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Molecular Phylogeny of Some Polychaete Annelids: An Initial Approach to the Atlantic–Mediterranean Speciation Problem

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Summary. The polychaete *Eupolymnia nebulosa* (family Terebellidae) displays two alternative modes of reproduction. In the Mediterranean, larvae are brooded in a mucous mass while in the Atlantic and English Channel, larvae follow a planktonic development. This paper attempts to discern whether this difference is expressed at the population, infraspecies, or species level. Specimens of *E. nebulosa* and representatives of a number of control species were sampled from Atlantic/English Channel and Mediterranean locations. Genetic sequencing of the Large-subunit ribosomal RNA 5′ end of six representative species allowed one to infer the relative position of *E. nebulosa* within the Terebellidae and the position of the latter within the animal kingdom. The relative genetic distances calculated between the different species were also used to approach the speciation problem raised by the differences between the Mediterranean and Atlantic/English Channel population of *E. nebulosa*. The genetic distance between Mediterranean and Atlantic populations of both *E. nebulosa* and *Lanice conchilega* are of the same order, suggesting that differences between the populations of *E. nebulosa* are infraspecific.

Key words: Terebellidae — Atlantic–Mediterranean relation — Speciation — Large-subunit ribosomal RNA — Direct sequencing

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Introduction

Studies of the reproductive biology of the terebellid polychaete *Eupolymnia nebulosa* (Montagu) at different points of its geographic range show the presence of two reproductive modes, the characteristics of which are summarized in Table 1. The most obvious difference is the presence or absence of brooding in mucous egg masses. Egg masses are present in Mediterranean populations of *E. nebulosa* (Milne-Edwards 1845, Claparède and Mecznikow 1868, 1869; Salensky 1883; Bhaud et al. 1987, Bhaud and Grémare 1991) but not in the Atlantic, as shown by observations in South Scandinavia (Holthe 1986a,b), Ireland (O'Connor, personal communication), Roscoff (Cabioch et al. 1968), Dinard (Lang 1986), and the Atlantic coast of Spain (Lopez-Jamar, personal communication). Two explanations of this phenomenon are possible. Either *E. nebulosa* exhibits poecilogony (Hoagland and Robertson 1988), i.e., the production of different larvae types, or *E. nebulosa* from the Atlantic and Mediterranean constitute two different species. In the latter case, morphological criteria are not powerful enough to justify separation into two distinct species (Lang 1986; Bhaud and Grémare 1988). The observed differences between these disparate populations are manifested only by biological criteria that are related to the lengths of the planktonic larval phase in correlation with the respective reproductive strategies (Menge 1975; Chia 1976; Pechenik 1979; Strathmann 1986). Thus, there would appear to be a case for poecilogony in this species,

Table 1. Comparison of some biological features between the two populations of *E. nebulosa*

	Features	Mediterranean Sea (Banyuls	Atlantic and English Channel (Dinard)
1	Length of spawning period	3 months: March → May	First part of July
2	Number of spawning period per season and per female	3–4	1
3	Growing period of oocytes	September → February	December → June
4	Values of temperature during this period	Decreasing	Increasing
5	Protection of eggs	Yes	No
6	Age of the first maturity of females	2 yr	1 yr

where reproductive polymorphism would be related to geographic separation reflecting a variation between distant populations. The divergence may either be a reversible adaptation or may represent a more unidirectional divergence associated with the vicissitudes of the Mediterranean settlement following the Messinian crisis (Stanley 1990) or with fluctuations connected with ice ages (Imbrie and Imbrie 1979). An attempt to address these problems has been made by quantifying the biochemical differences at the level of RNA between specimens living in the English Channel (Dinard, Roscoff) and those living in the Mediterranean Sea (Banyuls).

The biochemical basis of the comparison may be justified as follows. Ribosomal RNAs have been widely used as markers in phylogenetic studies, these molecules having been selected for many reasons:

- Because the translation messenger RNA's coding for proteins is universal and because rRNA sequences contain some totally homologous regions, they must have been present in the first forms of life. Therefore, their present structure reflects their evolution since life appeared on Earth (Clark 1987; Ragan 1987).
- They include strands ranging in length from 120 nucleotides to many thousands and each nucleotide may be considered as an independent character that may adopt four states (A, C, G, and U).
- The two longest rRNA (17–18S and 26–28S) molecules show a mosaic pattern with alternating conserved and variable regions, facilitating the inference of long-range phylogenies from the former and short-range phylogenies from the latter.
- Finally, the data base for rRNA sequences is growing dramatically and now includes sequences for many species representative of the major taxonomic groups.

Qu et al. (1989) have studied the evolution of the 5′ end (the first 360 nucleotides (nt)) of the Large-subunit ribosomal RNA (LSUrRNA) corresponding to the D1 variable domain and the flanking conserved domains. A time-related calibration of these regions has shown that the conserved sequences may be used to infer long-range phylogenies and that the variable domain plus the conserved domains may be used to infer short-range phylogenies, both with a good degree of reliability. This region has been selected for the present study.

The method most commonly used to infer phylogenies from sequences is based on a phenetic approach. This entails the calculation of the distance between each pair of organisms followed by the construction of a hierarchical tree using a suitable sorting method. It is therefore possible, by comparing sequences, to estimate a genetic distance between organisms. We have exploited this technique in order to investigate the speciation problem posed by *E. nebulosa* with its different reproductive modes in the Mediterranean and the Atlantic/English Channel.

Material and Methods

Biological Material. Eupolymnia nebulosa specimens used in these experiments were obtained from Roscoff (English Channel) and from Banyuls (Mediterranean Sea). Knowing that the difference eventually displayed by this comparison alone would not establish the level of the divergence (population, subspecies, species), specimens of *Lanice conchilega* (Pallas) from the English Channel and the Mediterranean were also analyzed to provide comparison for a control species which does not show the reproductive divergence of the *E. nebulosa* populations. From the morphological characters used by systematicians or the biological characteristics of ecologists, these geographical distant specimens of *Lanice* are deemed to belong to the same species. They do not show any differences in their spawning modalities. Thus *Lanice* is chosen as a control couple. In order to make the examination of the family even more complete, two other species were analyzed: *Eupolymnia nesidensis* (Delle Chiaje) and *Amphitrite edwardsi* (Quatrefages). These species constitute two additional controls, more or less distant (morphologically or biochemically) from the central species of this study. All four species belong to the subfamily Amphitritinae, family Terebellidae and order Terebellomorpha (Holthe 1986).

Eupolymnia nebulosa and *E. nesidensis* are distinguished by body color and the morphology of uncini: living specimens of the former are yellowish red with white spots and the latter are in uniform color; thoracic uncini of *E. nebulosa* have two large and 1–5 small teeth above rostrum; those of *E. nesidensis* have one large and three small teeth above rostrum. *Lanice* sp. are distinguished from *Eupolymnia* sp. by always having uncini in double rows. It is difficult to separate *Lanice* sp. (avicular uncini) from

Table 2. Geographical distribution and origin of the analyzed material

Species	Geographic distribution of material		Origin of material used	
	Medit.	Atl. + English Channel	Medit. (Banyuls)	English Channel (Roscoff)
Eupolymnia nebulosa (Montagu, 1818)	+	+	+	+
Eupolymnia nesidensis (Delle Chiaje, 1828)	+	+	+	
Lanice conchilega (Pallas, 1766)	+	+	+	+
Amphitrite edwardsi (Quatrefages, 1865)		+		+

Loimia sp. (pectinate uncini). Consequently, each selected specimen of *L. conchilega* was examined microscopically before testing. More detailed descriptions of these species can be found in Fauvel (1927), Day (1967), and Holthe (1986a,b) (Table 2).

Biochemical Processes

RNA Purification and Sequence Determination. Whole animals were squashed and homogenized in a potter tube containing 2.5 ml of 6 M guanidinium isocyanate, 5 mM sodium citrate (pH 7.0), 10 mM betamercapto-ethanol, and 0.5% sarkosyl, per gram of animal. Insoluble residues were discarded after centrifugation at 12 krpm for 30 min. Total RNA was purified by centrifugation through a 5.7 M CsCl cushion, solubilized in 10 mM Tris (pH 7.5), 1 mM EDTA, and washed by a phenol chloroform extraction. Polysaccharides were discarded by two LiCl precipitations (Maniatis et al. 1982).

Sequences from the LSUrRNA 5′ end were determined by reverse-transcriptase-mediated primer extension reaction on total rRNA (Qu et al. 1983; Lenaers et al. 1991). The ^{32}P-labeled primer is complementary to the conserved sequence region corresponding to the nucleotides 369–388 of the mouse LSUrRNA sequence; DNA fragments extending from the primer using a mix of the four deoxynucleotides and stopped by dideoxynucleotides incorporation (Sanger et al. 1977) were run on a 6% acrylamide gel for variable lengths of time.

Alignment of the annelid LSUrRNA D1 Sequences and Phylogenetic Inference. The efficient and rapid sequencing method based on direct cDNA synthesis on total RNA using a specific primer allowed the determination of some 360 nucleotides from the 5′ end of the LSUrRNA in one reaction (Fig. 1). The highly conserved sequence, corresponding to positions 1 to 128 and 274 to 362, can be easily aligned with that of other animals and the eucaryotes in general (Qu et al. 1989). On the other hand, the sequence in between, corresponding to the D1 variable domain, does not show any homology with species situated taxonomically outside of the annelid group. Therefore, large-range phylogeny is based on the conserved sequences (216 nt) and the annelid phylogeny is based on the maximum length sequence (362 nt). For both studies, phylogenetic distances were determined by counting nucleotide differences between each pair of sequences (Table 3). Deletions (single or multiple) were considered as events of the same weight as mutations. Hierarchical trees were constructed using the modified Neighbor-Joining method (Saitou and Nei 1987; Studier and Keppler 1988). The same tree topologies were obtained using the Fitch algorithm.

Results

Large-Range Animal Phylogeny

We have included in this study most of the animal sequences available for this part of the molecule (Qu et al. 1989) and have chosen *Saccharomyces cerevisiae* as the ancestor outgroup species. The tree topology presented in Fig. 2 clearly shows that the Terebellidae have emerged separately from, and later than, the helminth worms. Their origin appears to be shared with that of the Mollusca and is separate from the common origin of vertebrates and echinoderms. This is in good agreement with the morphologically based phylogenies (e.g., Barnes et al. 1988) and with the phylogeny inferred from the comparison of 17–18S rRNA total sequence (Lake 1990), thus, confirming the reliability of our phylogenetic marker. Concerning the Terebellidae phylogeny, the topology inferred only suggests that the *Lanice* species are opposed to a group formed by *Eupolymnia* and *Amphitrite*, a fact we will comment later. Among the group formed by *Eupolymnia* and *Amphitrite* species, the topology of the branchings is not significant, because it is based on phylogenetical distances which are too small. Therefore we added the phylogenetic information included in the D1-variable region, to specify the correct branching pattern among the group.

Short-Range Terebellidae Phylogeny

By including the D1-variable region in measuring the phylogenetic distances, on the one hand we increased the resolution of the Terebellidae tree topology but on the other hand we had to restrict the analysis to that group, because no significant homology has been found between Terebellidae D1 variable domain and that of other eucaryotes. Thus the topology inferred on the 362-nt long alignment suggests that *E. nesidensis* is more closely related to *E. nebulosa* than it is to *A. edwardsi*, which is in good agreement with classical rules of systematics. Because we arbitrarily positioned the root of this tree on the *Lanice* branching, according to the topology of the former tree, we have no direct measure for the exact length of the branch leading to the *Lanice* species in regard to the position of the root. Nevertheless, their distances relative to other Terebellidae species are significant and may be considered for the following speciation analysis.

The distance matrix isolates the two geographically distinct populations of *E. nebulosa* (seven dif-

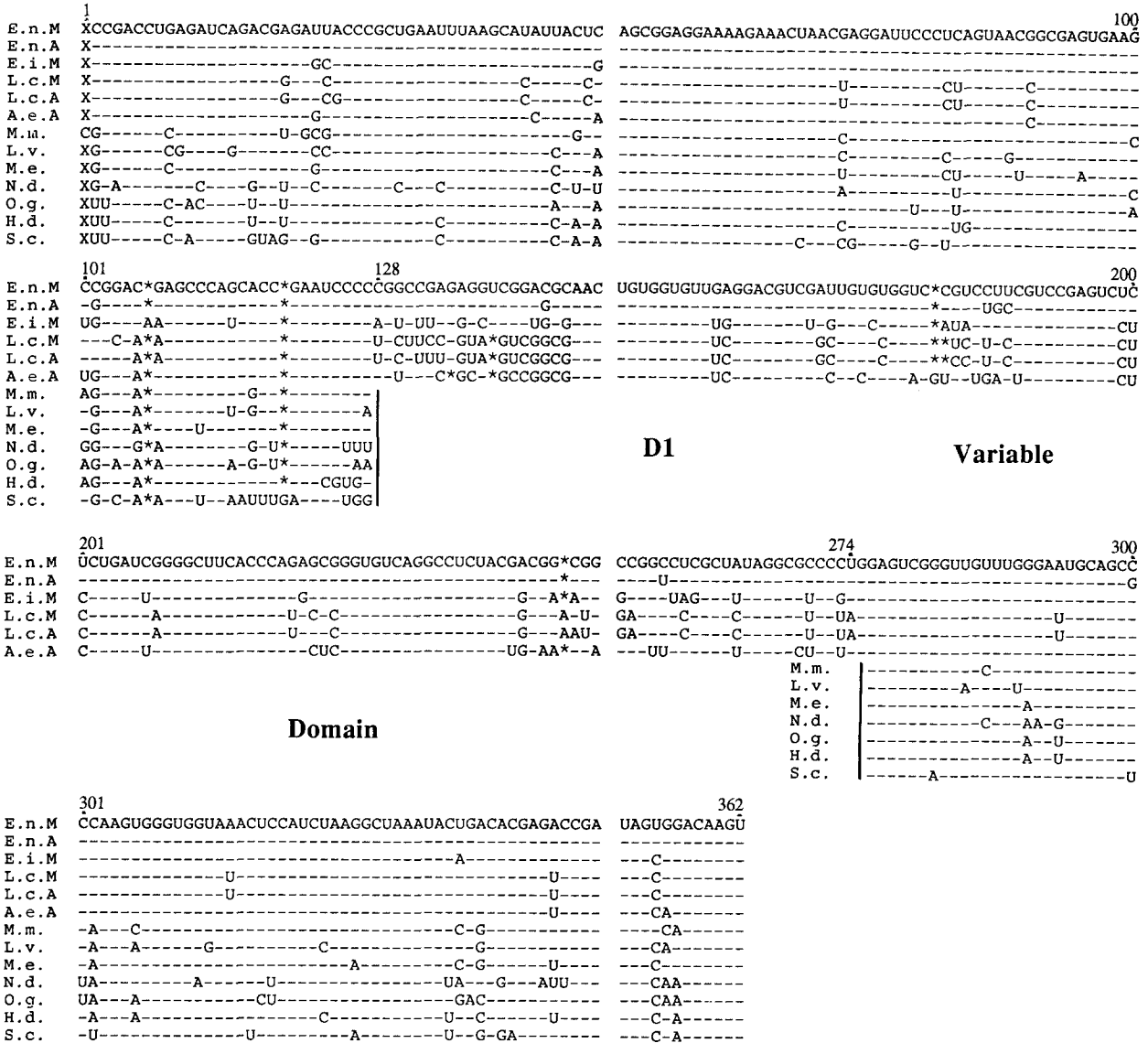

Fig. 1. Sequence alignment of the LSUrRNA 5′ end from the six terebellid specimens analyzed in this study compared with that of others animals and *Saccharomyces cerevisiae* (Qu et al. 1989). Nucleotides identical to the reference sequence (*E. nebulosa* Med) are indicated by dashes. The nucleotide positions that could not be determined are denoted by X. Deletions are represented by *. The region corresponding to the D1-variable domain is not alignable with the nonterebellid sequences. Numbering begins at the LSUrRNA 5′ end. Species abbreviations are as followed: E.n. = *Eupolymnia nebulosa*, E.i. = *E. nesidensis*, L.c. = *Lanice conchilega*, A.e. = *Amphitrite edwardsi*, M.m. = *Mus musculus*, L.v. = *Lithequinus variegatus*, M.e. = *Mytilus edulis*, N.d. = *Nematospiroides dubius*, O.g. = *Onchocerca gibsoni*, H.d. = *Hymenolepis diminuta*, S.c. = *Saccharomyces cerevisiae;* M = Mediterranean, A = Atlantic/Engl. Ch

ferences) and *L. conchilega* (eight differences) from each other and from the other species. Both of these species couples are separated by the same level of difference (Fig. 3 and Table 3). This result suggests that the difference between populations of *E. nebulosa* is infraspecific. In addition, the difference between *E. nebulosa* and *L. conchilega,* whatever the direction of comparison, is always of the same level: 58–60 mutations, which confirms that the units of each couple are, on a biochemical basis, very similar to each other.

It is also useful to consider the relative position of *E. nesidensis*. This species is morphologically very close to *E. nebulosa*. They are separated by 45 or 42 mutations depending on whether the comparison is based on specimens from Atlantic/Channel or from the Mediterranean sea. If the difference between two very close species (*E. nebulosa* and *E. nesidensis*), although very distinctly characterized by morphologists, is based on 42/45 mutations, a difference of seven mutations corresponds, very probably, to an infraspecific difference.

In approaching the problem of Mediterranean-Atlantic population divergence, one may at first examine the direction of evolution leading to the two kinds of reproductive characters. Is free spawning a

Table 3. Distance matrices inferred from the alignment: (A) reduced alignment (216 nt) including the 11 species; (B) complete alignment (362 nt) including only the Terebellidae

A						B						
						E.n.M.	*E.n.A.*	*E.i.M.*	*L.c.M.*	*L.c.A.*	*A.e.A.*	
E.n.M.	0					0	7	42	59	58	52	*E.n.M.*
E.i.M.	10	0					0	45	60	59	51	*E.n.A.*
L.c.M.	14	18	0					0	59	56	54	*E.i.M.*
A.e.A.	10	11	18	0					0	8	57	*L.c.M.*
M.m.	20	24	29	23	0					0	59	*L.c.A.*
L.v.	25	25	30	24	24	0					0	*A.e.A.*
M.e.	20	22	22	18	24	23	0					
N.d.	40	39	42	38	36	45	39	0				
O.g.	35	35	37	34	33	36	35	36	0			
H.d.	29	33	31	28	29	32	26	36	28	0		
S.c.	43	43	46	43	45	46	40	50	44	38	0	
	E.n.M.	*E.i.M.*	*L.c.M.*	*A.e.A.*	*M.m.*	*L.v.*	*M.e.*	*N.d.*	*O.g.*	*H.d.*	*S.c.*	

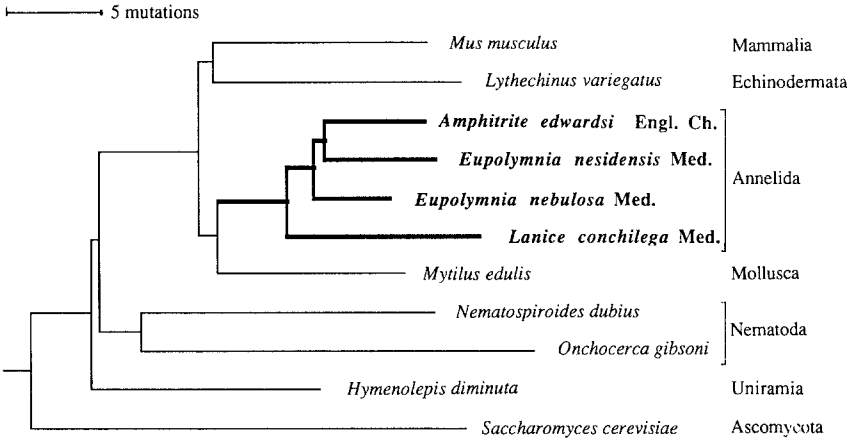

Fig. 2. Terebellidae polychaeta phylogenetic relationships within the animal kingdom. The large-range phylogeny is inferred on the reduced alignment including the conserved stretches (216 nt). The root was assigned to *S. cerevisiae*. Branches leading to the Terebellidae are in bold

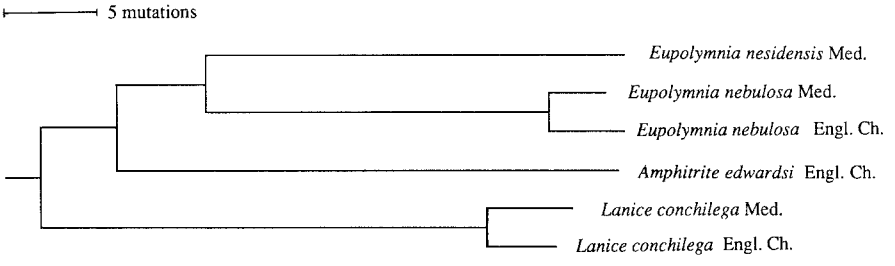

Fig. 3. Detailed phylogenetic relationships among terebellidae polychaeta. The short-range phylogeny is inferred on the complete alignment including only the terebellids (362 nt). The root was arbitrarily situated on the branch leading to the *Lanice* species, according to the position found in Fig. 2

derived character relative to brood protection in a mucous cocoon? A swift examination of reproductive patterns within the family indicates that development with a long planktonic larval phase occurs rarely (Wilson 1928; Bhaud 1986). It is only observed in the genera *Loimia* and *Lanice* (two genera out of 25). In spite of the limited biological data relating to development in this family (Herpin 1925; Duchêne 1983; Eckelbarger 1974, 1983; Smith 1989), the low frequency of this developmental pattern is real. A long larval life is easily detected in regular plankton sampling (Bhaud 1966, 1972). Direct development with telolecith eggs is the most commonly observed pattern in this family. Only one case of free development is known: i.e., *E. nebulosa* in the Atlantic and English Channel. The low frequency of this pattern suggests a derived nature relative to the protected development common in the rest of the family which is probably characteristic of the ancestral pattern. Indirectly, the sequences analysis is useful to specify the relative position of the two development patterns. The alternative scenario of a free larval development phase as the ancestral pattern, and persisting to the present day, while the derived brood-protected pattern is the dominant pattern in the family today,

seems unlikely; it would imply that *E. nebulosa* from the English Channel is closest to the ancestral type, but this is not supported by the biochemical data presented above.

Discussion and Conclusion

In the tested pool of Terebellidae, the tree topology suggests an opposition between *Lanice* sp. and other members of the family. This division may be parallel to, if not explained by, morphological and (or) biological differences. A way to go forward would be to get comparative data for *Loimia* sp. A clue similar to that found for *E. nebulosa* (same differences relative to other members of Terebellidae) would indicate that the indirect pattern of development (only encountered in *Lanice* and *Loimia* sp.) is a secondary evolutionary development. Morphologic analysis of the life cycle (Bhaud 1988) and comparison with first benthic stages of other species of the family suggest that the long pelagic larval stage corresponds to a secondary return to planktonic development which assumes an active evolutionary history possibly explained on a biochemical level by a higher number of events.

At the beginning of this work, taking into account the geographic distribution of the two reproductive patterns, it appeared that the characteristic observed in *E. nebulosa* (M) might represent a derived case. However, examination of the distribution of developmental types between species of the family, together with the results of the molecular biochemical analysis, suggest that *E. nebulosa* (A) is derived relative to *E. nebulosa* (M). It is possible to reconcile the present geographical distribution of the two reproductive patterns (i.e., brooding in the Mediterranean and broadcasting in the Atlantic) with an ancestral type producing an egg mass in the following scenario. During the last ice age, relict populations survived during a period of generally low sea temperatures in the Mediterranean Sea. During subsequent global warming in the postglacial period, *E. nebulosa* began a gradual colonization of Atlantic waters from its Mediterranean base. This extension of its geographical range occurred in tandem with the development of a new reproductive mode, which is now observed only outside the Mediterranean area. This scenario assumes several points:

1) The Mediterranean Sea acted as a sanctuary for species whose northward geographic boundary shifted toward the south (van den Hoek and Breeman 1990).
2) The post–ice-age expansion was not as rapid as the withdrawal phase; the Mediterranean phase was accompanied by physiological evolution but the reproductive pattern characteristic of the family was preserved; i.e., there was a degree of egg protection. Accordingly, this Mediterranean period was relatively conservative, and transformations were not as marked as the subsequent transformations, allowing the most recent expansion of the species. This difference in modification is probably related to the temperature pattern associated with ice-age cycles (Imbrie and Imbrie 1979): slow cooling, not modifying the species during its retirement to the Mediterranean Sea; and rapid warming following the end of the ice age leading to a higher rate of physiological transformation of the species.
3) The divergence observed in *E. nebulosa* between populations from high and temperate latitudes supports the idea of cooling affecting northward populations while species populations from low latitudes are not affected by ice-age fluctuations (Scavotto 1986; Ruddiman 1990). Therefore, in the case of *E. nebulosa* it is likely that intertropical populations of the species produce brooding egg masses.

This particularly speculative scenario may be confirmed by complementary approaches taking into account a larger biogeographical area of the species. This would include observations on life-cycle variations; further biochemical testing of specimens from geographically distant populations; a better understanding of the history of the Mediterranean Sea particularly, and of the mode of action of physical factors thought to have biological impact.

Final conclusions on the problem of the difference in *E. nebulosa* reproductive types in the Mediterranean and the Atlantic can now be presented. By determining the 360 nucleotides from the 5′ end of the LSUrRNA molecule, we can obtain enough information to provide both a phylogeny of the Terebellidae and to give reliable data concerning problems of speciation between animals sampled in different locations. This marker may be considered to be a powerful tool for use in solving many zoological problems that cannot be resolved through classical morphological comparisons because this RNA marker has evolved independently of morphological phenotypic changes (Qu et al. 1989). Furthermore, as shown here, both species couples, *E. nebulosa* and *L. conchilega,* sampled in the Atlantic/English Channel and the Mediterranean seas, present insufficient differences in the number of mutations to suggest specific differences at the taxonomic level; coming back to the question set in the introduction, geographic differences on reproductive mode of *E. nebulosa* are infraspecific.

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