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Short communication

Dinophilidae (Annelida) is most likely not a progenetic Eunicida: Evidence from 18S and 28S rDNA

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1. Introduction

Dinophilidae (e.g., *Dinophilus* O. Schmidt, 1848) is a typical meiofaunal polychaete taxon with a seemingly simple organisation, e.g., no parapodia, only a few segments (Rouse and Pleijel, 2001). Dinophilidae is thought to be an eunicidan taxon and is often presented as the classical example of progenetic evolution within annelids (Westheide, 1987). The retention of ancestral juvenile characters by adult stages of descendants (paedomorphosis) can arise either by a retardation of somatic development (neoteny) or by an acceleration of the sexual maturation (progenesis) (Gould, 1977). Due to convincing similarities to developmental stages of larger eunicidans and their relatively small size, the progenetic origin of Dinophilidae and other dorvilleids (e.g., *Parapodrilus*) within Eunicida has been repeatedly assumed (Fig. 1) (see Eibye-Jacobsen and Kristensen, 1994; Westheide, 1987).

Based on the possession of a ventral pharyngeal organ with specific jaw elements, Eunicida is a well-defined annelid taxon currently comprising "Dorvilleidae," Eunicidae, Hartmanniellidae, Histriobdellidae, Lumbrineridae, Oenonidae, and Onuphidae (Rouse and Pleijel, 2001). However, some meiofaunal taxa like *Parapodrilus* and a parasitic genus *Biborin* lack jaw apparatuses. Eunicidan species comprise both some of the smallest polychaetes (e.g., *Neotenotrocha*, 250 μm) and the largest polychaete up to 6 m in length (e.g., *Eunice*) (Eibye-Jacobsen and Kristensen, 1994; Rouse and Pleijel, 2001).

The most convincing evidence for a close relationship between dinophilids and eunicidans is from Åkesson's (1977) experiments demonstrating reciprocal infection with coelomic coccidia of the genus *Grellia*, parasites that are generally thought to be host-specific. In contrast, definitive morphological synapomorphies are lacking. For example, the ventral pharyngeal organs of Dinophilidae and Eunicida are different and most likely not homologous (Purschke, 1985, 1987). In a recent 18S rDNA study (Struck et al., 2002) Dinophilidae was not the sistergroup to any eunicidan taxon. However, data were not able to clearly refute Dinophilidae as derived eunicidans as judged by nodal support and statistical tests. Furthermore, in two of their analyses Dinophilidae were in close vicinity to Lumbrineridae and the dorvilleid *Pettiboneia urciensis*, the latter two taxa are usually closely related to each other in molecular analyses (e.g., Struck and Purschke, 2005). Thus, their possible progenetic origin within Eunicida is still controversial.

To address the phylogenetic relationship of Dinophilidae relative to Eunicida, the nuclear 28S rDNA was chosen as an additional molecular marker. Based on 18S rDNA results (Bleidorn et al., 2003; Struck and Purschke, 2005) concerning other possible placements for Dinophilidae, partial sequences of 28S rDNA and 18S rDNA of sabellidan, eunicidan, and dinophilid species were determined in this study. Combined analyses of the two genes (approximately 4 kb of data) were performed.

2. Materials and methods

2.1. DNA extraction, amplification, and sequencing

The 29 taxa employed in this study are listed in Table A1 in the Electronic Appendix A. DNA extraction,

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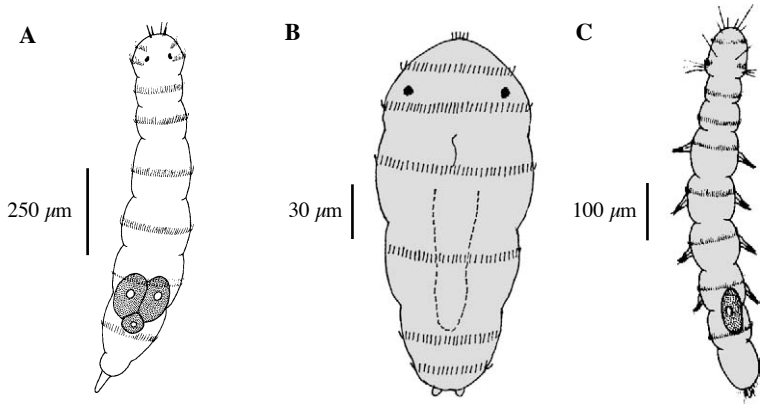

Fig. 1. (A) Adult of *Dinophilus gyrociliatus* (Dinophilidae); (B) early larva of *Schistomeringos rudolphi* ("Dorvilleidae"); (C) adult of *Parapodrilus psammophilus* (modified after Westheide, 1984). This figure exemplifies the similarity of adult dinophilids and early eunicidan larvae.

amplification, and collection of 28S sequences of one dinophilid (*Trilobodrilus heideri*) and three eunicidan species via PCR were performed according to Jördens et al. (2004) using primers from that paper and Passamaneck et al. (2004). Genomic DNA from the three sabellidan and another dinophilid species (*T. axi*) was extracted using Qiagen DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Hot Start-PCR was performed to amplify entire 18S (prerun: 3 min 94 °C; application of polymerase; 1 cycle: 3 min 94 °C; 40 cycles: 1 min 94 °C, 1 min 30 s 40 °C, 2 min 30 s 72 °C; 1 cycle: 7 min 72 °C; 25 μl reaction-mix: 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, ~1 ng/μl genomic DNA, 0.4 mM dNTPs, and 0.8 μM 18e and 18R1779), 0.03 U/μl *Taq* DNA Polymerase (Promega, Madison, WI) and entire 28S (prerun: 3 min 94 °C; application of polymerase; 1 cycle: 2 min 94 °C; 35 cycles: 30 s 94 °C, 30 s 48 °C, 12 min 70 °C; 1 cycle: 10 min 72 °C; 50 μl reaction-mix containing 7 μl of each 10× LA PCR Buffer II, 25 mM $MgCl_2$ and 10 mM dNTPs, 0.25 μl of 5 U/μl TaKaRa LA *Taq* (Takara Bio, Otsu, Japan) and 1 μl of 20 μM 28F63.2 and 28R3264.2). All products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The sequences were determined with either ABI Prism 377 (Perkin Elmer, Shelton, CT) or CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA).

2.2. Phylogenetic analyses

A brachiopod, phoronid, mollusc and nemertean were used as outgroups. A ~2.2 kb fragment of the 28S, common for all included species, and ~1.8 kb of the 18S were aligned with CLUSTAL W (CWA, Thompson et al., 1994) and subsequently corrected by hand in GeneDoc (Nicholas and Nicholas, 1997). We also employed an alignment based on the secondary structure of ribosomal RNA of bilaterians (SSR, Mallatt and Winchell, 2002). Ambiguous positions were excluded from the subsequent analyses. The alignments are available at TREEBASE (www.treebase.org).

To assess the phylogenetic signal for different regions of the nuclear rDNA genes preliminary analyses were conducted for both genes as described by Jördens et al. (2004) using the CWA data set. In these analyses saturation as well as mutation rates over 0.25 could be detected in the 50–100% class of variation of both genes (see Electronic Appendix A). Saturated positions in the CWA alignment were removed.

Combined analyses of the 28S and 18S data were conducted using either maximum likelihood (ML) in PAUP*4.0b (Swofford, 2002) or Bayesian inference (BI) with MrBayes 3.0B4 (Huelsenbeck and Ronquist, 2001). Appropriate models of sequence evolution for the combined dataset of the ML analyses were indicated by the hLRT criterion of Modeltest V 3.06 (Posada and Crandall, 1998): CWA, base frequencies—A = 0.2623, C = 0.2133, G = 0.2864, T = 0.2380, rate matrix—AC, AT, CG, GT = 1.0000, AG = 2.7284, CT = 5.8650, shape parameter $\alpha = 0.5841$, proportion of invariant sites = 0.4163; SSR, base frequencies—A, C, G, T = 0.2500, rate matrix—AC, AT, CG, GT = 1.0000, AG = 2.8443, CT = 4.5163, shape parameter $\alpha = 0.5573$, proportion of invariant sites = 0.4383. Heuristic searches were performed with TBR branch swapping and random taxon addition of 10 replicates. Reliability of nodes was estimated by 100 bootstrap replicates (BP; bootstrap proportion) with simple taxon addition (Felsenstein, 1985).

The hLRT criterion of MrModeltest 1.1b (Nylander, 2002) indicated SYM + I + Γ models for the 18S partition in both CWA alignments as well as for the 28S for SSR, and GTR + I + Γ for CWA. Model parameters of the two partitions were unlinked relative to each other. Each Markov chain, three heated and one cold, ran simultaneously for 10^6 generations, with trees being sampled every 100 generations for a total of 10,001 trees. Based on the convergence of the likelihood scores the first 1000 trees in both analyses were discarded as *burn*

in. The majority-rule consensus trees containing the posterior probabilities (PP) were determined from the remaining 9001 trees.

2.3. Significance testing

A particular a priori phylogenetic hypothesis and the best tree obtained (or an alternative a priori hypothesis) can be compared to each other to test whether the latter one is significantly better than the particular a priori hypothesis. Whereas BP and PP values only examine single nodes, significance tests of alternative topologies can evaluate signal along the entire tree. Thus, even when the support by BP or PP values is low or non-significant, explicit hypothesis testing procedures can allow discrimination between alternative hypotheses (Huelsenbeck and Rannala, 1997). Two such significance tests were performed under the ML criterion to evaluate the proposed progenetic origin of Dinophilidae within Eunicida. The two-tailed test of Kishino and Hasegawa (1989) (KH test) was used to compare two alternative a priori hypotheses, whether Dinophilidae is a subtaxon of monophyletic Eunicida or not. This test was performed with a RELL approximation. Furthermore, we also used the even more conservative SOWH test (Goldman et al., 2000) to compare the hypothesis that Dinophilidae is closely related to any eunicidan taxon (model tree) without constraining monophyletic Eunicida against the best solution. This test uses a parametric bootstrapping approach (for further details see Goldman et al., 2000). Using Seq-Gen V. 1.2.7 (Rambaut and Grassly, 1997) 1000 parametric bootstrap datasets were obtained. In the performed RELL approximation, substitution parameters for each dataset were optimized for the model tree and used for the heuristic search of the best solution.

3. Results

Aligned CWA data consisted of 4644 positions. Of the alignable 2821 positions, 976 were variable. In the case of the 4707 positions for SSR, 2816 were alignable and 980 variable. Resultant BI trees are very similar to ML trees for both alignments (Figs. 2A and B). PP values of BI are shown on the corresponding nodes. Differences between the BI and ML trees are mentioned below. Note that PP are generally higher than BP values (Huelsenbeck et al., 2002) and are less reliable measurement of support (e.g., Suzuki et al., 2002). Herein, the term "significant support" refers to results of significance tests.

In comparison to previous 18S only analyses (e.g., Bleidorn et al., 2003), monophyly of Annelida is recovered by all analyses, but only weakly supported (Fig. 2; CWA: PP = 0.95, SSR: PP = 0.60, BP < 50). Monophyletic Dinophilidae (CWA and SSR: BP = 100, PP = 1.00) was not closely related to any of the included eunicidan taxa, Clitellata or Sabellida. All analyses place Dinophilidae as part of a basal clade. In the ML CWA analysis, Dinophilidae (Fig. 2A) is closely related to *Protodriloides chaetifer*, a member of the former "Archiannelida," whereas in the BI of CWA and in both SSR based analyses Dinophilidae is sister to Chaetopteridae. In all cases nodal support is low. Similar results were obtained by ML and BI, when only 28S data were analysed (data not shown). The node comprising all eunicidan taxa also includes a syllid, but is weakly supported (Figs. 2A and B).

To more confidently evaluate competing hypotheses, we used KH and SOWH tests. Both tests show that the hypothesis of an origin of Dinophilidae within Eunicida is significantly worse from the alternative hypothesis or the best tree. The KH tests, comparing hypotheses of Dinophilidae within a monophyletic Eunicida or not, significantly rejected the former hypothesis (CWA: $P = 0.026$; SSR: $P = 0.008$). In SOWH tests, differences between the hypothesis that Dinophilidae is closely related to any eunicidan taxon and the best solution were significant in both cases ($P < 0.001$).

4. Discussion

Combined 18S and 28S rDNA data demonstrated that Dinophilidae is most likely not a progenetic eunicidan lineage, thereby calling into question morphological similarities between dinophilid adults and eunicidan larvae. Although BP and PP support values at any given node supporting exclusion were weak, KH and SOWH tests clearly reject the hypothesis of eunicidan origin. These tests take into account overall tree shape and thus are more powerful than evaluating the signal at any single node.

Struck et al. (2002) previously addressed this issue, but their results were inconclusive because the exclusion of Dinophilidae from Eunicida could not be statistically supported. The meiofaunal genera *Apharyngtus*, *Apodotrocha*, *Neotenotrocha*, and *Parapodrilus*, usually regarded as progenetic dorvilleids, were traditionally considered as closely related to Dinophilidae due to the possession of paedomorphic characters and an unpaired median pygidial appendage (Eibye-Jacobsen and Kristensen, 1994; Westheide and Riser, 1983). However, unpaired median appendages can also be found in several other dorvilleids, Amphinomidae, Nephtyidae, Paraonidae, Polygordiidae, and Sabellidae and are not present in *Trilobodrilus* (Eibye-Jacobsen and Kristensen, 1994; Rouse and Pleijel, 2001). Furthermore, the morphological phylogenetic assessment of paedomorphic species is often misled by widely distributed paedomorphic characters, lack of synapomorphic adult features and convergent evolution (Wiens et al., 2005). "Larval" and "juvenile" structures are usually widespread across

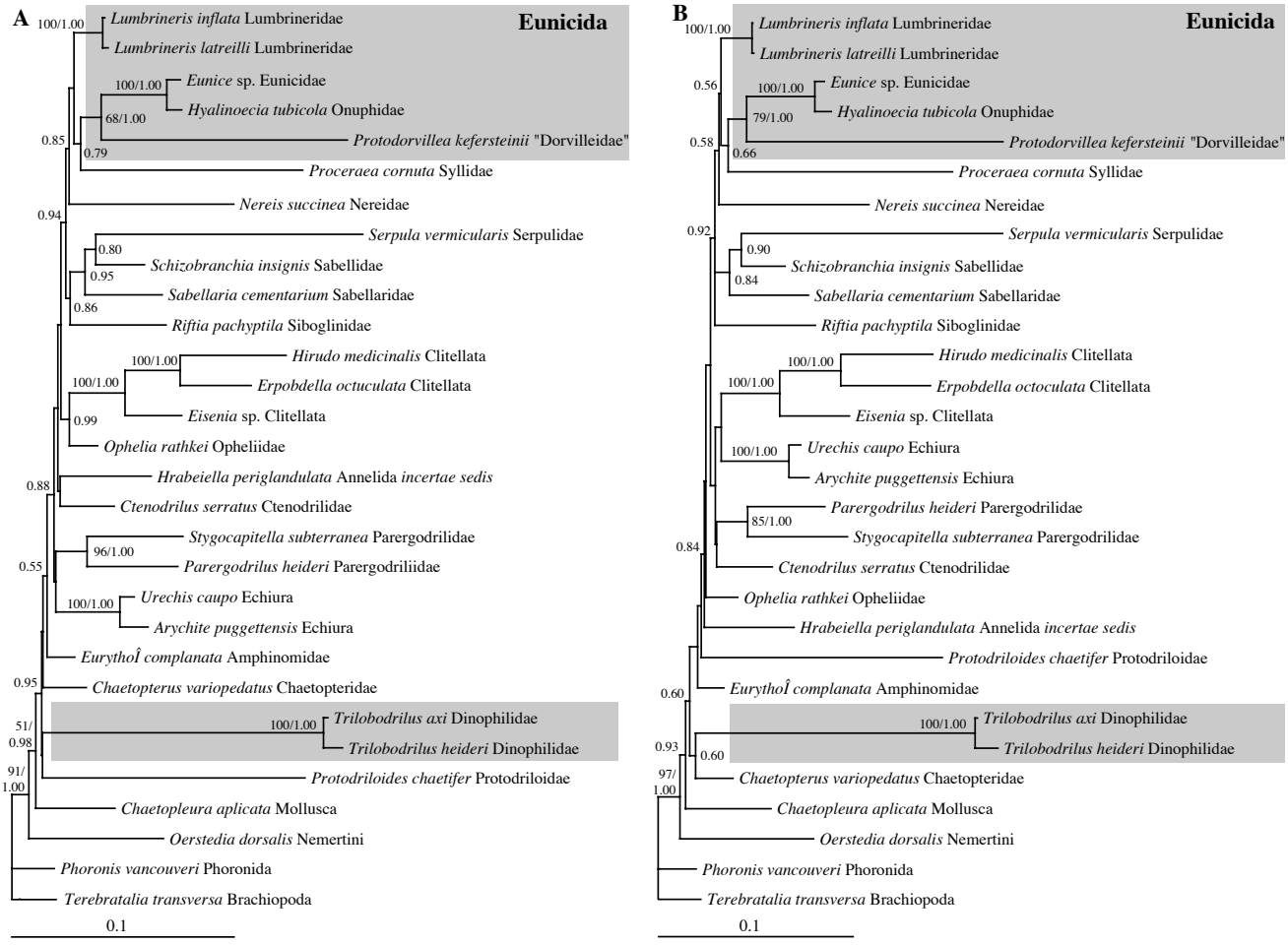

Fig. 2. ML trees based on: (A) the CWA alignment ($-\ln L = 16{,}609.26$) or (B) the SSR alignment ($-\ln L = 17{,}211.08$). Only BP and PP values above 50 or 0.50 shown, respectively. BP values at first position. For analyses parameters see text. Taxa of interest highlighted.

polychaetes, for example, larvae of the polytroch type can also be found in Orbiniidae and Cirratulidae (Rouse and Pleijel, 2001). Of the four genera mentioned above, only the inclusion of *Parapodrilus* and *Neotenotrocha* within dorvilleids is well established due to either molecular data (Struck et al., 2002) or the possession of jaw elements (Eibye-Jacobsen and Kristensen, 1994).

In light of the molecular data, evidence for a Dinophilidae/Eunicida relationship based on parasite–host experiments with the coelomic coccidia *Grellia* (Åkesson, 1977) need to be reconsidered. Of the eight genera (three dorvilleid, one dinophilid, and four non-eunicidan) considered, the four "outgroup" species are not interstitial fauna. Hence, it is possible that these results are indicative of a common environment rather than phylogenetic history. Furthermore, ultrastructural analyses of the ventral pharyngeal organs in these two lineages exhibit no synapomorphic features (Purschke, 1985, 1987). Thus, the progenetic origin of Dinophilidae within Eunicida is not supported by morphology or molecules, and is significantly rejected by molecular data.

In all molecular analyses of annelid phylogeny including Dinophilidae, a close relationship of Dinophilidae to any taxon with a polytroch-like larva has not been shown (Bleidorn et al., 2003; Struck and Purschke, 2005; Struck et al., 2002, present study). Cirratulidae, another taxon with a polytroch larvae, is represented here by the ctenodrilid *Ctenodrilus serratus* (Bleidorn et al., 2003). However, investigations on the nervous system of Dinophilidae clearly show larval and juvenile characters, which are commonly distributed in polychaetes (Müller and Westheide, 2002). Therefore, the issue of their possible progenetic origin remains controversial.

A suggested relationship of Dinophilidae to Sabellida or Clitellata (Bleidorn et al., 2003; Struck and Purschke, 2005) is not supported by the present analyses of 18S and 28S data. One ML analysis placed Dinophilidae near Protodriloidae at a basal position within Annelida, reminiscent of the Archiannelida hypothesis. However, taxon sampling is not representative for this issue and there is no nodal (BP < 50%) or statistical support for this placement. Dinophilidae was initially incorporated in "Archiannelida"

and considered to reflect the basic organisation of the Annelida (see Hermans, 1969). However, ultrastructural analyses (e.g., Purschke and Jouin, 1988), general arguments about annelid origins (e.g., Westheide, 1997), and 18S rDNA (Struck et al., 2002) changed this view. In other analyses, Dinophilidae is closely related to Chaetopteridae, but again without support. Therefore, the phylogenetic position of Dinophilidae within Annelida, except for the exclusion from Eunicida, is uncertain.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2005.07.010.

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