Cryptic Species in a Marine Polychaete and Their Independent Introduction from North America to Europe

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The vast body of ballast water carried across oceans by freight ships represents a major source for the introduction of foreign species into marine ecosystems. The worm *Marenzelleria viridis,* originally found only in North America, appeared in estuaries of the North Sea in 1979 and 6 years later also in the Baltic, where it has developed into a major faunal element. Two competing hypotheses are discussed here: either both populations owe their presence to a single introductory event in the North Sea, or each population originated from a separate introduction. Our phylogeographic analysis of Baltic, North Sea and American *Marenzelleria,* based on mitochondrial 16S rDNA sequences (326-bp segment) of 98 individuals from 17 localities on the North American, North Sea, and Baltic coasts not only favors the two-event hypothesis, but also separates the locations of origin for the introductions. Eighteen mitochondrial genotypes were identified altogether. In agreement with allozyme data, three lineages were identified: genotypes assigned to the same lineage differed from each other by up to 5 point mutations, and those assigned to different lineages differed by up to 17. The existence of three morphologically indistinguishable, and thus cryptic, species is therefore suggested. The individuals from the Baltic Sea probably originated from the Atlantic coast of the United States between Chesapeake Bay and Georgia, and the North Sea populations may stem from the U.S. coast region north of Chesapeake Bay to Nova Scotia. Despite their similar morphologies, the two European *Marenzelleria* species may differ ecologically with respect to their preference for habitat salinity. Assuming that transport via ballast water occurs quite frequently, we hypothesize that both European cryptic species of *Marenzelleria* may originally have been introduced to both the North Sea and the Baltic Sea but that neither of them was able to proliferate in both water bodies owing to their differential physiological performances at high and low salinities.

Introduction

Accidental introductions of nonindigenous species into foreign habitats are an inevitable consequence of human activity. Some instances, like the introduction of the nile perch into Lake Victoria (Witte et al. 1992) or of the North Pacific seastar *Asterias amurensis* into Australian waters (McLoughlin and Thresher 1994; Ward and Andrew 1995), proved to be detrimental to the endemic faunas; others did not have such effects. While species often expand their ranges of distribution along coasts, they can travel over long distances and across oceans when other organisms act as biogeographical vectors. Both range expansion and introduction can be identified by combinations of evidence furnished by biogeography, systematics, genetics, ecology, history, archeology, and paleontology (Carlton 1987). Many introduced species may have invaded and gone unrecognized or have been mistaken for a native species. Knowledge of the natural geographic distribution of a species before human-aided introduction is therefore fundamental for the recognition of ecological, biogeographical, and evolutionary relationships (Carlton and Geller 1993).

Successful introductions are particularly likely in marine organisms with many larvae and an extended planktonic stage. For marine organisms, ships have become the most effective carrier. While external fouling of the hull is only possible for sessile species, ballast water acts as a nonselective transport vector. It may contain both planktonic and benthic species. Water has been used for over 100 years in ocean-going ships, but the number of introductions has increased according to the size, number, and speed of ships (Carlton 1985; Carlton and Geller 1993). It has been estimated that, on any one day, several thousand species may be under way in ballast water carried by ocean liners around the world (Carlton and Geller 1993).

Marenzelleria viridis (Polychaeta: Spionidae) produces up to 21 million planktonic larvae per cubic meter of water during its reproductive season, and the planktotrophic larval stage lasts about 8 weeks (George 1966; Bochert and Bick 1995). This species lives in soft sediments in marine and brackish coastal habitats such as river estuaries, salt marshes, or sounds. The genus *Marenzelleria* is restricted to the northern hemisphere (George 1966; Dörjes and Howard 1975; Maciolek 1984). On the northern Pacific coast of the U.S.A., *Marenzelleria* has so far only been found in San Francisco Bay, perhaps also as a result of human introduction (Cohen and Carlton 1995; M. Kellogg, City and County of San Francisco, Department of Public Works, Bureau of Water Pollution Control, personal communication).

The first recent finding of *Marenzelleria* sp. (identified as *M. wireni*) in Europe was in 1979 (Elliott and Kingston 1987). Since its introduction, it has reached population densities of up to 270,000 juveniles and 8,500 adult individuals per square meter and thus has developed into a major faunal element in European marine and brackish habitats (Zettler, Bick, and Bochert 1995). Because the first record of *Marenzelleria* sp. was in the Forth Estuary near Edinburgh, Scotland (Elliott and Kingston 1987; McLusky, Hull, and Elliott 1993),

Key words: *Marenzelleria viridis,* DNA sequences, phylogeography, phylogeny.

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Mol. Biol. Evol. 15(2):97–103. 1998

Table 1
Sample Information and Collection Sites

Sampling Site	Abbreviation in Figures	Coordinates	Number of Individuals	Salinity (‰)	Origin and/or Museum Accession Number
Type I					
Ems Estuary, Netherlands	Ee	53°17′N, 07°11′E	9	20	Collected by the authors
Tay Estuary, Scotland	Ta	56°26′N, 03°03′W	5	16	Collected by the authors
Great Sippewisset Salt Marsh, U.S.A.	Gs	41°34′N, 70°39′W	4	30	Collected by the authors
Barnstable Harbor, U.S.A.	Bh	41°43′N, 70°23′W	6	22	Collected by the authors
Westport River, U.S.A.	Wr	41°30′N, 71°05′W	9	32	Collected by the authors
Cape Henlopen, U.S.A.	Ch	38°47′N, 75°07′W	9	32	Collected by the authors
Nova Scotia, Canada	Ns	43°06′N, 65°09′W	1	32[a]	Canadian Museum of Nature, ACC 1973-231
Type II					
Darss-Zingst Bodden, Germany	Dz	54°17′N, 12°26′E	5	5	Collected by the authors
Greifswalder Bodden, Germany	Gb	54°18′N, 13°24′E	3	8	Collected by the authors
Kurisches Haff, Lithuania	Kh	55°39′N, 21°08′E	4	1–2	Collected by the authors
Chesapeake Bay, Chester River, U.S.A.	Cr	39°05′N, 76°10′W	9	8	Collected by the authors
Chesapeake Bay, Trippe Bay, U.S.A.	Tb	38°04′N, 76°18′W	7	9	Collected by the authors
Ogeechee River, U.S.A.	Or	31°53′N, 81°16′W	8	1–2	Collected by the authors
Currituck Sound, U.S.A.	Cs	36°21′N, 75°48′W	5	4	Collected by the authors
New Hampshire, Durham, Fox Point, U.S.A.	Nh	43°07′N, 70°54′W	2	?	Smithsonian Institution, USNM 80 485, ACC 301700
Tuktoyaktuk Harbor, Northwest Territories, Canada	Th	69°29′N, 132°53′W	3	?[a]	Canadian Museum of Nature, ACC 1984-031
Type III					
Currituck Sound, U.S.A.	Cs	36°21′N, 75°48′W	9	4	Collected by the authors

[a] Personal communication by Judith A. Fournier, Canadian Museum of Nature; Tuktoyaktuk Harbor has reduced salinity, at least at the surface.

it was suggested that *Marenzelleria* was originally introduced into the North Sea and then spread into the Baltic Sea (Essink and Kleef 1993), where it appeared 6 years later (Bick and Burckhardt 1989). Larval transport could have occurred along the centerclockwise current system of the North Sea, by the steady water exchange of about 1,200 km^3/year with the Baltic (Otto et al. 1990), or by ships. An alternative hypothesis on the basis of allozyme data (Bastrop, Röhner, and Jürss 1995; Röhner, Bastrop, and Jürss 1996*a*, 1996*b*) suggests, however, that there have been at least two independent colonization events.

Materials and Methods

To test these alternative hypotheses for the colonization of the North Sea and the Baltic Sea by *Marenzelleria* spp., we sequenced a 326-bp segment of the mitochondrial 16S rDNA of 98 individuals from 17 localities on the North American, the North Sea and the Baltic Sea coasts (table 1). Total DNA was extracted from ethanol-preserved tissue samples using Chelex 100 (Bio-Rad no. 143-2832). Tissue was placed in 500 μl of 5% Chelex 100 in sterile H_2O and incubated at 56°C for 3 h. After brief vortexing, extracts were incubated at 95°C for 15 min and then stored at 4°C. Extracts were centrifuged at 13,000 rpm for 5 min before use. Aliquots (1 μl) of the supernatants were directly used for PCR. Two amplifications (one double-stranded and one single-stranded) were conducted as described elsewhere (Sturmbauer and Meyer 1993). The primers used were 5′-CGCCTGTTTATCAAAAACAT-3′ (16sar; Kessing et al. 1989) and 5′-CCGGTCTGAACTCAGATCACGT-3′ (16sbr; Kessing et al. 1989). Single-stranded amplification products were ultrafiltrated three times with 300 μl H_2O in spin colums (Millipore 30,000) before direct sequencing (Sequenase Images Non-Isotopic DNA Sequencing System; U.S. Biochemical; Sanger, Nicklen, and Coulson 1977). Both strands were sequenced using the 16sar and 16sbr primers, which were biotinylated at the 5′ end. The sequences were electrophoresed on 6% acrylamide-urea gels in Tris-borate-ethylenediamine-tetraacetate buffer (27 mM, pH 8.0) and visualized according to the manufacturer's protocol.

Phylogenetic Analyses

The sequences were entered and aligned by eye in ESEE (version 1.09; Cabot and Beckenbach 1989). No insertion/deletion events had to be infered in order to align the sequences. Phylogenetic analysis was performed by applying the parsimony and neighbor-joining methods in parallel using PAUP (version 3.1.1; Swofford 1993) and NJBOOT2 (Tamura 1993). The phylogeny is based on 26 phylogenetically informative positions and rooted using midpoint rooting. For parsimony analysis, the PAUP options heuristic search with random addition of taxa and 20 replications and ACCTRAN were chosen. All substitutions were weighted equally in parsimony analysis because of the small number of substitutions observed. Accordingly, Jukes-Cantor distances (Jukes 1980) were used for neighbor-joining. Phylogenies were subjected to bootstrap analysis (Felsenstein 1985) in both parsimony and neighbor-joining analyses. The DNA sequences of this study are available from EMBL under the accession numbers Z99778–Z99795.

Results and Discussion

The 98 sequenced individuals comprised 18 different mitochondrial genotypes. These genotypes were

Table 2
Genetic Distances Within and Among the Three Lineages of *Marenzelleria*

Type	I	II	III
I	*0.3%–1.5%* / 0.000–0.020	*2.2%–3.7%*	*4.3%–5.3%*
II.	0.995–1.127	*0.3%–0.9%* / 0.000–0.007	*3.4%–4.3%*
III	1.301–1.429	1.529–1.576	*0.3%* / NA

NOTE.—Minimum and maximum percentage differences within and among the three lineages of *Marenzelleria viridis* (types I–III). p distances among and within the three lineages of a 326-bp segment of the mitochondrial 16S rDNA appear above the diagonal in italic type. Below the diagonal are Nei's (1972) distances among and within populations of different lineages (Röhner, Bastrop, and Jürss 1996*b*). NA = not applicable.

grouped into three major mitochondrial lineages (table 2, upper section). Genotypes assigned to the same major lineage differed from each other by up to 5 point mutations (1.5%); those assigned to different major lineages differed by up to 17 mutations (5.3%). The lineages inferred from mitochondrial DNA sequences were congruent with the lineages inferred from Nei's genetic distances among populations (Röhner, Bastrop, and Jürss 1996*b*), summarized in table 2 (lower section).

The mitochondrial phylogeny (fig. 1*a*) is in agreement with the distance data (table 2) and with the phylogeny derived from allozyme data (fig. 1*b*; Röhner, Bastrop, and Jürss 1996*b*). The first lineage (named type I) comprises individuals from the North Sea and the North American East Coast from Nova Scotia south to Cape Henlopen, Delaware (figs. 1 and 2, circles). The second lineage (named type II) represents individuals sampled in the Baltic Sea and on the East Coast of the United States from Chesapeake Bay down to the Ogeechee River, Georgia (figs. 1 and 2, squares). Type II was also recorded at two other locations, one in New Hampshire and the other in the Arctic Ocean (The museum specimens from the Arctic were identified by J. Fournier as *Marenzelleria arctius*, Chamberlin 1920; see fig. 2). Foster (1971) also synonymized *Scolecolepides arctius* Chamberlin 1920 with *Scolecolepides viridis.* The third lineage has so far been found at a single location only, *viz.* Currituck Sound (North Carolina), and is named type III (see figs. 1 and 2, triangles). While type I and type II were never found in sympatry, type III has so far only been found sympatric with type II. Prior to the present study, *Marenzelleria* type III had been identified only by an analysis of electrophoretic allozyme data (Röhner, Bastrop, and Jürss 1996*b*). As yet, no morphological analysis has been carried out.

Static Morphology and Cryptic Species

The three identified lineages are nearly indistinguishable morphologically. The morphological characters presently used (i.e., numbers of segments with branchiae, shape of the prostomium) show too much variability to allow differentiation between lineages, at least for some life stages (Maciolek 1984; Bick and Zettler 1997), as has also been documented for another polychaete, *Capitella capitata* (Grassle and Grassle 1976). Bick and Zettler (1997) undertook a preliminary comparative morphological analysis of *Marenzelleria* types I and II in light of the population genetic analysis (Bastrop, Röhner, and Jürss 1995; Röhner, Bastrop, and Jürss 1996*a*, 1996*b*; results presented in this paper) and mostly corroborated the differentiation inferred by means of allozyme and/or sequence data. However, their methods require specimens of a certain size and in a satisfactory state of preservation. Size-dependent variations in certain morphological features have been found in both European *Marenzelleria* species, so larvae and juveniles can still not be identified with confidence. Contradictions between the results of morphological and genetic species determination still exist, however, particularly with respect to the specimens from New Hampshire (Nh; Durham, Fox Point). DNA sequence analysis has shown beyond doubt that these animals belong to *Marenzelleria* type II, whereas, on the basis of morphological features, Bick and Zettler (1997) would assign them to type I.

The congruence between mitochondrial markers and allozymes as nuclear markers documents the existence of separate gene pools, but does not prove the existence of three, instead of one, species. Only congruence between genetic data sets (DNA sequences and allozymes) and distinct biological characteristics allows precise delineation of species**.** The sympatry of two distinct lineages (types II and III), together with their genetic distinctness, does indicate their reproductive isolation. In contrast, types I and II have so far never been found in sympatry, but other biological characteristics of European populations suggest their reproductive isolation: North Sea populations of type I reproduce in early spring, whereas Baltic populations of type II reproduce in autumn (Atkins, Jones, and Garwood 1987; Bochert and Bick 1995).

The salinity data we obtained refractometrically or by conductivity measurements at the time of collection (low tide in tidal waters) show that populations of type I were found only in habitats of higher salinity (16‰–32‰) and that those of type II was found only at lower salinities (1‰–9‰; see table 1; exact data are not available for the sequenced museum specimens). Although this is not conclusive, it does suggest that type I is restricted to a higher salinity range than is type II.

Our analyses also indicate possible locations from which the introductions originated (fig. 2). The individuals from the Baltic Sea are likely to have originated from the Atlantic coast of the United States between Chesapeake Bay and Georgia. All sampled populations of type II, from the Baltic, the U.S. east coast including the isolated find in New Hampshire, and from the Arctic, share at least one genotype, which is also the most common. It seems likely that all of these populations have a common origin and have spread recently since they still contain identical genotypes. This is also supported by the results of allozyme studies (Röhner, Bastrop, and Jürss 1996*b*) showing that the gene flow (the number of migrants exchanged between populations per generation) between populations of type II (Nm = 25.1) is

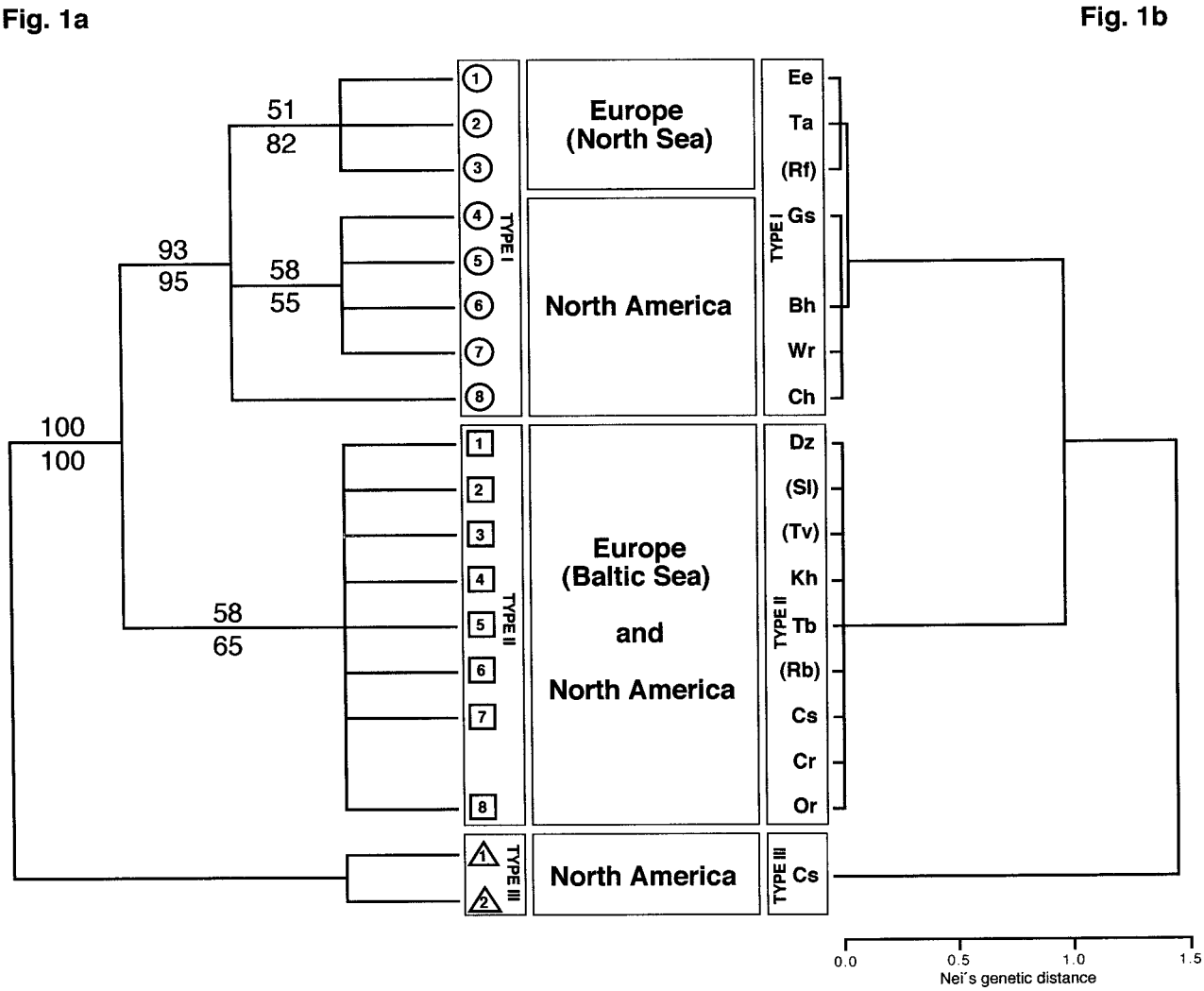

FIG. 1.—Molecular phylogeny of European and American populations of *Marenzelleria* spp. *a,* Bootstrap consensus tree (50% majority rule) of parsimony (500 replications) and neighbor-joining (1,000 replications), based on 16S ribosomal DNA sequences. Eighteen different genotypes, found among 98 sequenced individuals of 16 populations, were analyzed; no insertion/deletion events had to be inferred to align the sequences. The tree is based on 26 phylogenetically informative positions and rooted using midpoint rooting. All substitutions were weighted equally in parsimony analysis due to the small number of substitutions. Accordingly, Jukes-Cantor distances were used for neighbor-joining. Parsimony analysis (PAUP options heuristic search, random addition of taxa, 20 replications, ACCTRAN) resulted in 54 equally parsimonious trees of a length of 32 substitutions; the consistency index excluding uninformative sites was 0.77. Numbers above the branches are parsimony bootstraps; those under the branches are neighbor-joining bootstrap numbers. The three identified lineages are boxed and labeled as type I, type II, and type III, as they were defined in the allozyme study (Röhner, Bastrop, and Jürss 1996*b*). Type I is represented by circles, type II by squares, and type III by triangles. *b,* Dendrogram based on Nei's (1972) distances of 10 allozyme loci of 17 populations using BIOSYS-1 (Swofford and Selander 1989) and UPGMA. Taxon labels are abbreviations of sampled populations as given in table 1; no DNA sequences have been obtained from those populations in parentheses (Rf = Ringkobing Fjord, Denmark; Sl = Schlei River, Germany; Tv = Tvärminne area, Finland; Rb = Riga Bay, Estonia). The three identified lineages are again boxed and labeled as type I, type II, and type III. Biogeographic regions of sampled populations are given in the centers of the two phylogenetic trees.

almost twice as high as that for type I. Sample location has no significant effect among Baltic populations (*G*-test), so the animals can be regarded as forming a single large population. Röhner, Bastrop, and Jürss (1996*b*) suggest that the Baltic populations of *Marenzelleria* type II probably have their origin in Chesapeake Bay or Currituck Sound. Populations of type I, from the North Sea and the U.S. east coast and Nova Scotia, form two separate but closely related clades. Since the European and American populations do not share genotypes (fig. 1*a*), the exact founder population may not yet have been found.

In autumn 1996, specimens of *Marenzelleria* type II were discovered in the upper Elbe estuary in the North Sea (Germany; Bastrop et al. 1997; Bick and Zettler 1997). In Europe, type II has been found so far only in the Baltic Sea. Whether the two European *Marenzelleria* species (types I and II) occur sympatrically or parapatrically in the Elbe estuary will have to be investigated. The discovery of *Marenzelleria* type II in the upper Elbe estuary in 1996 allows the provisional conclusion that salinity differences as found in North Sea estuaries are crucial for the distribution of European *Marenzelleria* species. This seems to justify the as-

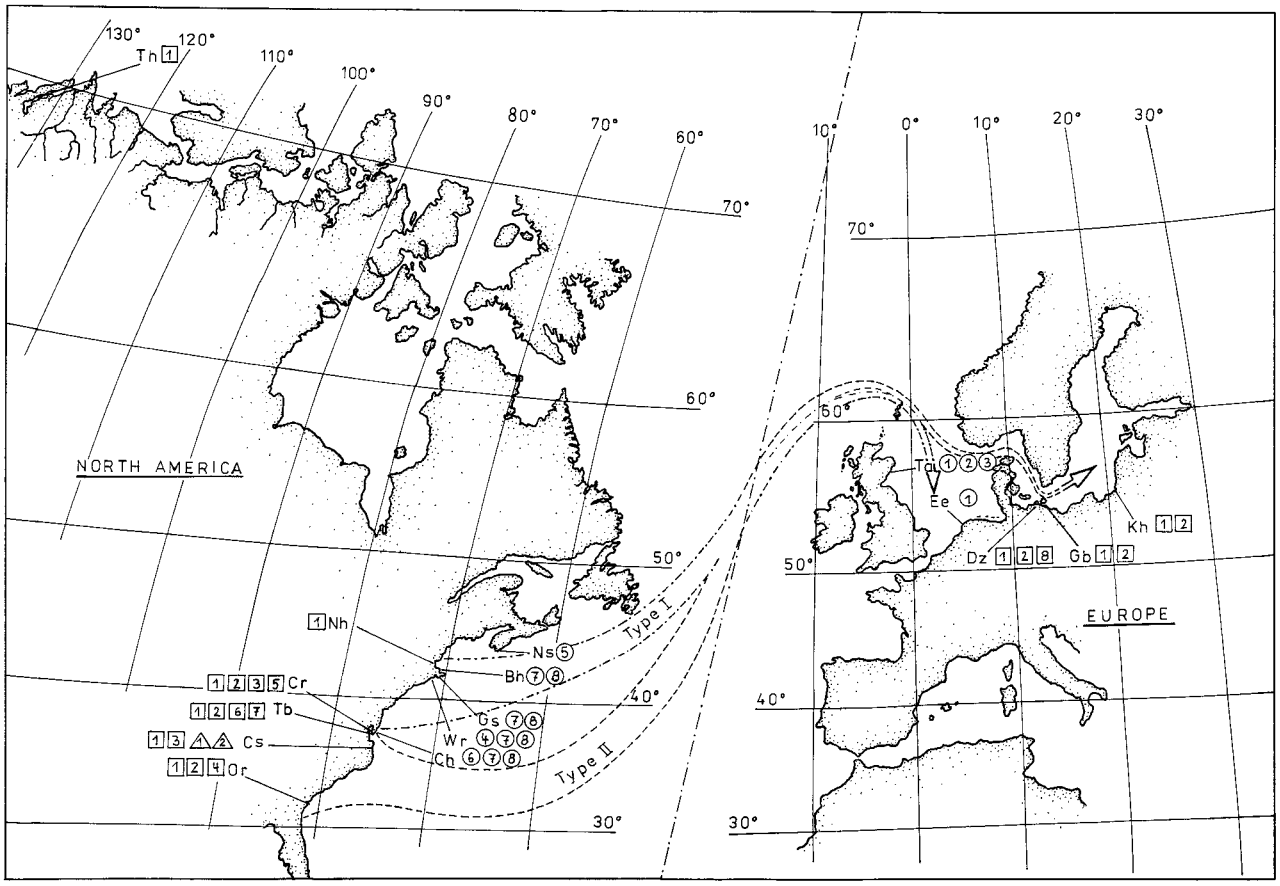

FIG. 2.—Map of the Atlantic coasts of the U.S.A. and Europe with the distribution of the three identified major lineages of *Marenzelleria* spp. The locations of sampled populations are abbreviated according to table 1 and figure 1. The three lineages are labeled by the symbols defined in the phylogenetic tree shown in figure 1*a* (circle = type I; square = type II; triangle = type III). Numbers within the symbols refer to the mitochondrial genotypes. The stippled arrows indicate the hypothesized locations of origin of the two separate introductions, of type I into the North Sea and of type II into the Baltic Sea.

sumption of a high-salinity lineage and a low-salinity lineage (Bastrop et al. 1997). Naturally, it is impossible to be sure that type I never arrived in the Baltic, but, as no specimen has been found there we can be fairly certain that if it did, it failed to become established.

Species Distribution and Taxonomy

Our genetic analysis casts doubt on the validity of the present species assignment of the genus *Marenzelleria,* since none of the three identified lineages can be unambiguously assigned to a single described species. If *Marenzelleria* type II would represent the species *Marenzelleria viridis* (originally named *Scolecolepis viridis* Verrill 1873 and *Scolecolepides viridis* Maciolek 1984; Bick and Zettler 1997), its area of distribution would have to be redefined, as there can be no doubt that the museum specimens of *Marenzelleria arctius* (Tuktoyaktuk Harbor, Th) also belong to *Marenzelleria* type II. Moreover, there are discrepancies between Maciolek's (1984) assignment to species and ours, in that she assigns the animals from Barnstable Harbor (Bh; Massachusetts) and New Hampshire (Nh; Fox Point in Durham) to a single species (*Marenzelleria viridis*), whereas according to our results and those of Röhner, Bastrop, and Jürss (1996*b*), these animals belong to different lineages (Bh to type I and Nh to type II). Also, neither we nor Röhner, Bastrop, and Jürss (1996*b*) were able to find *Marenzelleria jonesi* specimens in Cape Henlopen (Ch; Delaware), although Maciolek (1984) reported that it is found only there. In our opinion the species status of *M. jonesi* is very doubtful. Rodi and Dauer (1996) were unable to follow Maciolek's (1984) description on the basis of morphological criteria. Our genetic findings suggest that *Marenzelleria* type I occurs in that area and that no other species of this genus is present there.

Finally, the taxonomic problems caused by the use of morphological characters only may also apply to *M. wireni.* This species cannot be consistently delineated on the basis of morphology (Maciolek 1984). Maciolek (1984) described the occurrence of *M. wireni* as largely restricted to the Arctic Circle, but also included the North Sea in their original area of distribution. *Marenzelleria wireni* has been found in the waddens of the island Sylt in the southern North Sea (Wohlenberg 1937; Otte 1979) and, in 1979, in the Forth Estuary in Scotland (Elliott and Kingston 1987). On the basis of these findings, Atkins, Jones, and Garwood (1987) suggested that the number of introductions of *Marenzelleria* may

even be larger. They noted: ''If, however, the two forms [North Sea *Marenzelleria viridis* and *Marenzelleria wireni*] are synonymous, then an origin in the European Arctic is possible, and natural processes may be sufficient to account for the presence of the Tay population . . .'' If *Marenzelleria* type I were synonymous with *Marenzelleria wireni* (Bick and Zettler 1997), the reported distribution range (Arctic) of the species (Maciolek 1984) would require major revision. If so, the Arctic would also be a potential location of origin for the European *Marenzelleria* type I.

Conclusions

The phylogeographic pattern derived from the mtDNA phylogeny clearly favors the two-event hypothesis of introduction of *Marenzelleria* spp. to Europe. From the congruence between mtDNA, allozymes, and biological characteristics we suggest, in contradiction to the present taxonomy, that three cryptic species exist. Assuming that transport via ballast water occurs quite frequently, we hypothesize that both European cryptic species of *Marenzelleria* may originally have been introduced to both the North Sea and the Baltic Sea but that neither was able to proliferate in both water bodies owing to constraints on their physiological performances at high and low salinities.

Our investigations, together with those published by Bastrop, Röhner, and Jürss (1995) and Röhner, Bastrop, and Jürss (1996*a*, 1996*b*) demonstrate that knowledge of phylogeny and natural geographic distributions are of paramount importance for species delineation and interpretation of patterns in ecology and biogeography (Carlton and Geller 1993). Molecular data can help to identify reproductive units masked by static morphology.

We feel that the data for the genus *Marenzelleria* available at present (bibliography about the genus *Marenzelleria* of about 233 sources; Zettler 1997) are not yet complete enough to fully understand the patterns observed. More genetic data are necessary for a conclusive discussion of phylogeograpy, and additional species may be discovered in the future. Genetic methods will remain necessary for a definitive species assignment of all life stages, and more rapid screening methods based on PCR should be developed. Future studies should relate morphological characters to genotypes and address intraspecific variability of morphological features. Finally, comparative ecophysiological studies of the two European *Marenzelleria* species should shed light on the specific environmental requirements and identify specific ecological and life history characteristics of these so far cryptic species.

Acknowledgments

This work was supported by the Bundesministerium für Forschung und Technologie (FRG), FKZ 03F0031A. We gratefully acknowledge U. Schöttler, R. Rieger, and W. Wieser for discussion, and the many colleagues who helped us before and during sampling: C. Avila, D. M. Dauer, D. Daunys, K. Essink, J. P. Grassle, J. Hardege, D. Miller, S. Olenin, P. Rodi, M. Röhner, D. Russell, R. Sarda, D. Schiedek, J. T. Turner, I. Valiela, and J. R. Weinberg. We also thank L. A. Ward and K. Fauchauld, Smithsonian Institution, Washington, D.C., and J. A. Fournier, Canadian Museum of Nature, Ottawa, for providing museum samples.

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CARO-BETH STEWART, reviewing editor

Accepted October 21, 1997