genus.

A fragment of the mitochondrial cytochrome oxidase 3 gene (*cox3*) was chosen for this investigation. Mitochondrial markers have frequently been used for phylogeographic analysis; however, because mitochondrial DNA replicates without recombination in most taxa, the mitochondrial genome constitutes a single molecular marker (Avise 2004). A number of processes may cause the mitochondrial marker to reflect a history different from the nuclear genes of an organism, including selection, incomplete lineage sorting, and introgression (Avise 2004). To obtain a more complete view of the history of and relationships among populations, nuclear ISSR markers were included in the study. ISSRs, or inter-simple sequence repeats, have been used in a number of population genetic and systematic studies (Zietkiewicz et al. 1994; Wolfe et al. 1998; Reddy et al. 1999; Kostia et al. 2000). Although ISSRs are dominant markers and do not provide direct information about allele frequencies, they allow a quick method of measuring diversity at multiple

nuclear loci and are able to detect gene flow, differentiation, and hybridization in populations at varying levels of divergence (reviewed in Wolfe et al. 1998).

In addition to providing an independent data source, nuclear markers considered together with mitochondrial markers may reveal occurrences of introgression -- for example, of nuclear alleles from a genetically distant lineage (e.g., Morando et al 2004, Goodman et al 1999). Such an event could represent historical hybridization between morphologically distinct populations, giving rise to a new local variety. A long history of repeated hybridization events could reveal itself as incongruence between the phylogeographic patterns apparent in mitochondrial versus nuclear gene trees (e.g., Morando et al 2004).

Finally, while the genetic distance separating clusters of similar mitochondrial haplotypes has been used as a criterion for diagnosing cryptic species (e.g., Huvla et al. 2004), the addition of nuclear markers provides a more robust means of doing so. Populations that are distinct based on both mitochondrial and nuclear gene trees are likely to have been separate long enough for lineage sorting to occur in both markers, and may represent cryptic species. The occurrence of such populations in sympatry would be a good indication of reproductive isolation (e.g., Derycke et al. 2005, Castro-Longoria et al. 2003).

In addition to molecular data, morphological measurements were collected to detect the presence of quantitative phenotypic variation. The dimensions of the stylet and the middle chamber of the proboscis are among several characters that have been observed to

vary among local populations within morphotypes (Envall & Norenburg 2001). These two structures were measured for individuals from one Gulf Coast and three East Coast populations.

Methods

Sample collection and cox3 sequencing

Individuals were collected from the intertidal zone at nine coastal locations in North America and the Caribbean. Sampling locations are shown on the map in Figure 1. Latitude/longitude and sample size for each location are listed in Table 1. The sites were selected opportunistically, but with the intent of sampling both closely spaced and distantly spaced locations. Florida was sampled more densely than New England, since *Ototyphlonemertes* occur with greater diversity in the former (Envall & Norenburg 2001). The coast of North America between northeastern Florida and Long Island was not sampled because the intertidal sediment is too fine-grained to support *Ototyphlonemertes*, and subtidal sediment was not sampled because Fila is not known to exist subtidally (Norenburg 1988). Samples were sorted by morphotype using the criteria in Envall & Norenburg (2001).

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 *cox3* gene was amplified using one of the following primer sets: forward TGATGGCGGGATGTGGTTCGTGAAGG and reverse ACAAAATGCCAATATCAAGCAGC or forward

CAGTG A T G G C G G G A T G T G G T T C G T G A and reverse ACAAAGTGTCAGTATCAGGCAGC. PCR reactions 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 1000 automated DNA sequencer.

Cox3 data analysis

Nucleotide sequences were aligned using MAFFT (Katoh et al. 2002). *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.

Neutral evolution of *cox3* was tested using Tajima's D statistic (1989). Since this statistic is known to be sensitive to population subdivision and historical changes in population size (Simonsen et al. 1995), additional neutrality tests using Fu & Li's F* (1993), and McDonald-Kreitman (1991) were conducted for each phylogenetic lineage and the results compared to mismatch distributions in order to differentiate between demographic history and the effects of selection. For the McDonald-Kreitman test, the major mitochondrial lineages were treated as separate species for the purpose of comparing synonymous and nonsynonymous substitutions. Two pairwise comparisons were made, each between a

group that showed violation of neutrality by other tests and a group that did not. Neutrality tests and mismatch analyses were performed with the software DNASP (Rozas & Rozas 1999, Rozas et al. 2003). Tests for substitution saturation were performed for sequences within the Fila morphotype and for sequences across *Ototyphlonemertes* using the software DAMBE (Xia & Xie 2001). The F84 model of nucleotide substitution (Felsenstein & Churchill 1996) was used for this test, since of the models available in the software, it was most similar to the optimal models (HKY85 within Fila and GTR within *Ototyphlonemertes*, discussed below).

Because of the large diversity of haplotypes present at most sites, it is likely that the sampling strategy used in this study did not capture all haplotypes present at all sites. For this reason, estimates of F_{st} based on haplotype frequencies may be misleading, showing high levels of subdivision between populations whose sampled haplotypes differ, but are interspersed in the haplotype network. Therefore, Hudson's (2000) S_{nn} was used as the measure of population differentiation, since it accounts for the occurrence of "nearest neighbor" haplotypes in the populations examined. S_{nn}, as well as Hudson, Slatkin & Maddison's (1992) method of estimating F_{st} from sequence data, was performed using DNASP.

Cox3 sequences were tested for clock-like evolution using the log-likelihood ratio test (Felsenstein 1981). The null hypothesis of a molecular clock was not rejected, so a UPGMA tree was constructed for unique *cox3* haplotypes using PAUP* version 4.0b10

(Swofford 2003). MrModeltest version 2.2 (Nylander 2004; Posada & Crandall 1998) was used to select a model of nucleotide substitution for *cox3* sequences within Fila and across the genus *Ototyphlonemertes*. The selected model (HKY+G within Fila and GTR+I+G across *Ototyphlonemertes*) was used to calculate maximum-likelihood distances between Fila individuals and for Bayesian analysis of phylogeny at the genus level. Bayesian analysis of nucleotide sequences was completed using MrBayes version 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) with 2.5 million generations. Bayesian analysis of amino acid sequences was also performed, using the mtREV+I+G model (Adachi & Hasegawa 1996) and 250,000 generations. Maximum parsimony analysis at the genus level was performed in PAUP* using a heuristic search with 100 random addition replicates and TBR branch swapping, and bootstrapped using 1000 replicates. A representative of the nemertean genus *Nipponemertes* was used as the outgroup for phylogenetic analyses at the genus level.

A 95% statistical parsimony network (Templeton et al. 1992) was constructed using the software TCS (Clement et al. 2000) to describe relationships between *cox3* haplotypes within Fila. Nested clade analysis (NCA; Templeton 1998) was performed with the software GeoDis (Posada et al. 2000), using 10,000 permutations to test the significance of association between geography and haplotype distribution. The nesting design for NCA was constructed from the parsimony network using the rules of Templeton et al. (1987) and Templeton & Sing (1993).

ISSR amplification and data analysis

ISSRs were amplified using the primers $(GA)_8T$ and $(CA)_8G$ at an annealing temperature of 46°C and 50°C respectively. The PCR profile was as follows: 94°C denaturation for 1 min 30 sec, then 37 cycles of 94°C denaturation for 25 sec, annealing for 40 sec, and 68°C extension for 1 min 20 sec, followed by a final extension of 10 min at 68°C. The bands were visualized on 1.5% agarose gel run at 1-2 V/cm for 6 to 12 hours. Approximately 1/4 of the amplifications were repeated to test the reproducibility of bands.

For ISSR markers, F_{st} is not an appropriate statistic, since traditional methods of calculation depend on knowledge about allele frequencies (Lynch & Milligan 1994). Since ISSRs are a dominant marker, allele frequencies typically cannot be determined without assuming Hardy-Weinberg equilibrium, which is frequently violated in natural populations. Calculation of Θ_B, a Bayesian estimate of F_{st} that accounts for the random effects of population sampling, was performed in Hickory (Holsinger et al. 2002). However, the 95% credible intervals were wide, probably due to the low number of loci scored (data not shown). Therefore, ISSR-based population structure is described here using AMOVA (Excoffier et al. 1992) and distance-based tree methods. Tree construction was performed in PAUP* under the minimum evolution criterion using a heuristic search with 100 random addition replicates and TBR branch swapping. This was found to return a shorter tree than other search strategies (such as starting with a single neighbor-joining tree).

The Nei-Li distance measure implemented in PAUP* is not strictly appropriate for ISSR markers, as this measure estimates nucleotide sequence divergence from restriction enzyme banding patterns assuming a recognition sequence of given length. For this reason, the Dice-Sørensen (Dice 1945; Sørensen 1948) distance was also obtained, using the program FAMD (Schlüter & Harris 2006), and input into PAUP* as a user-specified distance matrix. In this case, however, the trees obtained using the two distance measures were identical except for branch lengths. The trees obtained using the Dice-Sørensen distance are reported.

AMOVA was performed with the software GenAlX (Peakall & Smouse 2006), using 1000 permutations to test the significance of pairwise Φ_{st} values. The Dice-Sørensen distance was used in place of the Euclidian metric. Although AMOVA was originally designed to work with a Euclidian distance metric, non-Euclidian metrics are also acceptable (Excoffier et al. 1992). Using a Euclidian metric is undesirable for ISSRs, because it will count shared band absences as positive matches, when in reality shared absences are unlikely to be homologous (Wolfe et al. 1998).

Morphological measurements

Prior to preservation of samples, digital photomicrographs were taken for use in comparing morphological characters and obtaining measurements. Measurements of the stylet and middle proboscis chamber were obtained from the digital images using the software Carnoy (Schols & Smets 2001). The total stylet length was measured as the combined length of the base and tip, and the width of the stylet was measured at the widest part of the base. In addition, the length of the stylet tip was measured as the distance from the end of the tip to the point of attachment to the base. The width of the middle chamber of the proboscis was taken at its widest point, roughly parallel to its base where it attaches to the posterior proboscis. The length of the middle chamber was taken perpendicular to the width measurement.

Morphometric relationships were visualized as plots of the ratios of total stylet length to stylet tip length, total stylet length to stylet width, and total stylet length to middle chamber length. The middle chamber width measurements were discarded in favor of the length measurements, since the widths displayed a higher variance, raising the possibility that they were confounded to a greater degree by differential "squashing" of the organism on the microscope slide. Regression lines were calculated using the least-squares method, and the regressions for different sample sets were compared using one-way analysis of covariance (ANCOVA) to test for differences in the slopes and y-intercepts.

Results

Cox3 diversity and population differentiation

Sequence data were obtained for 494 bp of *cox3* from a total of 156 individuals of the Fila morphotype representing nine geographical locations (Table 1). In addition, sequence data were obtained for representatives of other morphotypes collected at sites in Florida and California. Within Fila, 66 unique haplotypes were identified. These haplotypes grouped into three major clades by both distance (Fig. 2) and statistical parsimony (Fig. 3, discussed in a later section). Two of the clades further divided into two sub-clades each of moderate sequence divergence. Sequence divergence between the three major clades of Fila was 16-18% (uncorrected). The first of the three major clades consists of two sub-clades, which will be referred to as "Gulf" and "New England". The second of the three major clades consists of two sub-clades which will be referred to as "Florida 1" and "Florida 2". The remaining clade will be referred to as “Caribbean”. Surprisingly, the clades Florida 1 and Florida 2, which occur in sympatry at all east coast Florida locations sampled, are separated by approximately the same genetic distance (around 3%) as the Gulf and New England clades, which are separated by a geographic distance of at least 2400 km.

The test for substitution saturation indicated that saturation was not an issue at low to moderate genetic distances such as those within each of the three major clades, but that substitutions approached saturation when considering the most distant individuals between the major clades (Fig. 4). Resolving the relationships among the major clades proved difficult, likely due to substitution saturation. Attempts were made with Bayesian analysis and analysis of amino acid sequences, discussed below.

Hudson's (2000) S_{nn}, as well as Hudson, Slatkin & Maddison's (1992) F_{st}, shows lack of differentiation between the two Gulf Coast populations, between the two New England populations, and between the three east coast Florida populations at Bathtub Beach, Ocean Reef, and Miami Beach. The population at Sebastian Inlet is significantly differentiated from other Floridian and Gulf populations, probably due to the approximately equal frequencies of Floridian and Gulf mitochondrial clades at this location. S_{nn} and F_{st} values (and N_m estimated from F_{st}) are shown in Table 2. Naples and Sanibel samples were combined due to the low sample size at Sanibel. Pairwise comparison of the two populations gave an S_{nn} of 0.622 ($p=0.645$) and an F_{st} of 0.0084 ($N_m=59.3$).

Selective neutrality and mismatch distribution

Results of neutrality tests are shown in Table 3. Tajima's D test applied to all Fila haplotypes together showed significant deviation from neutral expectations; however, Fu & Li's F* test did not. The Gulf clade deviated from neutrality using Tajima's D. If the

Naples/Sanibel population is analyzed by itself (excluding the Gulf-clade individuals on the Atlantic coast), deviation from neutrality is seen with both D and F*. The Florida 1 and Florida 2 clades together do not show deviation from neutrality, but if Florida 1 is considered by itself, it deviates under both measures. The McDonald-Kreitman test found no deviation from neutrality in the populations that deviated by the above measures.

Mismatch distribution plotted against expected values under a model of constant population size and a model of population expansion are shown in Figure 5. The Naples/Sanibel population displays the unimodal distribution and low raggedness statistic (r) characteristic of population growth (Harpending 1994). The Florida 1 and Florida 2 clades together show a bi-modal distribution that may be indicative of a subdivided population (Marjoram & Donnelly 1994). If the two clades are analyzed separately, Florida 1 shows a distribution that conforms to the constant population size model, and the Florida 2 distribution displays the unimodal distribution associated with population growth.

Phylogenetic analysis

Attempts were made to construct a partial phylogeny of *Ototyphlonemertes* using representatives from different morphotypes (Envall & Norenburg 2001), in order to place the relationships between the Fila lineages into context and to compare their levels of divergence to the divergence between other species in the genus. Trees derived from

maximum parsimony and Bayesian analysis of *cox3* nucleotide sequences are shown in Figures 6a and 6b, respectively.

The lack of resolution and low bootstrap values and posterior probabilities suggest that *cox3* evolves too quickly to resolve relationships between *Ototyphlonemertes* species. The transition/transversion plot (Fig. 7a) indicates thorough substitution saturation at the 230 variable *cox3* sites (out of 494 total sites). When third codon positions are discarded, saturation is less evident, although the ratio of transitions to transversions still shows a decrease above a corrected distance of 0.08 (Fig. 7b). Furthermore, the estimate for α, which describes the distribution of among-site rate heterogeneity, is 0.14 for first and second codon positions, indicating that most substitutions occur at a small proportion of the variable sites. The translated amino acid sequence appears relatively conserved, with 48 variable residues (34 parsimony-informative) out of 165. In addition, it is likely that the number of viable substitutions at the variable sites is limited by conservation at the protein level, so that much of the observed variation may be non-neutral (Kimura, 1986).

Bayesian analysis of first and second codon positions gave poor resolution (Fig. 8a), while analysis of translated amino acid sequences (Fig. 8b) gave results that are unrealistic given the morphology of the genus and disagree with previous findings based on morphology (Envall & Norenburg, 2001; Envall 1996). Parsimony analyses of first and second codon positions and amino acid sequences show low (< 60%) bootstrap support for most branches and are not shown.

Despite the problems at the genus level, all of the preceding methods recover a monophyletic Fila, supporting the morphological taxonomy of Envall & Norenburg (2001). In addition, Bayesian analysis of the translated *cox3* amino acid sequences recovered a monophyletic non-helicophoran clade with 94% posterior probability, supporting the findings of Envall (1996) and Envall & Sundberg (1998).

Haplotype network and NCA

The 95% statistical parsimony network of *cox3* haplotypes is shown in Figure 3. The 11-step connections between the Gulf and New England clades and between the Florida 1 and Florida 2 clades were not within the 95% confidence limit (but were correct within a 90% confidence limit); therefore, these connections are more than 5% likely to be incorrect by one or more steps. In particular, the New England clade is more likely to connect to the Gulf clade at the interior haplotype #34 than at the tip nodes (Excoffier & Smouse 1994, reviewed in Posada & Crandall 2001), and the Florida 2 clade is likely to connect to the Florida 1 clade at haplotype #1 for the same reason. The relationships between the three major clades cannot be determined unambiguously by parsimony methods. However, maximum parsimony analysis of the *Ototyphlonemertes* genus indicates that the Caribbean clade is more closely related to the Florida 1 and Florida 2 clades than to the Gulf or New England clades with 77% bootstrap support, and Bayesian analysis reaches the same conclusion with 97% posterior probability. Therefore, the relationships shown in Figure 6a

will be treated as accurate for purposes of joining high-level networks for nested clade analysis (Templeton & Sing 1993).

The geographical distributions of haplotypes are color-coded on the haplotype map (Fig. 3). The Gulf clade occurs at Gulf Coast locations and in smaller numbers on the east coast of Florida. The related New England clade occurs only at New England locations. The Florida 1 and Florida 2 clades occur in similar proportions at all four locations on the east coast of Florida, although a few individuals from Florida 1 are found at St. Barthélemy approximately 2000 km away. The Caribbean clade is found primarily at St. Barthélemy, although a single individual from this clade was found at Miami Beach. The Gulf and Florida 1 clades each show a strong star-like radiation, with a central node of high frequency surrounded by numerous descendant nodes (most represented by a single individual). The individuals of the Gulf clade that were found on the east coast of Florida all had identical *cox3* sequence.

NCA rejected the null hypothesis of no association of between geography and haplotype distribution for clades at several levels of nesting. These clades are shown in Table 4 with significant statistics indicated. The nesting arrangement (with clade numbers) used for the analysis is shown in Figures 9a and 9b. The inference key from Templeton (2004) interprets the significant values as follows. In clade 1-33, consisting of haplotypes from both New England populations, restricted gene flow with isolation by distance. In clade 3-5, consisting of haplotypes from Gulf Coast populations as well as Gulf-clade individuals

on the East Coast, long distance colonization possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion. In clade 5-3, consisting of the Gulf and New England clades, inconclusive, since there is no way to determine interior/tip status; however, if either clade is treated as interior and the other as a tip, the same inference is drawn: allopatric fragmentation. In clade 6-1, consisting of the Florida 1, Florida 2, and Caribbean clades, inconclusive, since tip/interior status cannot be determined. In this case, however, if we treat nested clade 5-1 (Florida 1 and Florida 2 combined) as the interior, the inference is range expansion, and if clade 5-2 (Caribbean) is treated as interior, the key indicates that sampling is inadequate to distinguish between fragmentation and isolation by distance. For the total cladogram, i.e., the two level 6 clades, the outcome is inconclusive.

ISSR diversity

Banding patterns were obtained for 114 of the 156 Fila individuals in the study. Individuals from the Sanibel Island populations showed faint or no bands in amplifications with either of the ISSR primers used (probably due to DNA degradation, as these were older samples); thus, the Sanibel population is not represented in the ISSR data. In addition, several individuals from the New England populations amplified poorly. To maintain an adequate sample size, both New England populations were treated as a single population in the analysis of ISSR data.

After discarding faint or non-reproducible bands, as well as bands that were so similar in size that they were likely to give a high error rate in scoring from agarose gels, 15 unique bands remained, which were assumed to represent independent loci. These bands identified 37 unique genotypes among Fila populations. The minimum evolution tree based on Dice-Sørensen distances shows three major clusters (Fig. 10). At this level, there is complete concordance between the nuclear and mitochondrial genealogies; i.e., every individual from each of the three ISSR clusters belongs to the corresponding high-level mitochondrial clade. This pattern includes sympatric individuals from divergent lineages; for instance, the few Florida-clade individuals found at St. Barthélemy show ISSR markers concordant with other Florida-clade individuals, and the single Caribbean-clade individual sampled at Miami Beach shows ISSR markers concordant with those of its relatives at St. Barthélemy.

Within each of the three ISSR clusters, however, there is no such concordance -- individuals from Florida 1 and Florida 2 *cox3* clades do not form separate clusters based on the nuclear data, nor do individuals from Gulf and New England *cox3* clades. The Gulf clade individuals from the east coast of Florida do cluster together based on ISSR data. Within this cluster, there is very little genotypic diversity, with all but two of the individuals displaying identical nuclear genotypes based on the 15 loci examined.

Although Gulf and New England individuals do not form separate clusters based on ISSR data, they do differ in terms of genotype frequencies. Out of 14 genotypes, 5 occur exclusively among Gulf-clade individuals, and 8 are exclusive to New England (Fig. 10).

One genotype is shared between Gulf and New England individuals; however, this genotype, which comprises the majority of Gulf individuals, consists of only a single positive band among the 15 scorable loci. Several New England individuals have this band in addition to one or more others. Since bands can be lost for a number of reasons other than shared ancestry or gene flow (Wolfe et al. 1998), the sharing of this genotype among the distant populations may be due to homoplasy resulting from the low number of scorable bands among Gulf individuals.

AMOVA

Pairwise Φ_{st} values (using Dice-Sørensen distances) were obtained using mitochondrial lineages as populations to test for gene flow (Table 5), as well as with geographical populations to detect any additional structuring that may not be evident from looking at mitochondrial haplotypes (Table 6). The Florida 1 and Florida 2 mitochondrial lineages showed no differentiation based on the ISSR data (Φ_{st}=0, P=0.38), indicating panmixis between these lineages where they are sympatric (all locations on the east coast of Florida). These lineages were combined for subsequent analysis by AMOVA. Additionally, because the Gulf-clade individuals on the East Coast form a distinct cluster based on ISSR data, these were treated as a separate clade to examine the relationship of these individuals to each mitochondrial lineage.

Overall pairwise Φ_{st} values are high, indicating low gene flow. The Gulf and New England clades show lower Φ_{st} values between each other than either does with any of the more distantly related clades. The Gulf-clade individuals on the East Coast show a high level of differentiation from all mitochondrial lineages (Φ_{st} = 0.452 to 0.887), as might be expected given its unique ISSR banding pattern and low internal variability. For these individuals, the lowest pairwise Φ_{st} (0.452) is with the New England clade. The pairwise Φ_{st} with the combined Florida 1 and Florida 2 clades is high (0.755), despite their presence in sympatry. The Caribbean clade shows a lower Φ_{st} (0.521) with the New England clade than with the other clades. This is somewhat misleading, as there are no shared bands between them. However, since there is relatively high variation within each of these groups, a relatively lower proportion of the variation is between them, and it is this proportion that Φ_{st} measures.

AMOVA using geographical populations reveals roughly the same pattern obtained from mitochondrial haplotypes. The St. Barthélemy population is highly differentiated from all other populations (Φ_{st} = 0.419 to 0.742), the lowest pairwise value occurring with the New England populations (for the same reasons as the corresponding mitochondrial lineages above). Bathtub Beach, Ocean Reef, and Miami Beach do not have pairwise Φ_{st}'s significantly above 0 (P = 0.11 to 0.39), indicating panmixis. Sebastian Inlet, which contains a high frequency of Gulf-clade individuals, is differentiated from the above three populations, while showing a relatively low pairwise Φ_{st} (0.197) with the New England populations.

Overall, mitochondrial clade affiliation is better than geographical location at explaining variation in the ISSR data, as would be expected given the concordance between mitochondrial and ISSR genealogies. AMOVA using clades as populations indicates that 70% of total variation is explained by variation among clades, while AMOVA using geographical populations indicates that 56% of total variation is explained by variation among locales.

Band frequencies

The frequency of positive ISSR bands at each locus for each mitochondrial lineage is shown in Table 7. These values reveal possible incidents (in bold type) of gene flow between sympatric lineages that are too rare to be reflected in the Φ_{st} statistic or to alter the genotype tree. These frequencies correspond to three individuals, found at Bathtub Beach, Ocean Reef, and Miami Beach, that belong to the Florida 1 and Florida 2 clades but show a band at a locus that is common among Caribbean-clade individuals; one Florida 2 clade individual at Miami Beach that shows a band at a different locus that is common among the Caribbean clade; one Caribbean-clade individual from the St. Barthélemy population that has a band common in Florida populations; and three individuals (two from Ocean Reef and one from Miami Beach) that belong to the Florida 1 and Florida 2 clades but show a band common among the Gulf clade individuals occurring on the East Coast.

Morphology

No qualitative difference in the morphology of the proboscis or the anterior features of the organism was evident among the Gulf, Florida 1, and Florida 2 clades. Morphological measurements were obtained for 25 individuals of the Gulf clade from the Naples and Sebastian Inlet populations, and 25 individuals of the Florida 1 and Florida 2 clades from the Bathtub Beach, Sebastian Inlet, and Ocean Reef populations. Morphometric relationships between stylet length and stylet width and between stylet length and middle chamber length show possible distinctions between Gulf clade and Florida clade individuals (Figs. 11b and 11c), which were found significant by one-way ANCOVA (Table 8). The comparisons involving stylet width are somewhat less reliable, as the smaller measurement is more difficult to obtain precisely when measuring image pixels; in addition, the width is irregular along the stylet making it difficult to determine the widest point consistently. However, the pattern is the same in the case of both significant results: there is no difference between the slopes of the regressions, but a significant difference in the y-intercept, indicating a difference in the adjusted means of the ratios. No significant difference is found when comparing Gulf-clade individuals from Naples to those from Sebastian Inlet, nor when comparing individuals of either clade collected in March to those collected in August (Table 8).

Discussion

Selective neutrality and demographic history

Deviation from neutrality is found in the overall set of Fila *cox3* sequences, in the Naples/Sanibel population, and in the Florida 1 clade; however, this does not necessarily mean that *cox3* is under the influence of selection in these clades. Large positive values of D have been associated with population subdivision (Simonsen et al. 1995), which is concordant with expectations for the overall haplotype set, and large negative values (Naples population and Florida 1 clade) are associated with population growth. In addition, the McDonald-Kreitman test does not support the results obtained with D and F*. This makes sense if we consider that out of the 494 nucleotide sites sequenced for *cox3*, 140 were variable, whereas only 18 amino acid positions were variable. No two ingroup individuals differed by more than 10 amino acids. Therefore, silent substitutions (which are likely to be neutral barring selection on the organellar level (Ballard & Whitlock 2004)) greatly outnumber nonsynonymous substitutions in all sample sets.

Mismatch analysis shows past population expansion in the Naples population, which may explain the large negative values obtained for D and F*. However, it cannot explain the observed deviation from neutrality in the Florida 1 clade, which shows no such evidence of

expansion. Therefore, it is possible that selection has influenced the frequency of haplotypes within this clade. Negative values of D are associated with directional selection (Simonsen et al. 1995). In the Florida 1 clade, the geographically widespread haplotype #1 (Fig. 3) occurs with much higher frequency than the central node of any of the other clade radiations, suggesting that it may have been favored by selection. However, there are no differences in the amino acid sequence between this haplotype and several of the haplotypes in the Florida 2 clade, and the McDonald-Kreitman test does not detect any non-neutrality based on non-synonymous substitutions. A possible explanation is "hitchhiking" (Maynard Smith & Haigh 1974). Since the mitochondrial genome does not normally recombine, an advantageous mutation in one gene will result in positive selection on the entire genome. Eventually, the favored genome will replace the pre-existing diversity in the population (Bazin et al. 2006). Tajima's D test has been shown to have good statistical power in detecting this kind of event (Simonsen et al. 1995).

If a selective sweep of the mitochondrial genome has occurred in the Florida 1 clade, then it will appear to be younger and less genetically diverse than it actually is. Nonetheless, there is no strong evidence that phylogeographic relationships between the clades have been obscured. The Florida 1 clade is separated from the Florida 2 clade by eleven synonymous nucleotide substitutions, representing a long branch in the haplotype network (Fig. 3). A past selective sweep may have eliminated some intermediate haplotypes on the Florida 1 side of the branch, but there is no reason to believe that such a sweep would have preferentially eliminated intermediate haplotypes. It should also be noted that selective

sweeps of the mitochondrial genome occur due to hitchhiking in all lineages (Bazin et al. 2006), and this process contributes to lineage sorting and divergence of allopatric populations (Avise et al. 1987). It may be that the Florida 1 clade merely underwent such a sweep more recently than the other clades.

Dispersal mechanisms: panmixis in Florida vs. isolation by distance in New England.

Nested clade analysis indicated reduced gene flow with isolation by distance for the two New England populations. The mitochondrial haplotype that yielded the significant statistic is exclusive to the East Chop population, where it occurs in relatively high frequency. Unfortunately, a sufficient sample size of ISSR data is not available to confirm whether the gene flow at other loci is reduced; nonetheless, the inference is a statistically sound measure of structuring that is not evident from one-dimensional measures of differentiation such as F_{st}. The New England populations exhibiting isolation by distance are separated by approximately 125 km. By contrast, populations on the east coast of Florida that are separated by larger distances show evidence of panmixis based on mitochondrial and ISSR data. Furthermore, all *cox3* haplotypes that occur in more than one individual on the east coast of Florida were found in multiple populations. It appears that the Florida and New England coasts differ fundamentally with regard to dispersal of meiofauna. One possible explanation is that the coast of Florida between Sebastian Inlet and Miami Beach consists largely of coarse-sediment beaches that are excellent habitat for *Ototyphlonemertes* (Norenburg 1988), whereas Martha's Vineyard is separated from Long

Island by open water and the fine-grained sand beaches of the Rhode Island coast, neither of which is suitable habitat for the obligately intertidal members of the genus. However, the presence of shared mitochondrial haplotypes between Miami Beach and St. Barthélemy in the eastern Caribbean suggest that open water is not necessarily an obstacle to dispersal.

Another possible explanation is the higher frequency of hurricanes in Florida and the Caribbean. Hurricanes can cause massive quantities of sediment to be suspended in the water column, which is then subject to movement by oceanic currents (Chang et al. 2001). Such storm activity has been implicated in the dispersal of meiofaunal copepods (Bouck 2003), and it is likely that it is an important factor in the dispersal of meiofaunal nemerteans (Envall et al., in prep.). The combination of storms and currents can also explain the apparent transport of individuals between St. Barthélemy and the east coast of Florida, at least in the direction of the east-to-west currents of the Caribbean (Gyory et al. 2005; Rowe et al. 2005). The presence of Florida 1 haplotypes at St. Barthélemy is more difficult to explain by this mechanism. However, since the region between Florida and the eastern Caribbean was not sampled in this study, there is no reason to conclude that the above haplotypes originated in the Florida populations (nor that the clade labeled "Florida 1" has the center of its diversity in Florida).

Deep divergence between the Florida 1 and Florida 2 clades.

One unusual feature of the mitochondrial network in the presence of two moderately (approximately 3%) diverged clades existing in sympatry on the east coast of Florida. The observed level of divergence is similar to that seen between the allopatric clades on the Gulf Coast and New England, and depending on the substitution rate (discussed below), it represents about 100,000 to 1 million years of divergence in allopatry. NCA cannot offer an explanation of the origins of these clades, since there are no related clades against which to establish a tip/interior relationship. It is possible that Florida 1 and 2 actually comprise a single clade where lineage sorting has resulted in the extinction of a range of related haplotypes, but that is unlikely, even given evidence of a possible selective sweep in one of the clades. When populations are separated by vicariance or by a long distance colonization event, their individual mitochondrial lineages begin to diverge due to a combination of lineage sorting and the accumulation of new mutations, so that over time they attain a state of reciprocal monophyly (Avise et al. 1987). In the absence of new mutations, lineage sorting would over time eliminate all but one haplotype in a population (Avise et al. 1987). The result is the type of diversity pattern seen in the Florida 1 clade: a central, relatively widespread "ancestral" haplotype surrounded by its mutational descendants.

Avise et al. (1987) give two possible explanations for the existence of divergent clades in sympatry: intrinsic reproductive isolation (i.e., sympatric speciation) and secondary contact

between previously allopatric populations. The first possibility can be eliminated on the basis of the ISSR data; thus, the observed pattern is likely due to secondary contact. The secondary contact must be relatively recent in evolutionary terms; otherwise, evidence of divergence would have been eliminated by lineage sorting. The question that remains is the cause of the original divergence between the two clades -- where did they exist in allopatry? A couple of possibilities can be imagined, although the present data cannot distinguish between them:

1. Both clades are endemic to the Atlantic coast of Florida, but conditions in the distant past did not favor admixture. Thus, the clades diverged due either to hydrogeographic barriers to gene flow (e.g., Reeb & Avise 1990, Collin 2001) or due to the combined effects of isolation by distance and climatic fluctuation (Caudill & Bucklin 2004). Following secondary contact in the recent past, lineage sorting has eliminated haplotypes from the Florida 2 clade, resulting in the relatively reduced numbers and diversity represented in the sample.

2. One clade is endemic to Florida, while the other clade's presence represents range expansion from a population not sampled in this study. In this case, the Florida 1 clade is likely to be the endemic population and Florida 2 the recent arrival, as mismatch analysis suggests stable size in the former and range expansion in the latter. This may be the more likely explanation, as there are several unsampled areas that are connected by currents to the Atlantic coast of Florida. The Yucatan peninsula, connected to Florida by the Loop

Current (Gyory et al. 2005), is one possible place to look for the source of the divergent clade, and the region along the Antilles current between Florida and the West Indies is another (Rowe et al. 2005). Weak dispersal by the latter current is already demonstrated by the sporadic presence of St. Barthélemy haplotypes at Miami Beach. The past allopatry of the Florida clades may be due to diversion of the connecting current by land masses during the prolonged periods of low sea level in the Pleistocene (Reeb & Avise 1990), but locating the missing population is necessary before such a hypothesis could be tested.

Gulf clade population at Sebastian Inlet.

An isolated population of Gulf clade individuals is found on the east coast of Florida, sympatric with individuals belonging to the Florida 1 and Florida 2 clades. Fifteen ISSR loci show minimal to no gene flow between the divergent clades. This does not mean that gene flow does not take place; however, if mating were random then some shared bands would be expected, at least at the Sebastian Inlet population where the two clades occur in approximately equal numbers. A possible explanation is that the Gulf-clade individuals are recent migrants that arrived after the last reproductive season, but that is unlikely, since they are present in relatively high numbers at Sebastian Inlet yet show evidence of being closely related. Another explanation is that some degree of reproductive isolation exists between the divergent clades.

If the clades are interpreted as separate biological species, then the presence of the Gulf clade individuals is concordant with the predominant phylogeographic pattern for marine species in the region as discussed below. NCA indicates that the observed distribution of Gulf clade haplotypes is either the result of long-distance colonization of the East Coast sites from the Gulf Coast population, or fragmentation of an ancestral range that spanned both coasts. In this case, the extremely low genetic diversity in the isolated population, reflected in both the nuclear and mitochondrial markers, is consistent with the pattern expected if the population were founded by a single individual and sufficient time had not yet passed for new mutations to arise (Avise et al. 1984). In benthic marine species with no larval dispersal, the migration of a single gravid female is often enough to establish a new population (Johannesson 1988; Cunningham & Collins 1998). These observations support long-distance colonization as the population's origin. Alternately, a recent and severe bottleneck may explain the low genetic diversity in the population (Nei et al. 1975).

Why the population is almost entirely restricted to the Sebastian Inlet location remains unexplained given the lack of such restriction among either Florida 1 or Florida 2 haplotypes. However, if the colonization was a recent singular event, significant dispersal may not have yet occurred. Additionally, competitive exclusion (Gause 1934) with the other clade is a possibility given the evidence of reproductive isolation. Competitive exclusion may also explain why no evidence of additional past colonization events was found.

Surprisingly, the ISSR genotypes found in the Gulf clade population on the East Coast are not similar to any of the genotypes seen at the Gulf Coast locations. Furthermore, although the *cox3* haplotype of the East Coast population is nested within the Gulf clade, the exact haplotype was not found at either Gulf Coast location sampled. These observations raise questions about the geographical origin of its founders. Gene flow with members of the Florida 1 and Florida 2 clades is low to non-existent, which makes introgression an unlikely explanation for its unique nuclear genotype. It is possible that additional geographic structuring exists on the Gulf Coast, and the members of the isolated population are representatives of a population that was not sampled. However, the population at Naples/Sanibel is highly diverse, consisting of haplotypes that are between 1 and 6 mutational steps away from the central haplotype in the network. This diversity is not reflected in the ISSR data not because of reduced nuclear gene diversity, but because many of the bands that were variable in this population were deemed too faint or potentially homoplasious, and not scored. Therefore, it is possible that sampling failed to reveal the isolated population's alleles in the highly diverse ancestral population.

Comparative phylogeography, divergence between Gulf and New England clades.

The continuity between the Gulf-clade populations at Naples and Sebastian Inlet, as well as the divergence between the Gulf and New England clades, are concordant with a pattern of North American coastal phylogeography that applies to a diverse range of marine species with different modes of dispersal (e.g. Reeb & Avise 1990, Collin 2001). The

general pattern is a break between Gulf and Atlantic lineages that occurs in northeastern Florida around the Cape Canaveral area (though some temperate species have a boundary at the southern tip of Florida, e.g. Young et al. 2002). The cause for this common pattern of structuring is uncertain, and may differ from species to species (reviewed in Reeb & Avise 1990). Possibilities include ancient vicariance caused by the emergence of Florida in the Pliocene, more recent vicariance caused by the repeated rise and fall of sea levels during the Pleistocene, mediation of dispersal by coastal flow patterns with boundaries occurring where major ocean currents meet (Gaylord & Gaines 2000), and a transition between subtropical and temperate marine biomes that occurs around the Cape Canaveral area that may limit the distribution of species or locally adapted populations that are sensitive to temperature and coastal geography (Briggs 1974).

In the absence of a taxon-specific molecular clock, and lack of knowledge about effective population size which could allow substitution rates to be estimated by coalescent methods (Kuhner et al. 1995), it is difficult to determine the timing of the split between Gulf and New England clades and correlate it with geological events, but some preliminary efforts can be made. Reeb & Avise (1990) proposed a concordance-based method for differentiating between structuring due to extrinsic factors such as barriers to gene flow, and stochastic factors such as extinction of intermediate lineages. If structuring is due to a geographical barrier to gene flow, it will have acted on all markers concurrently, and there should be concordance between phylogeographic relationships revealed by independent markers. If structuring is due to stochastic processes, however, there may be discordance

between markers with different population histories. In the case of the Gulf and New England clades of Fila, lack of concordance between the *cox3* and ISSR gene trees may indicate that extinction of intermediate lineages over time has been a factor in their divergence. Alternately, this lack of concordance could be an artifact of poor resolution by ISSR markers due to an insufficient number of loci scored.

A study of the estuarine copepod *Acartia tonsa* suggested that the presence of deeply diverged, reciprocally monophyletic clades in the absence of extrinsic barriers to gene flow may result from repeated extinction of lineages due to climatic fluctuations (Caudill & Bucklin 2004). A similar process may have occurred in the ancestor of the Gulf and New England lineages during the Pleistocene due to fluctuating sea levels. An alternate vicariant hypothesis for the same time period may be the partial isolation of the Gulf of Mexico due to exposed land masses (Petuch 1982, reviewed in Reeb & Avise 1990). A vicariant hypothesis for an earlier divergence is the emergence of the Florida peninsula, mentioned above.

During the last glacial maximum, ice cover in eastern North America extended as far south as Long Island. Given that present-day *Ototyphlonemertes* are not generally found above 60 degrees north latitude (Envall & Norenburg 2001), we can assume that the region presently occupied by the New England populations was colonized after the glacial retreat, approximately 15,000 years ago. Since *Ototyphlonemertes* are passive adult dispersers, such a colonization event probably occurred by long-distance dispersal of a single

mitochondrial haplotype, so we can assume that the present genetic diversity within the New England clade is approximately 15,000 years old. Using an average maximum likelihood distance of 0.3% within the clade, our age estimate requires a *cox3* substitution rate of approximately 10% per million years. That is not an unreasonable estimate, given that the substitution rate of *cox1* in invertebrates has been estimated as high as 3.1%/m.y. in sea urchins (Lessios et al. 1999) and 6.0%/m.y. in hermit crabs (Young et al. 2002), and that *cox3* generally evolves faster than the more conserved *cox1*.

Using the 10%/m.y. estimate and an ML distance between the Gulf and New England clades of 2.7% (after applying Nei & Li's (1979) correction for average divergence within the clades), a divergence time of approximately 135,000 years is obtained. This places the timing of the divergence during the lower sea levels and climatic fluctuations of the Pleistocene. Using a more traditional estimate of 2%/m.y. divergence time for mitochondrial DNA (Brown et al. 1979), the divergence time is estimated at 675,000 years ago, also within the Pleistocene. The same tentative estimate of substitution rate can be used to estimate the start of the inferred population expansion in the Florida 2 clade discussed above. Using the estimate of $\tau = 2.4$ from mismatch analysis, where τ is twice the time since expansion multiplied by the per-locus substitution rate (Rozas et al. 2003), the start of the expansion is dated at approximately 24,000 years ago. This is close to the end of the Pleistocene, when rising sea levels may have removed the barriers to a hypothetical expansion by current transport.

Cryptic species, genetic structuring among meiofauna.

Nuclear markers give some evidence for gene flow between the Gulf clade and sympatric populations of the Florida 1 and Florida 2 clades, as well between the Caribbean clade and the Florida 1 and Florida 2 clades, based on the infrequent presence of shared ISSR bands. Since determining homology is problematic with ISSR markers and the probability that similarly sized bands are homoplasious increases with increasing genetic divergence (Peakall et al. 1998), such incidents of shared bands are not necessarily reliable evidence of gene flow. However, they cast some doubt on the existence of reproductive isolation between the mitochondrial lineages, especially between the Caribbean and Florida 1/Florida 2 lineages, since a total of five individuals (three from locations where the lineages are sympatric) show evidence of having received an allele from the other lineage. Between the Gulf lineages and the Florida 1/Florida 2 lineages, none of the Florida-clade individuals from the area of greatest sympatry show evidence of introgressed alleles, and none of the Gulf individuals show any of the bands common in the Florida populations. In this case, it is more likely that the shared bands represent false homologies.

Combined with the high genetic distance observed between the mitochondrial sequences, the nuclear data suggests that the Gulf clade may be a distinct biological species from the Florida 1 and Florida 2 clades. Furthermore, the absence of the either Florida clade from the Gulf Coast, as well as the low diversity of the Gulf clade on the Atlantic Coast, is

suggestive of competitive exclusion, which may be expected if the clades are two different species with no difference in ecological requirements.

Some evidence exists for a morphological distinction between the two clades, amounting to a slight difference in the shape (length to width ratio) of the stylet. The lack of a significant difference between the Gulf Coast population of the Gulf clade and the Atlantic population of the Gulf clade suggests that the distinction is between the Gulf and Florida mitochondrial clades and not between the geographical locations. Likewise, the lack of difference between individuals collected in March and individuals collected in August suggests that the difference in morphology is not due to the organisms' growth in the intervening months.

However, since the ratios of animal body features within a species necessarily change with a change in size (Gould 1966), the difference in stylet shape could simply be the result of differences in body size. Exact body size is difficult to measure in *Ototyphlonemertes* because of the organism's ability to elongate and contract by muscle activity, and because of differential "squashing" on a microscope slide; however, if the middle chamber length is taken as a proxy for body size, and plotted against the measure of stylet shape (Fig. 11d), it appears that longer, narrower stylets are weakly associated with larger body size. Thus, it is possible that the morphological difference between the two clades can be explained by a difference in body size, which by itself does not appear to be a phylogenetically reliable character in this genus (Envall & Sundberg 1998).

Other studies have revealed the presence of cryptic species among benthic meiofaunal taxa (e.g., Derycke et al. 2005, Castro-Longoria et al. 2003, Casu & Curini-Galletti 2006, Glatzel & Königshoff 2005). In addition, Lee (2000) found that morphological stasis and high within-lineage variability in the planktonic microcrustacean *Eurytemora affinis* masked the existence of deeply divergent, reproductively isolated species. *Ototyphlonemertes* appears to support this pattern of meiofaunal diversity, though morphological variability within and between the populations examined in this study was minimal. In the case of *O. fila*, as in the above studies, dispersal over significant distances is not impossible; thus, poor dispersal ability alone does not appear to be sufficient to explain the high incidence of cryptic species among meiofauna.

Future directions in Ototyphlonemertes research.

Morphological variation. The possibility of hybridization as an explanation for the apparent lack of strong morphological boundaries in the genus has not been excluded; however, given the morphological conservatism and high genetic distances seen between Fila populations, it is also possible that taxa that have diverged enough to be morphologically distinct would have diverged enough genetically so that they are unable to hybridize. Geographical variation in morphology may be due to local variation within each species of *Ototyphlonemertes*, since restricted gene flow in the presence of morphological selection would favor local adaptation (Wright 1943). In such cases, the scale that would

be considered "local" may vary greatly depending on hydrogeographic conditions, so that relatively distant, morphologically distinct populations may belong to a single species. At the same time, other morphologically distinct, as well as indistinct, populations occurring in relative proximity may represent reproductively isolated species. Phylogeographic analysis of morphological and molecular variation in a large sample of populations on multiple scales may be necessary to distinguish between these possibilities.

Phylogeny of *Ototyphlonemertes*. An accurate phylogeny of the genus is crucial for testing evolutionary hypotheses. *Cox3*, though an excellent marker for studying intraspecific variation, is inadequate for this purpose, as evidenced by the poor trees it yields when applied to relationships between morphotypes. Substitution saturation is reached even between sister species, and the amino acid sequence is too highly conserved to be of help. Cytochrome oxidase 1 (*cox1*) has shown promise for phylogenetic analysis at the generic and higher levels, and evidence from Fila suggests that the *cox1* nucleotide sequence is much more highly conserved than *cox3* in *Ototyphlonemertes* (unpublished data). For closely related species, however, any single marker is likely to be inadequate, and a consideration of nuclear, mitochondrial, and morphological variation will be necessary.

Phylogeography of the Caribbean. The exact relationship between the east coast Florida and St. Barthélemy mitochondrial clades remains uncertain, and without sampling between these locations there is insufficient evidence to differentiate between allopatric fragmentation and gradual isolation by distance (Templeton 2004). One possible

explanation for the co-existence of divergent clades on the Atlantic coast of Florida is range-expansion from the west, discussed above. In that case, it is possible that limited gene flow between islands has resulted in a range of intermediate lineages between Florida and St. Barthélemy. Alternately, the Caribbean may contain divergent, wide-ranging lineages like those seen in North America. Sampling populations from the Bahamas and Greater Antilles would potentially resolve this issue and offer insight into the phylogeography of the Caribbean as well as the origins of *Ototyphlonemertes* diversity in North America.

Literature Cited

Literature Cited

Adachi J, Hasegawa M (1996) MOLPHY version 2.3: programs for molecular phylogenetics based on maximum likelihood. Computer Science Monographs of Institute of Statistical Mathematics 28:1-150.

Altschul SF, Gish W, Miller W et al. (1990) Basic local alignment search tool. *J. Mol. Biol.*, **215**, 403-410.

Avise JC (2004) *Molecular Markers, Natural History, and Evolution*. Chapman & Hall, New York.

Avise JC, Arnold J, Ball RM et al. (1987) Intraspecific Phylogeography: The Mitochondrial DNA Bridge Between Population Genetics and Systematics. *Annual Review of Ecology and Systematics*, **18**, 489-522.

Avise JC, Neigel JE, Arnold J (1984) Demographic influences on mitochondrial DNA lineage survivorship in animal populations. *J. Mol. Evol.*, **20**, 99–105.

Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. *Molecular Ecology*, **13**, 729-744.

Bazin E, Glémin S, Galtier N (2006) Population size does not influence mitochondrial genetic diversity in animals. *Science*, **312**, 570-576.

Bouck, L (2003) *Two new Diosaccids (Copepoda, Harpacticoida) from the northern Gulf of Mexico and their responses to winter storms*. PhD Thesis, Florida State University, Tallhassee, FL

Briggs JC (1974) *Marine zoogeography*. McGraw-Hill, New York.

Brown W Jr, George M Jr, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. USA,* **76**, 1967–1971.

Castro-Longoria E, Alvarez-Borrego J, Rocha-Olivares A et al. (2003) Power of a multidisciplinary approach: use of morphological, molecular and digital methods in the study of harpacticoid cryptic species. *Mar. Ecol. Prog. Ser.*, **249**, 297-303.

Casu M, Curini-Galletti M (2006) Genetic evidence for the existence of cryptic species in the mesopsammic flatworm *Pseudomonocelis ophiocephala* (Rhabditophora: Proseriata). *Biological Journal of the Linnean Society*, **87**, 553–576.

Caudill CC, Bucklin A (2004) Molecular phylogeography and evolutionary history of the estuarine copepod, *Acartia tonsa*, on the Northwest Atlantic coast. *Hydrobiologia*, **511**, 91–102.

Chang GC, Dickey TD, Williams AJ III (2001) Sediment resuspension over a continental shelf during Hurricanes Edouard and Hortense. *Journal of Geophysical Research*, **106**, 9517–9531.

Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657-1660.

Collin R (2001) The effects of mode of development on phylogeography and population structure of North Atlantic *Crepidula* (Gastropoda: Calyptraeidae). *Molecular Ecology*, **10**, 2249-2262.

Cunningham, CW, Collins TM (1998) Beyond area relationships: Extinction and recolonization in molecular marine biogeography. In: *Molecular Approaches to Ecology and Evolution* (eds DeSalle R, Schierwater B), pp. 297–321. Birkhauser Verlag, Basel, Switzerland.

Derycke S, Remerie T, Vierstraete A, Backeljau T et al. (2005) Mitochondrial DNA variation and cryptic speciation within the free-living marine nematode *Pellioditis marina*. *Mar. Ecol. Prog. Ser.*, **300**, 91-103.

Dice LR (1945) Measures of the amount of ecologic association between species. *Ecology,* **26,** 297.

Envall M (1996) *Ototyphlonemertes correae* sp. n. and a redescription of *O. duplex* Bürger 1895 (Nemertea, Monostilifera, Ototyphlonemertidae), with a phylogenetic consideration of the genus. *Journal of Zoology, London*, **238**, 253–277.

Envall M, Norenburg JL (2001) Morphology and systematics in mesopsammic nemerteans of the genus *Ototyphlonemertes* (Nemertea, Hoplonemertea, Ototyphlonemertidae). *Hydrobiologia*, **456**, 145-163.

Envall M, Sundberg P (1998) Phylogenetic relationships and genetic distances between some monostiliferous interstitial nemerteans (*Ototyphlonemertes*, Hoplonemertea, Nemertea) indicated from the 16S rRNA gene. *Zoological Journal of the Linnean Society*, **123**, 105-115.

Excoffier L, Smouse PE (1994) Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: molecular variance parsimony. *Genetics*, **136**, 343-359.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction sites. *Genetics*, **131**, 479-491.

Felsenstein, J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol*, **17**, 368-376.

Felsenstein J, Churchill GA (1996) A Hidden Markov Model approach to variation among sites in rate of evolution. *Mol. Biol. Evol.*, **13**, 93-104.

Fu Y-X, Li W-H (1993) Statistical tests of neutrality of mutations. *Genetics*, **133**, 693-709.

Gause, GF (1934). *The struggle for existence*. Baltimore, MD: Williams & Wilkins.

Gaylord B, Gaines SD (2000) Temperature or transport? Range limits in marine species mediated solely by flow. *American Naturalist*, **155**, 769–789.

Glatzel T, Königshoff D (2005) Cross-breeding experiments among different populations of the "cosmopolitan" species *Phyllognathopus viguieri* (Copepoda: Harpacticoida), *Hydrobiologia*, **534**, 141-149.

Goodman SJ, Barton NH, Swanson G et al. (1999) Introgression Through Rare Hybridization: A Genetic Study of a Hybrid Zone Between Red and Sika Deer (Genus *Cervus*) in Argyll, Scotland. *Genetics*, **152**, 355-371.

Gould, SJ (1966) Allometry and size in ontogeny and phylogeny. *Biological Reviews*, **41**, 587-640.

Gyory J, Mariano AJ, Ryan EH (2005) The Caribbean Current. *Ocean Surface Currents* [internet]. Available from: http://oceancurrents.rsmas.miami.edu/caribbean/caribbean.html [cited 20 August 2006].

Hanken J, Wake DB (1993) Miniaturization of Body Size: Organismal Consequences and Evolutionary Significance. *Annual Review of Ecology and Systematics*, **24**, 501-519.

Harpending MM (1994) Signature of ancient populations growth in a low resolution mitochondrial DNA mismatch distribution. *Human Biology*, **66**, 591–600.

Hasegawa M, Kishino H, Yano T (1985) Dating the human-ape split by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, **22**, 160-174.

Higgins RP, Thiel H (1988) *Introduction to the Study of Meiofauna*. Smithsonian Institution Press, Washington, DC.

Holsinger KE, Lewis PO, Dey DK (2002) A Bayesian approach to inferring population structure from dominant markers. *Molecular Ecology*, **11**, 1157-1164.

Hudson RR (2000) A new statistic for detecting genetic differentiation. *Genetics*, **155**, 2011–2014.

Hudson RR, Slatkin M, Maddison WP (1992) Estimation of levels of gene flow from DNA sequence data. *Genetics*, **132**, 583–589

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics*, **17**, 754-755.

Huvla P, Horá_ek I, Strelkov P, Benda P (2004). Molecular architecture of *Pipistrellus pipistrellus/Pipistrellus pygmaeus* complex (Chiroptera: Vespertilionidae): further cryptic species and Mediterranean origin of the divergence. *Molecular Phylogenetics and Evolution*, **32**, 1023-1035.

Johannesson, K (1988) The paradox of Rockall: Why is a brooding gastropod (Littorina saxatilis) more widespread than one having a planktonic larval dispersal stage (L. littorea)? *Mar. Biol*, **99**, 507–513.

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.*, **30**, 3059-3066.

Kimura, M (1986) DNA and the neutral theory. *Phil. Trans. R. Soc. Lond. B,* **312**, 343-354.

Knowlton N (2000) Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia*, **420**, 73-90.

Kostia S, Ruohonen-Lehto M, Vainola R, Varvio SL (2000) Phylogenetic information in inter-SINE and inter-SSR fingerprints of the Artiodactyla and evolution of the Bov-tA SINE. *Heredity,* **84**, 37-45.

Kuhner MK, Yamato J, Felsenstein J (1995) Estimating effective population size and mutation rate from sequence data using Metropolis-Hastings sampling. *Genetics*, **140**, 1421-1430.

Lee CE (2000) Global phylogeography of a cryptic copepod species complex and reproductive isolation between genetically proximate "populations". *Evolution*, **54**, 2014-2027.

Lessios HA, Kessing BD, Robertson DR, Paulay G (1999) Phylogeography of the pantropical sea urchin *Eucidaris* in relation to land barriers and ocean currents. *Evolution*, **53**, 806–817

Lynch M, Milligan BG (1994) Analysis of population genetic structure with RAPD markers. *Molecular Ecology,* **3**, 91-99.

Marjoram P, Donnelly P (1994) Pairwise Comparisons of Mitochondrial DNA Sequences in Subdivided Populations and Implications for Early Human Evolution. *Genetics,* **136**, 673-683.

Maynard Smith J, Haigh J (1974) The hitch-hiking effect of a favourable gene. *Genetical Research,* **23**, 23-35.

McDonald JH, Kreitman M (1991) Adaptive evolution at the *Adh* locus in *Drosophila*. *Nature,* **351,** 652-654.

Morando M, Avila LJ, Baker J, Sites JW (2004) Phylogeny And Phylogeography Of The *Liolaemus Darwinii* Complex (Squamata: Liolaemidae): Evidence For Introgression And Incomplete Lineage Sorting. *Evolution*, **58**, 842-861.

Nei M, Li W-H (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA*, **76**, 5269–5273.

Nei M, Maruyama T, Chakraborty R (1975) The bottleneck effect and genetic variability in populations. *Evolution*, **29**, 1-10.

Norenburg, JL (1998) Nemertina. In: *Introduction to the Study of Meiofauna* (eds Higgins RP, Thiel H) pp 287-292. Smithsonian Institution Press, Washington, DC.

Nylander, JAA (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Peakall R, Gilmore S, Keys W et al. (1998) Cross-species amplification of soybean (*Glycine max*) simple sequence repeats (SSRs) within the genus and other legume genera: implications for the transferability of SSRs in plants. *Mol Biol Evol*, **15**, 1275-87.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes,* **6**, 288-295.

Petuch, EJ (1982) Geographical heterochrony: contemporaneous coexistence of Neogene and Recent molluscan faunas in the Americas. *Paleogeogr. Paleoclimatol. Paleoecol.*, **37**, 277-312.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**, 817-818.

Posada D, Crandall KA (2001) Intraspecific gene genealogies: trees grafting into networks. *Trends in Ecology and Evolution*, **16**, 37-45.

Posada D, Crandall KA, Templeton AR (2000) GeoDis: A program for the Cladistic Nested Analysis of the Geographical Distribution of Genetic Haplotypes. *Molecular Ecology,* **9**, 487-488.

Reddy, KD, Nagaraju J, Abraham EG (1999) Genetic characterization of the silkworm Bombyx mori by simple sequence repeat (SSR)-anchored PCR. *Heredity,* **83**, 681-687.

Reeb CA, Avise JC (1990) A genetic discontinuity in a continuously distributed species: Mitochondrial DNA in the american oyster, *Crassostrea virginica*. *Genetics*, **124**, 397–406.

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572-1574.

Rowe E, Mariano AJ, Ryan EH (2005) The Antilles Current. *Ocean Surface Currents* [internet]. Available from: http://oceancurrents.rsmas.miami.edu/atlantic/antilles.html [cited 20 August 2006].

Rozas J, Rozas R (1999) DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, **15**, 174-175.

Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19:** 2496-2497.

Schizas NV, Street GT, Coull BC, Chandler GT, Quattro JM (1999) Molecular population structure of the marine benthic copepod *Microarthridion littorale* along the southeastern and Gulf coasts of the United States. *Marine Biology*, **135**, 399–405.

Schlüter PM, Harris SA (2006) Analysis of multilocus fingerprinting data sets containing missing data.

Schols P, Smets E (2001) Carnoy: analysis software for LM, SEM and TEM images. Leuven: distributed by the authors. http://www.carnoy.org

Simonsen KL, Churchill GA, Aquadro CF (1995) Properties of Statistical Tests of Neutrality for DNA Polymorphism Data. *Genetics*, **141**, 413-429.

Sørensen T (1948) A method of establishing groups of equal amplitude in plant sociology based on similarity of species conent and its application to analyses of the vegetation on Danish commons. *Vidensk Selsk Biol Skr,* **5**, 1-34.

Swofford, DL (2003) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics,* **123**, 585-595.

Templeton AR (1998) Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology*, **7**, 381-397.

Templeton AR (2004) Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology,* **13**, 789-809.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, **132**, 619-633.

Templeton AR, Sing CF (1993) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics*, **134**, 659–669.

Thollesson M, Norenburg JL (2003) Ribbon worm relationships: a phylogeny of the phylum Nemertea. *Proc. R. Soc. Lond.*,**270**, 1471-2954.

Westheide W, Haß-Cordes E, Krabusch M, Müller MCM (2003) *Ctenodrilus serratus* (Polychaeta: Ctenodrilidae) is a truly amphi-Atlantic meiofauna species - evidence from molecular data. *Marine Biology*, **142**, 637-642.

Wolfe AD, Xiang Q-Y, Kephart SR (1998) Assessing hybridization in natural populations of *Penstemon* (Scrophulariaceae) using hypervariable inter simple sequence repeat markers. *Molecular Ecology*, **7**, 1107-1125.

Wright, S (1943) Isolation by distance. *Genetics,* **28**, 114-138.

Xia X, Xie Z (2001) DAMBE: Data analysis in molecular biology and evolution. *Journal of Heredity*, **92**, 371-373.

Young AM, Torres C, Mack JE, Cunningham CW (2002) Morphological and genetic evidence for vicariance and refugium in Atlantic and Gulf of Mexico populations of the hermit crab *Pagurus longicarpus. Marine Biology*, **140**, 1059-1066.

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics,* **20**, 176-183.

APPENDIX A

Figures

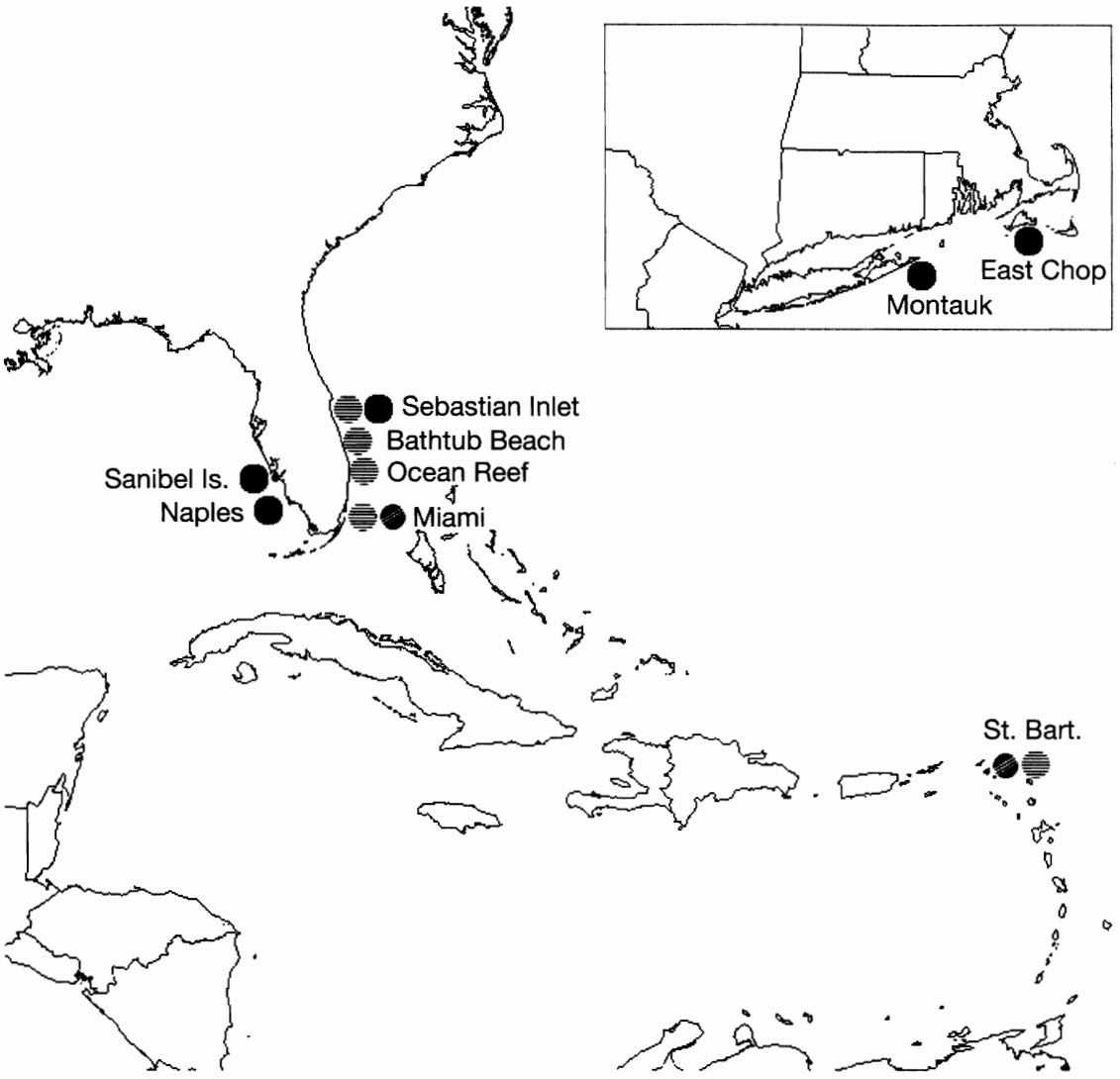

Fig. 1 Fila sampling locations and distributions of the three major *cox3* mitochondrial clades. ● = Clade 1, showing a distribution from the Gulf Coast to New England; ◍ = Clade 2, found on the east coast of Florida and in smaller numbers at St. Barthélemy; ◕ = Clade 3, found at St. Barthélemy with isolated individuals occurring at Miami Beach. Additional structuring within these clades is illustrated in Figs. 2 and 3. The region between Northeastern Florida and Long Island was not sampled due to an absence of suitable habitat. Sample sizes and lat./long. coordinates of sites are listed in Table 1.

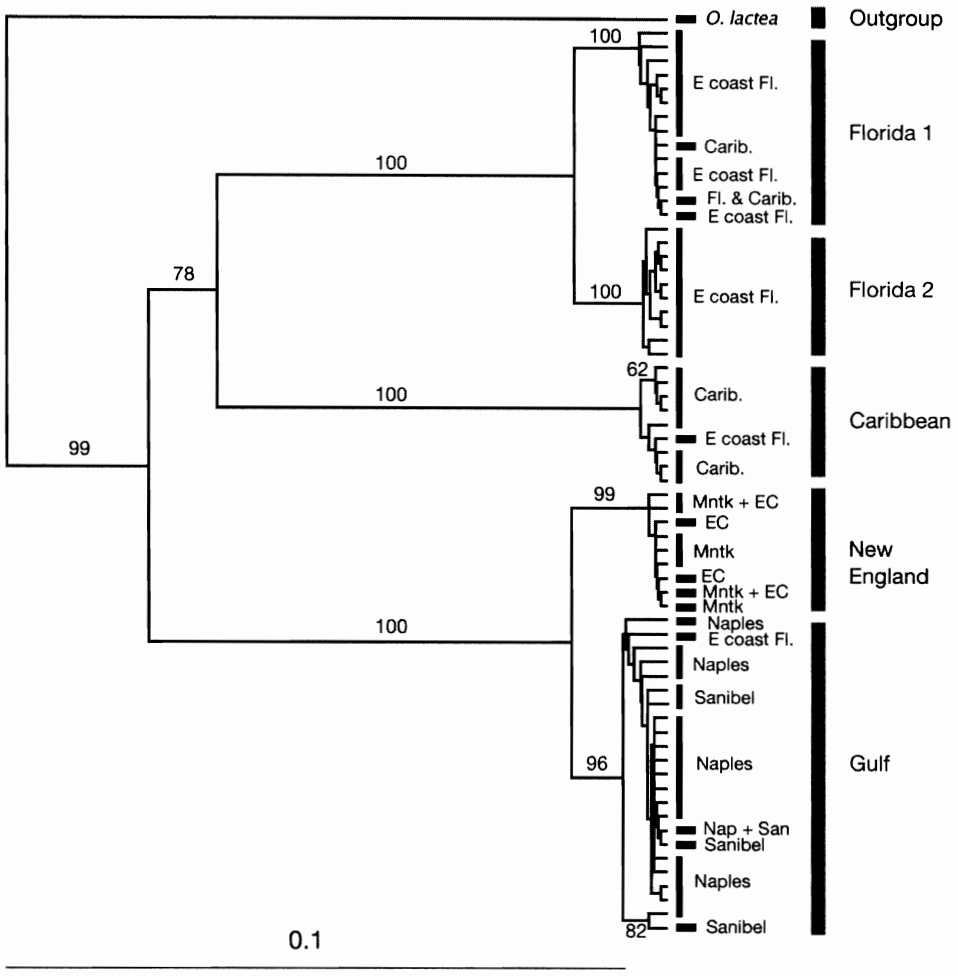

Fig. 2 UPGMA tree of Fila *cox3* nucleotide sequences based on uncorrected distances, with geographical distributions of the haplotypes shown near the leaves, and mitochondrial group monikers on the right. Clock-like evolution of *cox3* was confirmed using the log-likelihood ratio test. Bootstrap support is based on 1000 replicates. Abbreviations: Mtnk = Montauk, NY; EC = East Chop, MA; Nap = Naples, FL; San = Sanibel Island, FL.

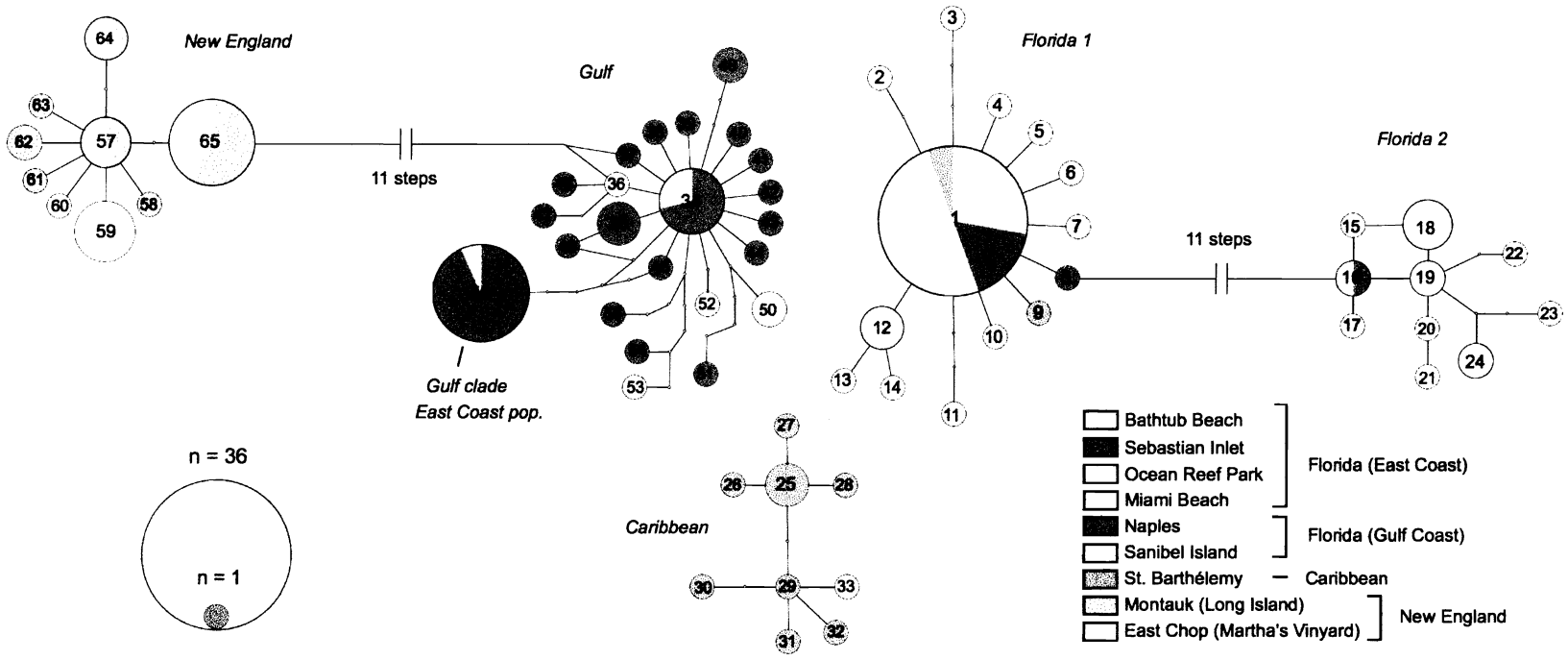

Fig. 3 Statistical parsimony network of Fila *cox3* mitochondrial haplotypes, color coded by geographic distribution. Clade names are indicated next to each haplotype group. New England and Gulf clades together correspond to Clade 1 in Fig. 1; Florida 1 and Florida 2 clades correspond to Clade 2 in Fig. 1; Caribbean clade corresponds to clade 3 in Fig. 1. Area of circles is proportional to sample size. All connections between nodes in the network are within 95% confidence limits of statistical parsimony, except for the 11-step connections between New England and Gulf and between Florida 1 and Florida 2, which are within 90% confidence limits. The relationships among the three high-level groups are uncertain and no connections are drawn between them.

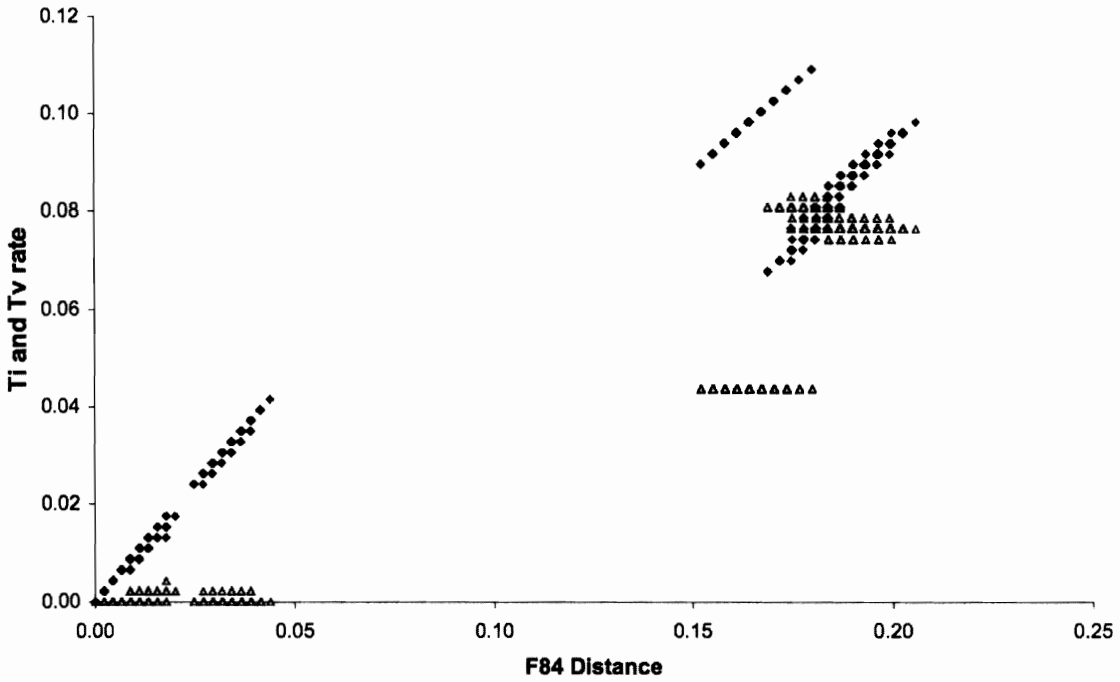

Fig. 4 Plot of transition rate (◆) and transversion rate (▲) vs. corrected (F84) distance for Fila *cox3* sequences. A decrease in the ratio of transitions to transversions suggests substitution saturation above a distance of approximately 0.18.

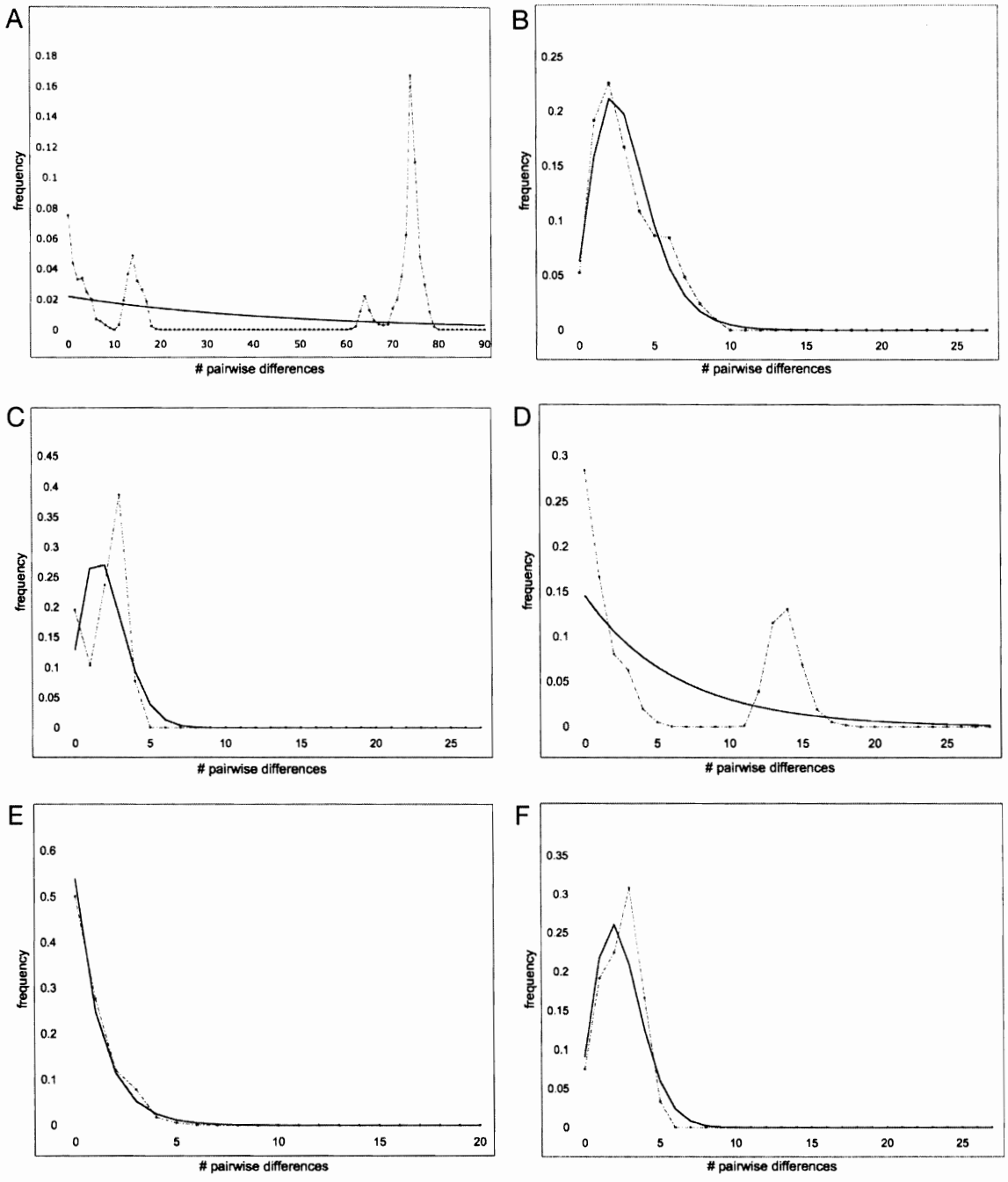

Fig. 5 Mismatch distributions among *cox3* nucleotide sequences plotted with theoretical distributions under a model of either constant population size or population expansion. Dashed lines correspond to observed distributions. Solid lines correspond to theoretical distributions. A = all Fila individuals vs. constant model. B = Naples, FL population vs. expansion model. C = combined New England populations vs. expansion model. D = all individuals from Florida 1 and Florida 2 mitochondrial clades vs. constant model. E = Florida 1 mitochondrial clade vs. constant model. F = Florida 2 clade vs. expansion model.

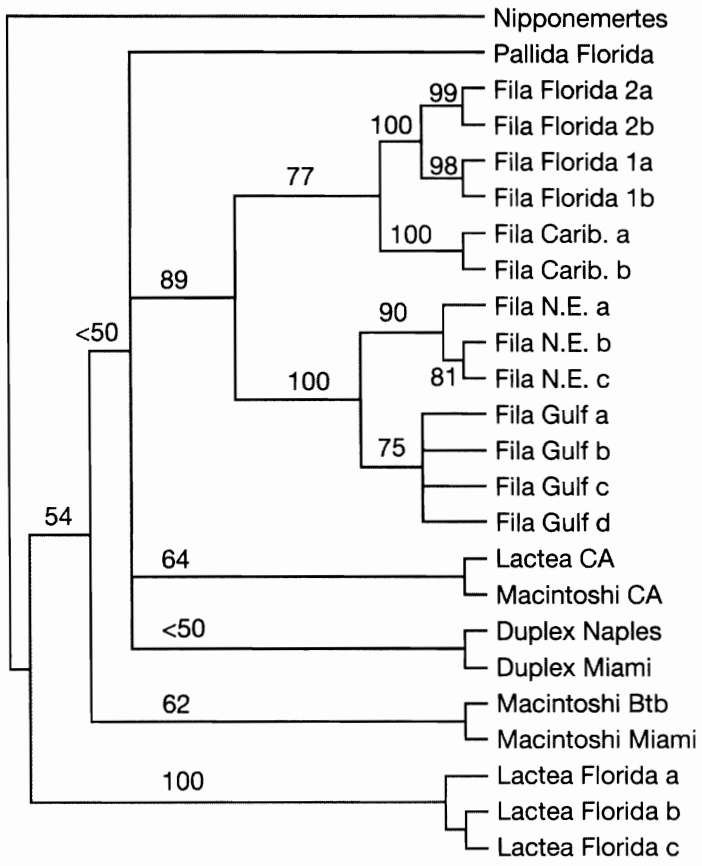

Fig. 6a Maximum parsimony analysis of *cox3* nucleotide sequences for representatives of genus *Ototyphlonemertes* using a representative of genus *Nipponemertes* as an outgroup. Analysis was performed in PAUP* using a heuristic search with 100 random addition replicates and TBR branch swapping. Taxon names refer to morphotypes described in Envall & Norenburg 2001. Tree shown is a strict consensus of 6 trees with a length of 712. Bootstrap values are based on 1000 replicates. Abbreviations: CA = California; N.E. = New England; Btb = Bathtub Beach

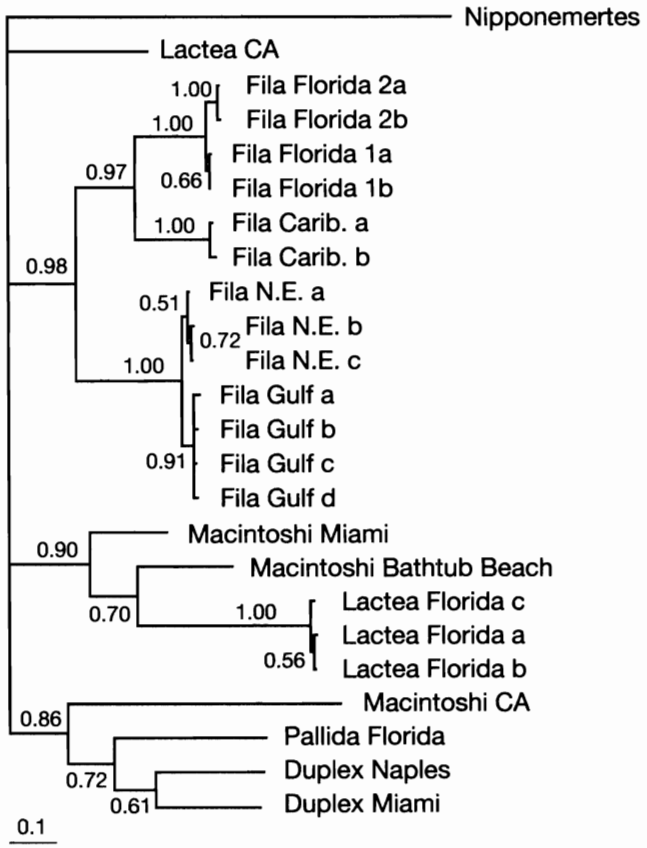

Fig. 6b Bayesian analysis of *cox3* nucleotide sequences for representatives of genus *Ototyphlonemertes* with posterior probability values shown, using a GTR+I+G model of substitution and 2.5 million generations. Taxon names refer to morphotypes described in Envall & Norenburg (2001). A representative of nemertean genus *Nipponemertes* was used as an outgroup. Abbreviations: CA = California; N.E. = New England

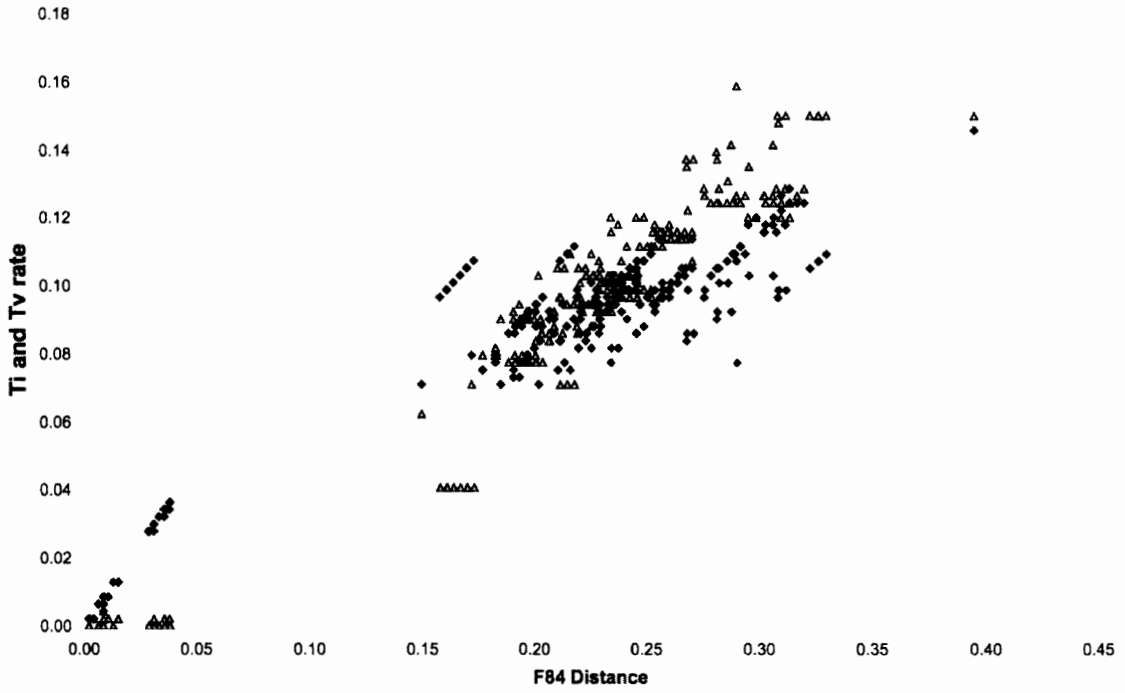

Fig. 7a Plot of transition rate (◆) and transversion rate (▲) vs. corrected (F84) distance for *cox3* nucleotide sequences from representatives of genus *Ototyphlonemertes*. A decrease in the ratio of transitions to transversions suggests substitution saturation above a distance of approximately 0.18.

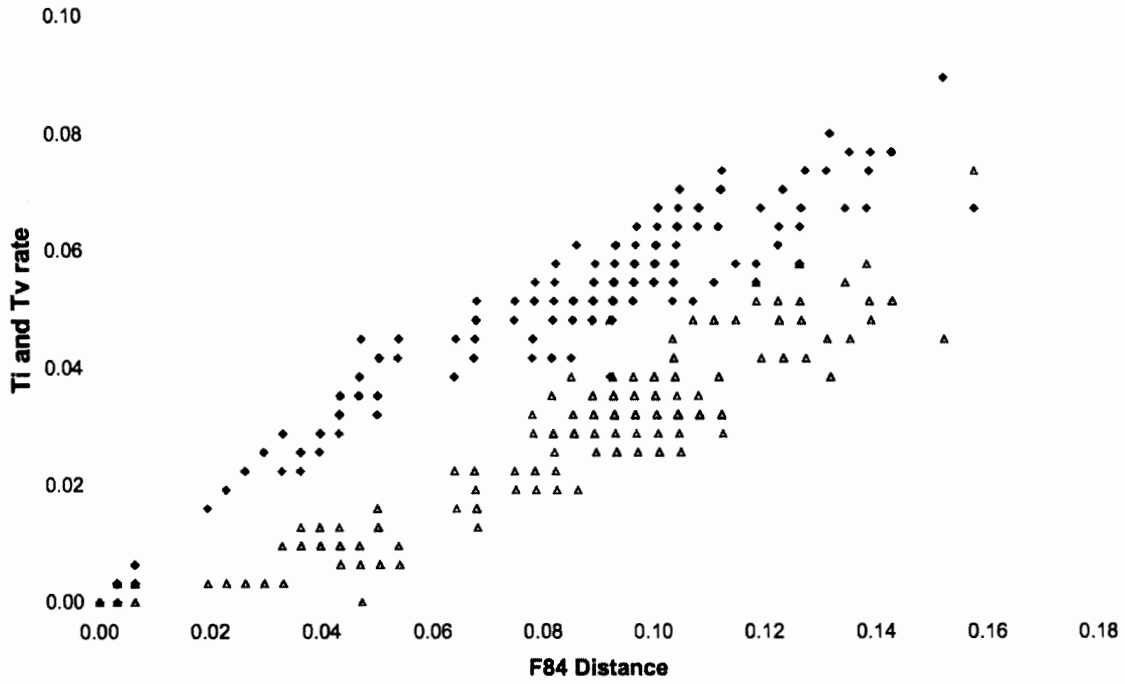

Fig. 7b Plot of transition rate (◆) and transversion rate (▲) vs. corrected (F84) distance for *cox3* nucleotide sequences from representatives of genus *Otoyphlonemertes*, excluding third codon positions. A decrease in the ratio of transitions to transversions suggests substitution saturation above a distance of approximately 0.08.

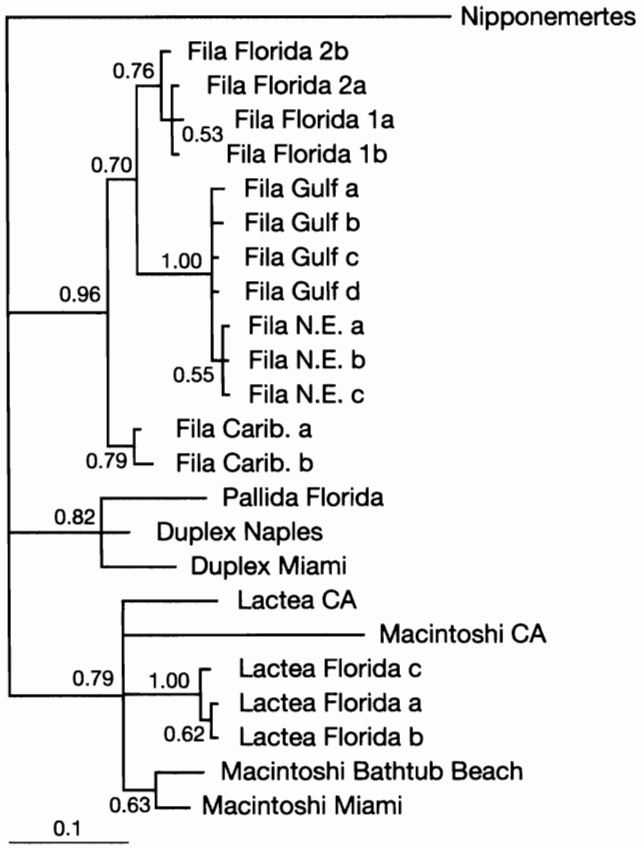

Fig. 8a Bayesian analysis of *cox3* nucleotide sequences for representatives of genus *Ototyphlonemertes* excluding third codon positions, using a GTR+I+G model of substitution and 2.5 million generations. Taxon names refer to morphotypes described in Envall & Norenburg (2001). A representative of nemertean genus *Nipponemertes* was used as an outgroup. Abbreviations: CA = California; N.E. = New England

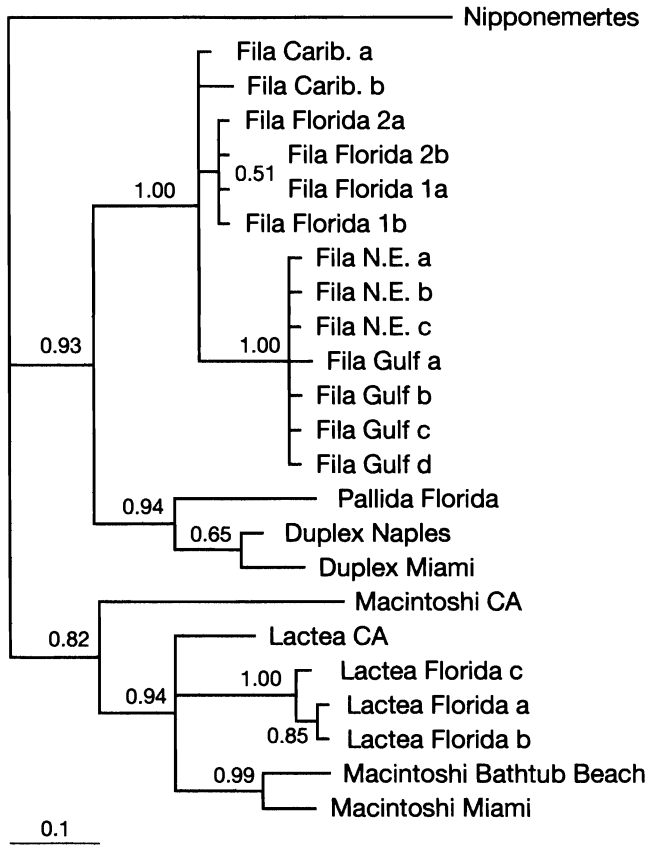

Fig. 8b Bayesian analysis of translated *cox3* amino acid sequences for representatives of genus *Ototyphlonemertes*, using an mtREV+I+G model of amino acid change and 250,000 generations. Taxon names refer to morphotypes described in Envall & Norenburg (2001). A representative of nemertean genus *Nipponemertes* was used as an outgroup. Abbreviations: CA = California; N.E. = New England

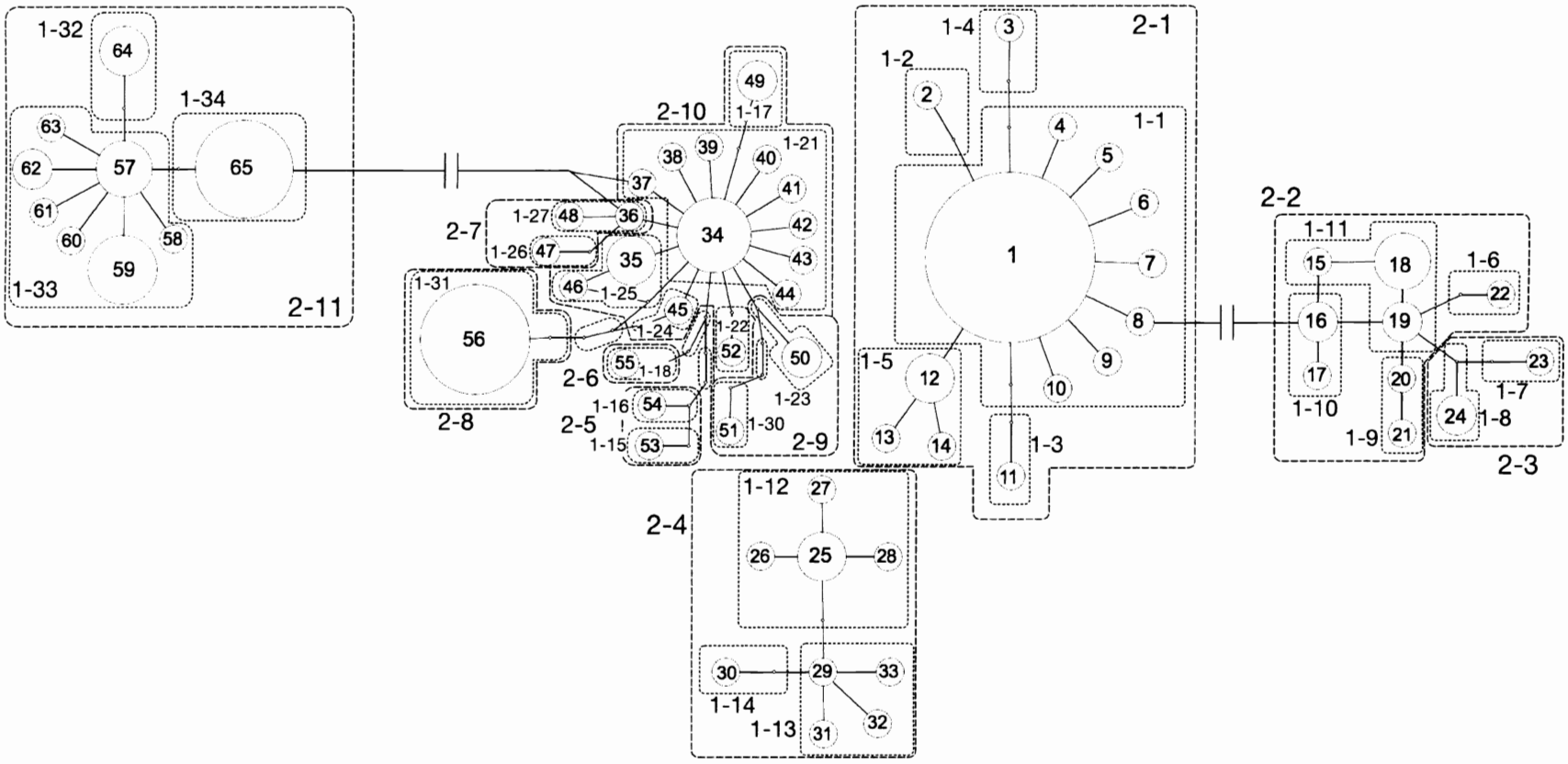

Fig. 9a Nested clade design used in nested clade analysis, following the nesting rules of Templeton et al. (1987) and Templeton & Sing (1993). First two nesting levels are shown here. Levels 3 – 6 are shown in Fig. 9b.

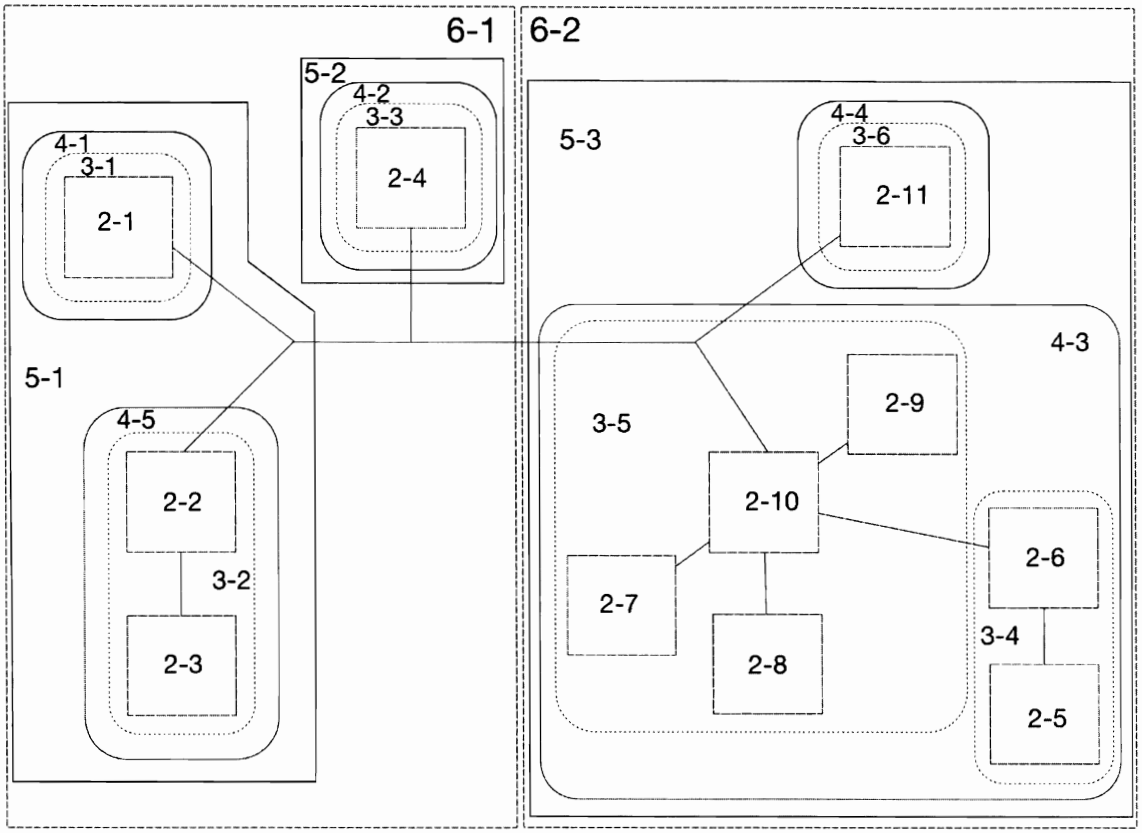

Fig. 9b Nested clade design used in nested clade analysis, following the nesting rules of Templeton et al. (1987) and Templeton & Sing (1993). Levels 3 through 6 are shown here. Levels 1 and 2 are shown in Fig. 9a.

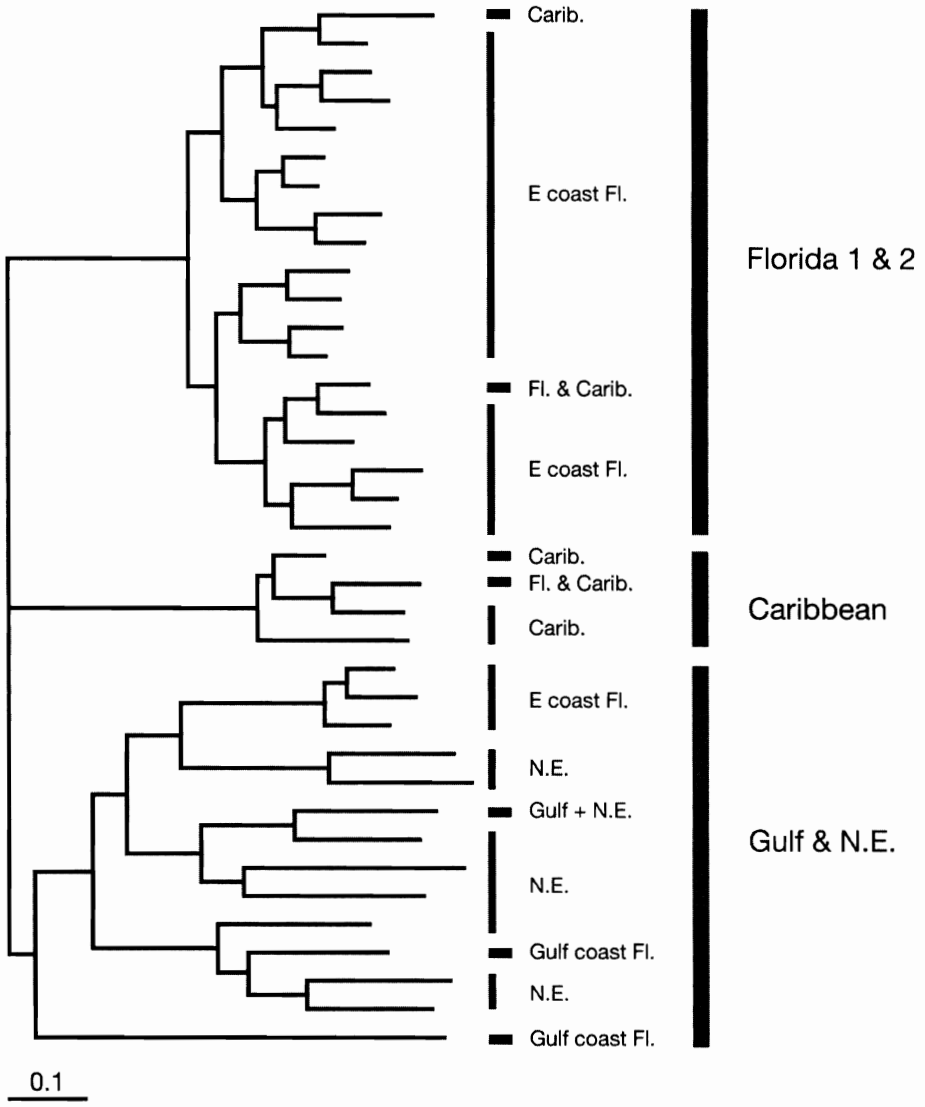

Fig. 10 Minimum evolution tree of Fila ISSR genotypes based on Dice-Sørensen distances calculated from banding patterns. Analysis was performed in PAUP* using a heuristic search with 100 random addition replicates and TBR branch swapping. Geographic distribution of each genotype is shown next to the leaves. On the right are the *cox3* mitochondrial clades associated with each ISSR cluster; i.e., all individuals from the topmost cluster belong to either Florida 1 or Florida 2 mitochondrial clades, all individuals from the middle cluster belong to the Caribbean clade, and all from the lower cluster belong to either the Gulf or New England clades.

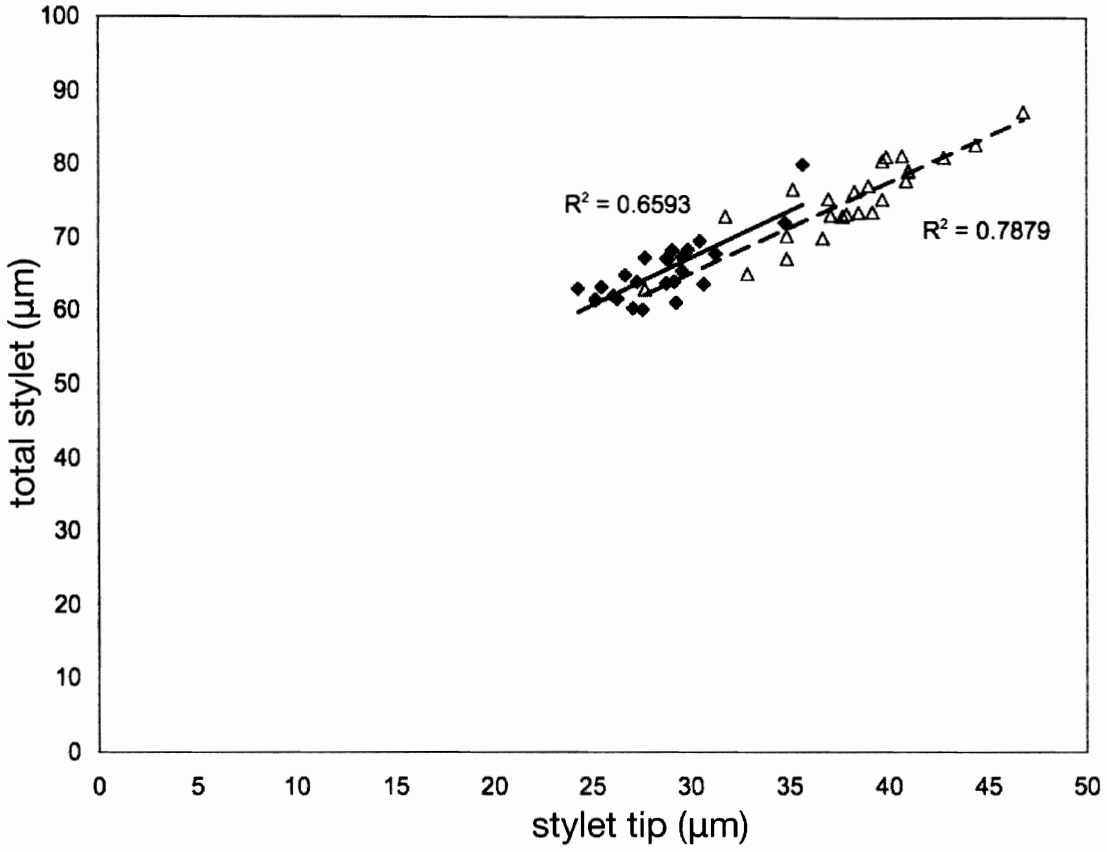

Fig. 11a Total stylet length vs. stylet tip length for Gulf-clade (◆) and Florida 1- and 2-clade (▲) individuals. The least-squares regression line represents the allometric relationship between the two measurements. No difference between the regressions was found using ANCOVA (Table 8).

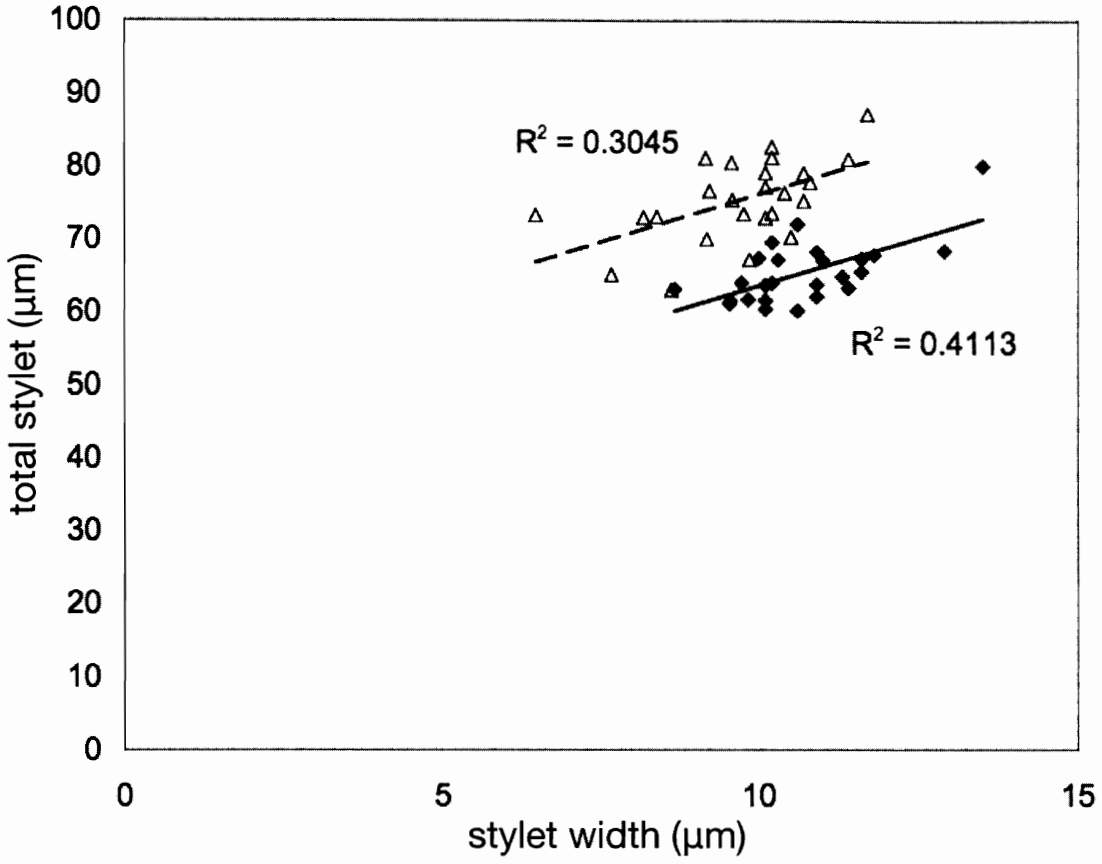

Fig. 11b Total stylet length vs. stylet width for Gulf-clade (◆) and Florida 1- and 2- clade (▲) individuals, with the least-squares regression line shown as in Fig. 11a. ANCOVA indicates a significant difference between the y-intersects, but not the slopes, of the regressions (Table 8).

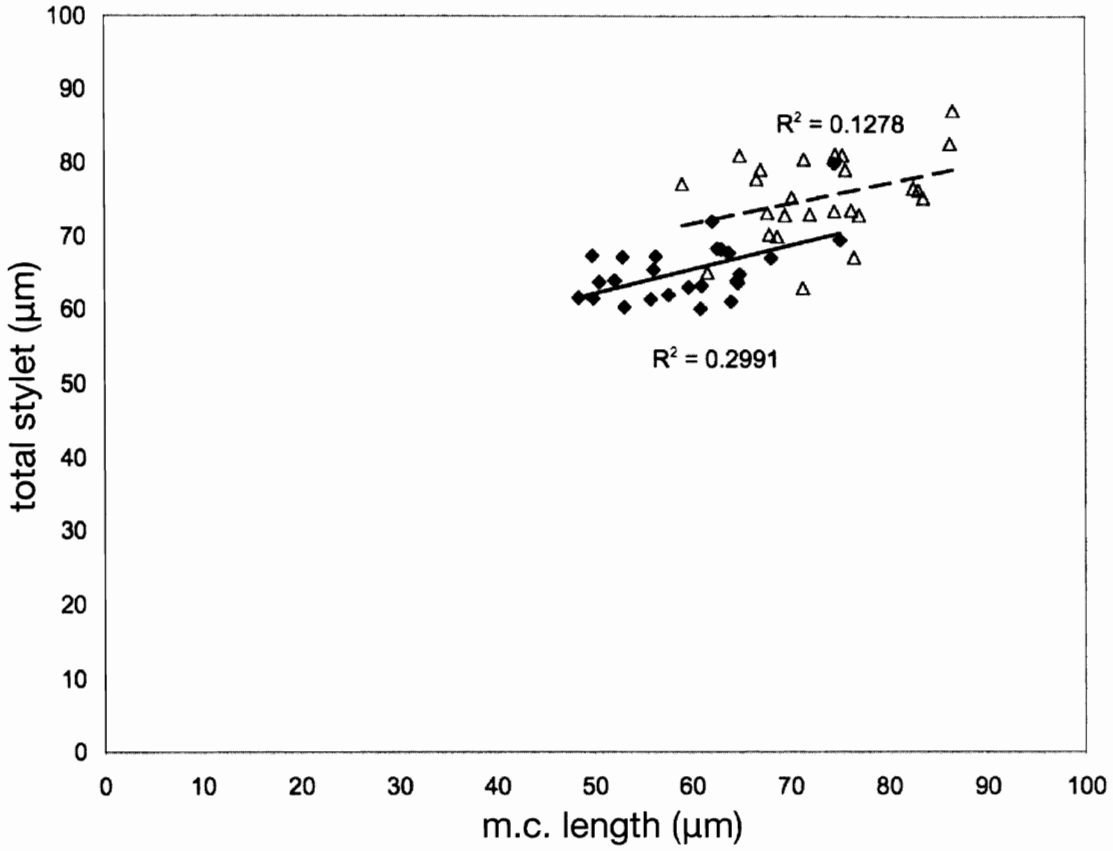

Fig. 11c Stylet length vs. middle chamber length for Gulf-clade (◆) and Florida 1- and 2-clade (▲) individuals, with the least-squares regression line shown as in Fig. 11a. ANCOVA indicates a significant difference between the y-intersects, but not the slopes, of the regressions (Table 8).

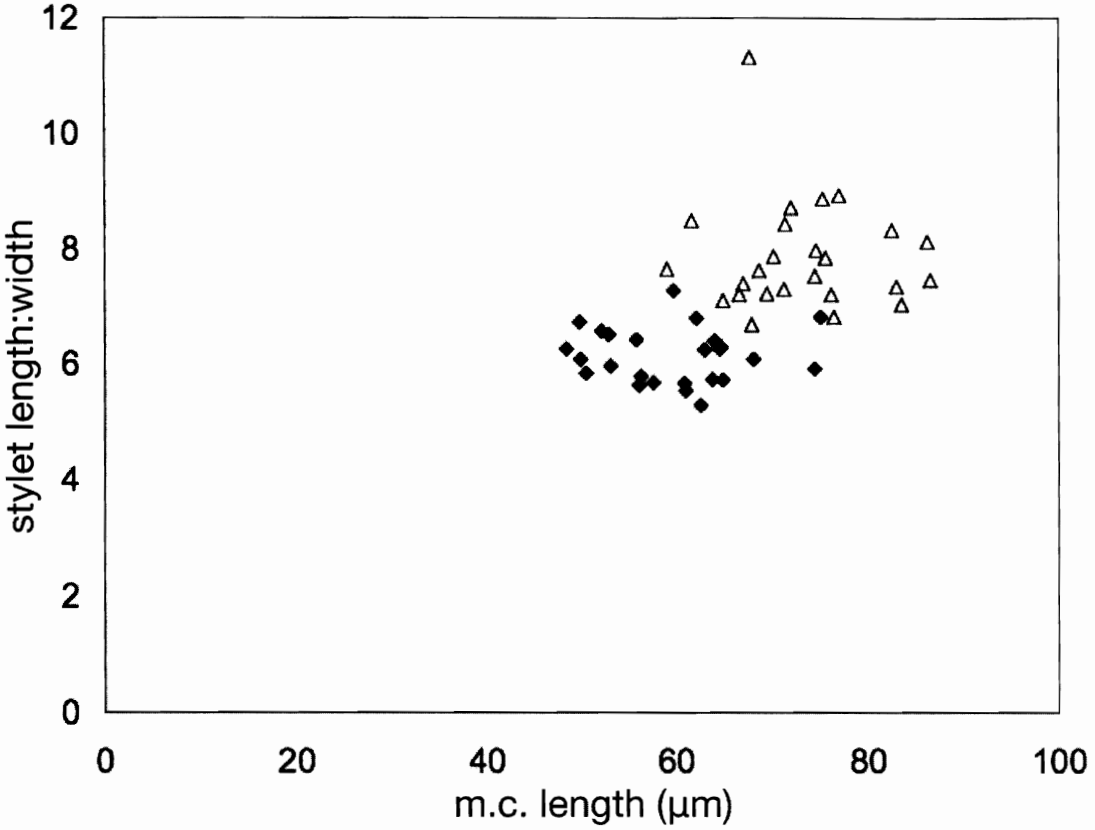

Fig. 11d Stylet length:width ratio vs. middle chamber length for Gulf-clade (◆) and Florida 1- and 2- clade (▲) individuals. A weak linear relationship was found in the data (correlation coefficient $\rho = 0.49$; $R^2 = 0.24$)

APPENDIX B

Tables

Table 1 Sampling sites used in the study including latitude/longitude, and sample size at each site.

Site	n	Lat.	Long.
Bathtub Beach, FL	19	27°11'19.56"N	80° 9'36.96"W
Sebastian Inlet, FL	22	27°51'45.05"N	80°26'50.44"W
Ocean Reef Park, FL	19	26°47'36.42"N	80° 1'55.94"W
Miami Beach, FL	20	25°46'29.06"N	80° 7'48.33"W
Naples, FL	25	26° 8'25.39"N	81°48'26.80"W
Sanibel Island, FL	7	26°25'18.83"N	82° 4'48.50"W
St. Barthélemy	13	17°54'25.70"N	62°49'25.07"W
Montauk, NY	16	41° 4'41.31"N	71°56'5.79"W
East Chop, MA	15	41°28'0.60"N	70°34'34.24"W

Table 2 Population differentiation based on *cox3* mitochondrial sequences. Below diagonal: pairwise Hudson's (2000) S_{nn} and significance. Above diagonal: F_{st} calculated using the method of Hudson, Slatkin, & Maddison (1992) and N_m as estimated from F_{st}. Lack of differentiation is indicated in bold type. All other pairs of populations show significant differentiation based on 1000 permutations of Hudson's S_{nn} statistic. Site abbreviations: St. B = St. Barthélemy; Btb = Bathtub Beach, FL; OR = Ocean Reef Park, FL; Mia = Miami Beach, FL; Seb = Sebastian Inlet, FL; Mntk = Montauk, NY; EC = East Chop, MA; Nap/San = combined samples from Naples, FL and Sanibel Island, FL

	St. B	Btb	OR	Mia	Seb	Mntk	EC	Nap/San
St. B	--	0.685 (Nm=0.23)	0.608 (Nm=0.32)	0.622 (Nm=0.30)	0.513 (Nm=0.47)	0.798 (Nm=0.13)	0.801 (Nm=0.12)	0.800 (Nm=0.13)
Btb	0.849 (p=0.000)	--	**0.037 (Nm=13.0)**	**0.028 (Nm=18.3)**	0.573 (Nm=0.37)	0.951 (Nm=0.03)	0.953 (Nm=0.02)	0.945 (Nm=0.03)
OR	0.807 (p=0.000)	**0.509 (p=0.334)**	--	**0.032 (Nm=15.0)**	0.477 (Nm=0.55)	0.886 (Nm=0.06)	0.888 (Nm=0.06)	0.876 (Nm=0.07)
Mia	0.809 (p=0.000)	**0.471 (p=.624)**	**0.462 (p=0.662)**	--	0.532 (Nm=0.44)	0.918 (Nm=0.04)	0.920 (Nm=0.04)	0.912 (Nm=0.05)
Seb	0.873 (p=0.000)	0.640 (p=0.001)	0.677 (p=0.001)	0.727 (p=0.000)	--	0.480 (Nm=0.54)	0.491 (Nm=0.52)	0.368 (Nm=0.86)
Mntk	1.00 (p=0.000)	1.00 (p=0.000)	0.971 (p=0.000)	1.00 (p=0.000)	1.00 (p=0.000)	--	**0.044 (Nm=10.9)**	0.814 (Nm=0.11)
EC	1.00 (p=0.000)	1.00 (p=0.000)	0.971 (p=0.000)	1.00 (p=0.000)	1.00 (p=0.000)	**0.580 (p=0.065)**	--	0.830 (Nm=0.10)
Nap /San	1.00 (p=0.000)	1.00 (p=0.000)	0.980 (p=0.000)	1.00 (p=0.000)	1.00 (p=0.000)	1.00 (p=0.000)	1.00 (p=0.000)	--

Table 3 Tajima's D, Fu and Li's F*, and McDonald-Kreitman tests for neutral evolution of *cox3* in each mitochondrial clade, in the subset of the Gulf clade corresponding to the Naples, FL population, and in all Fila individuals. The McDonald-Kreitman test was performed only for clades deviating from neutrality based on D and F*, using a clade showing neutral evolution as the outgroup. Significant deviation from neutrality is indicated in bold type.

					McDonald-Kreitman	
Clade	Tajima's D	P-value	Fu & Li's F*	P-value	N.I.	P-value
all Fila	**2.996**	**(< 0.01)**	1.193	(> 0.10)		
Gulf	-1.497	(> 0.10)	**-2.631**	**(< 0.05)**		
Gulf (Naples pop.)	**-2.169**	**(< 0.05)**	**-3.018**	**(< 0.05)**	0.977	1.00
New England	-0.5726	(> 0.10)	-0.8282	(> 0.10)		
Caribbean	-1.083	(> 0.10)	-1.687	(> 0.10)		
Florida 1 + 2	-0.5871	(> 0.10)	-1.977	(> 0.05)		
Florida 1	**-2.440**	**(< 0.01)**	**-4.893**	**(< 0.02)**	2.374	0.136
Florida 2	-1.043	(> 0.10)	-1.167	(> 0.10)		

Table 4 Results from nested clade analysis, with significantly large or small values of D_c and D_n indicated in bold. Clades without significant (based on 10,000 permutations) χ^2 values for geographic structuring of haplotype distribution are not shown. Nested clade assignments are shown in Figs. 9a and 9b.

Clade	chi-sq P-value	Nested clades	Tip/interior	*Dc*		*Dn*	
1-33	0.015	57	Interior	100.0		68.97	
		62	Tip	0.000		77.59	
		58	Tip	0.000		75.00	
		63	Tip	0.000		75.00	
		61	Tip	0.000		75.00	
		60	Tip	0.000		58.33	
		59	Tip	**0.000**	**(p< = 0.012)**	70.00	
			I-T	**100.0**	**(p> = 0.002)**	-2.577	
3-5	0.000	2-10	Interior	**0.000**	**(p< = 0.000)**	244.5	
		2-7	Tip	0.000		186.3	
		2-8	Tip	**29.65**	**(p< = 0.000)**	**415.9**	**(p> = 0.000)**
		2-9	Tip	0.000		186.3	
			I-T	-21.18		**-105.8**	**(p< = 0.000)**
5-3	0.000	4-3	Interior	**320.2**	**(p< = 0.000)**	**1269**	**(p< = 0.001)**
		4-4	Tip	**85.10**	**(p< = 0.000)**	**1574**	**(p> = 0.000)**
			I-T	**235.1**	**(p> = 0.013)**	**-305.0**	**(p< = 0.000)**
6-1	0.000	5-1	Tip	**299.9**	**(p< = 0.000)**	**546.5**	**(p< = 0.000)**
		5-2	Tip	615.4		**1749**	**(p> = 0.000)**
Total	0.000	6-1	Tip	**730.6**	**(p< = 0.000)**	**1056**	**(p< = 0.008)**
Cladogram		6-2	Tip	**1395**	**(p> = 0.031)**	**1368**	**(p> = 0.000)**

Table 5 Results from AMOVA of ISSR data (Dice-Sørensen distances) using mitochondrial clades as populations. Below diagonal: pairwise Φ_{st} values. Above diagonal: p-value for null hypothesis of $\Phi_{st} = 0$ based on 1000 permutations. Clade abbreviations: Carib. = Caribbean clade; N.E. = New England clade; Fl. 1 + 2 = combined samples from Florida 1 & Florida 2 clades; Gulf (E. Coast pop.) = subset of Gulf clade individuals occurring on the Atlantic coast of Florida.

	Carib.	N.E.	Fl. 1 + 2	Gulf (E. Coast pop.)	Gulf
Carib.	--	0.001	0.001	0.001	0.001
N.E.	0.521	--	0.001	0.001	0.001
Fl. 1 + 2	0.697	0.537	--	0.001	0.001
Gulf (E. Coast pop.)	0.887	0.452	0.755	--	0.001
Gulf	0.826	0.333	0.733	0.828	--

Table 6 Population differentiation as indicated by AMOVA of ISSR data (using Dice-Sørensen distances). Below diagonal: pairwise Φ_{st} values. Above diagonal: p-value for null hypothesis of $\Phi_{st} = 0$ based on 1000 permutations. Lack of differentiation is indicated in bold type.

	St. Bart.	Bathtub B.	Ocean Reef	Miami	Seb. Inlet	Montauk + E. Chop	Naples + Sanibel
St. Bart.	--	0.001	0.001	0.001	0.001	0.001	0.001
Bathtub B.	0.596	--	**0.428**	**0.107**	0.001	0.001	0.001
Ocean Reef	0.572	**0.000**	--	**0.211**	0.001	0.001	0.001
Miami	0.481	**0.051**	**0.020**	--	0.001	0.001	0.001
Seb. Inlet	0.515	0.455	0.395	0.376	--	0.001	0.001
Montauk + E Chop	0.419	0.470	0.447	0.393	0.197	--	0.001
Naples + Sanibel	0.742	0.797	0.750	0.697	0.550	0.333	--

Table 7 ISSR band frequencies per locus for each mitochondrial clade. Data from Gulf-clade individuals is separated into East Coast and Gulf Coast populations due to different banding patterns between these two sample sets. Bold type indicates possible gene flow between sympatric populations of divergent clades. n = number of individuals genotyped; N.E. = New England clade

		ISSR Locus														
clade	n	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Caribbean	12	**1.00**	0	0	0	**0.83**	0	0.50	0	**0.08**	0	0	0	0	0	0
N.E.	11	0	0.18	0	0.27	0	0	0	0.45	0	0.09	0	0.27	0.27	0	0
Florida 1	39	**0.05**	0	0.41	0	0	0.97	0	**0.03**	**0.79**	0.59	0.10	0	0	0	0.62
Florida 2	16	**0.06**	**0.06**	0.5	0	**0.06**	1.00	0	0	**0.88**	0.75	0.13	0	0	**0.06**	0.50
Gulf (E coast pop)	15	0	**1.00**	0	0.93	0	0	0	**1.00**	0	0	0	0.07	0	**1.00**	0
Gulf (Gulf pops)	21	0	0	0	0.10	0	0	0	0.95	0	0	0	0	0	0	0

Table 8 One-way ANCOVA comparing linear regressions of morphometric ratios for Gulf-clade individuals with those for Florida 1- and Florida 2-clade individuals (plots with regressions are shown in Figs. 11a – 11c). Also listed are comparisons between Gulf Coast and East Coast populations of Gulf-clade individuals, and between samples collected in March and those collected in August.

		null hypothesis	
		same	same
regression	samples	slope	intersect
total stylet length vs. stylet tip	Gulf and Florida 1+2	0.88	0.097
	Gulf (gulf pop.) and Gulf (e. coast pop)	0.91	0.77
	Mar. and Aug. collections	0.95	0.69
stylet length vs. middle chamber length	Gulf and Florida 1+2	0.78	**0.0019**
	Gulf (gulf pop.) and Gulf (e. coast pop)	0.13	0.22
	Mar. and Aug. collections	0.46	0.34
stylet length vs. stylet width	Gulf and Florida 1+2	0.97	**5.80E-13**
	Gulf (gulf pop.) and Gulf (e. coast pop)	0.55	0.21
	Mar. and Aug. collections	0.34	0.74

VITA

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