5. Phylogenetic Relationships and Evolution of Orbiniidae (Annelida, Polychaeta) based on Molecular Data

Abstract - The phylogenetic relationships of orbiniid taxa were reconstructed based on sequence data of the mitochondrial 16S rRNA gene and the nuclear 18S rRNA gene. Both genes were analysed separately and in combination using Maximum Likelihood, Bayesian inference, and Maximum Parsimony. Regardless the method used a clade consisting of the investigated Orbiniidae, *Methanoaricia dendrobranchiata*, and *Questa* is strongly supported by the 18S dataset. The presence of camarated chaetae in all these taxa supports the monophyly of this clade from the morphological side. The analysis of the combined dataset suggests an inclusion of *Methanoaricia dendrobranchiata* in the Orbiniidae with a close relationship to species of *Orbinia* and *Phylo*, rather than as being the sister taxon of all other orbiniids. Evidence is given for a paraphyletic status of *Leitoscoloplos*, *Naineris*, *Orbinia*, *Phylo* and *Scoloplos*, which represent the most species rich genera of the Orbiniidae. Thus it can be reasoned that the morphological characters which are presently used for genus diagnosis are not informative for cladistic analysis. No support is found for the hypotheses that taxa of the Protoariciinae represent juveniles of Orbiniinae, instead in the case of *Protoaricia oerstedi* strong support for a progenetic origin is given.

5.1 Introduction

Masses of specimens of a large polychaete have been found in association with hydrocarbon cold seeps in the Gulf of Mexico and were first noticed by MacDonald (1990). These so called "seepworms" bear an unusual combination of characters which makes it difficult to place them into a known polychaete family. Blake (2000) described this species as *Methanoaricia dendrobranchiata* (fig. 1B) and included it in the Orbiniidae, a classification which was not obvious since the discovery of this worm.

The Orbiniidae comprise a group of world-wide distributed deposit feeding polychaetes. Approximately 150 species have been described in 18 genera (Glasby, 2000). The taxonomic history of this taxon was extensively reviewed by Hartman (1957) and in this contribution the classification of orbiniid worms in Protoariciinae and Orbiniinae was established. Protoariciinae are characterised as small and slender worms which possess two (or more) peristomal rings (fig. 1A), whereas most of the Orbiniinae are medium
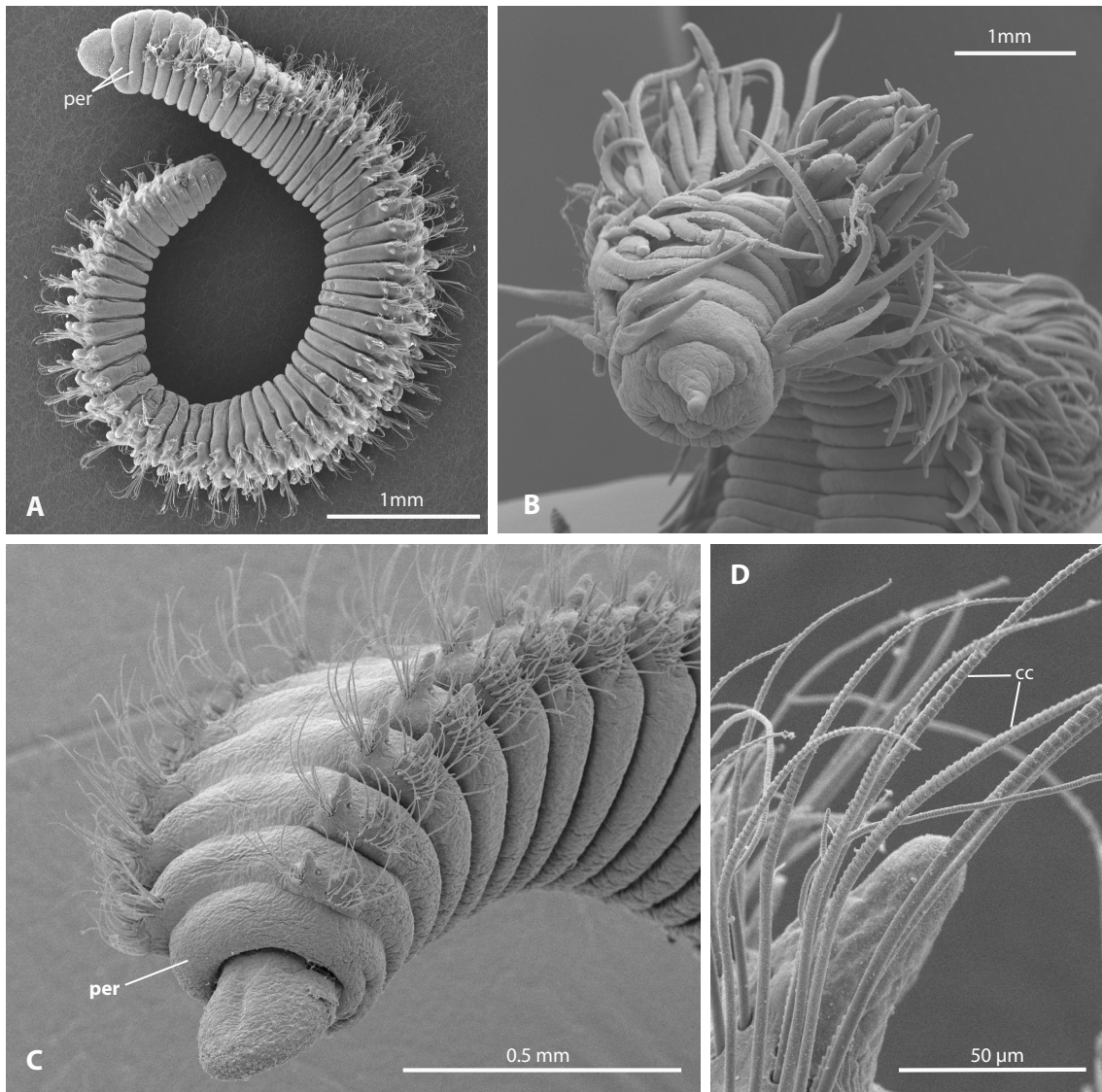
Figure 1. (A) *Protoaricia oerstedi*, lateral view; per = peristomal ring; (B) *Methanoaricia dendrobranchiata*, anterior end; (C) *Naineris dendritica*, anterior end; per = peristomal ring; (D) *Naineris dendritica*, notopodium with camerated chaetae (cc).

sized to large species with only one peristomial ring (fig. 1C). Development and larval morphology are only known for few orbiniid species. The development of *Phylo foetida* is described in Eisig (1914) and Anderson (1959, 1961) describes the development of *Scoloplos armiger* and *Scoloplos simplex* (referred to as *Haploscoloplos fragilis* in the paper). A description of the larval development of *Leitoscoloplos pugettensis* and *Scoloplos acmeceps* is provided by Blake (1980). All these investigations on members of the Orbiniinae are concordant with an early establishment of one peristomal ring during ontogenetic development. Blake (see Blake & Hilbig 1990) was the first who mentioned that there is evidence that some species within the Orbiniinae, e.g. *Naineris laevigata* (see Giangrande & Petraroli, 1991), show two achaetous rings in early development, whereas the transition to a single ring occurs later. These observations give rise to the hypothesis

that many of the currently assigned Protoariciinae might be juveniles of taxa of the Orbiniinae (Blake, 1996). The alternative hypotheses would be to assume heterochronic evolution in the Protoariciinae. First ideas of a progenetic origin of *Protoaricia oerstedi* go back to Eisig (1914), who observed that the ventral pharyngeal organ (see Purschke, 1988), the pygidial cirri, and the shape of the thoracic neuropodia show a high similarity to the corresponding structures in juvenile specimens of *Naineris* or *Phylo*.

In a recent cladistic investigation of the phylogenetic interrelationships of the genera of Orbiniidae (Blake, 2000) characters concerning the numbers of peristomial rings were excluded and a data matrix consisting of 23 morphological absent/present characters was analysed. According to this analysis, *Methanoaricia dendrobranchiata,* the deep sea orbiniid which has been described in the same paper is the sister taxon of all other orbiniids. Furthermore the Orbiniidae are classified in Microrbiinae (*Microorbinia*, *Orbiniella*, *Falklandiella*, and *Proscoloplos*) and a new combined Orbiniinae (the rest), which now comprehends a lot of the former Protoariciinae (e.g. *Protoaricia*). The presence of distinct body regions is assessed as an autapomorphy for the Orbiniinae, whereas the lack of these regions characterise the Microrbiinae. Nevertheless, the support for these clades is very weak and the monophyly of some of the genera used in this study is doubtful.

Another taxon with uncertain affinities are the Questidae. They comprise a group of interstitial species with "oligochaetoid morphology" (Giere and Rieser, 1981) and are, by some authors, regarded to represent the sister group of the Clitellata (Almeida *et al.*, 2003). This is contradicted by molecular (Bleidorn, Vogt & Bartolomaeus, 2003a, 2003b), as well as morphological (Rouse & Fauchald, 1997) studies, which both recover a closer relationship to the Orbiniidae.

The attempt of the present study is to reconstruct orbiniid ingroup relationships (including the Questidae), as well as the question of the phylogenetic position of *Methanoaricia*, using mitochondrial 16S rRNA gene and nuclear 18S rRNA gene sequences. Several studies have shown that these genes are suitable for unravelling ingroup relationships of annelid taxa (Dahlgren *et al.*, 2001; Jamieson *et al.*, 2002; Nygren & Sundberg, 2002; Borda & Siddall, 2004).

5.2 Materials and methods

Choice of taxa

The investigated orbiniid taxa (Appendix A) represent a variety of all major taxonomic groups. Outgroups (Appendix A) represent putative sister taxa and have been chosen on basis of hypotheses derived from morphological (Rouse & Fauchald, 1997) as well as molecular data (Bleidorn *et al.*, 2003a). Therefore representatives of all scolecid families

and the Parergodrilidae are included. The errant polychaete *Eunice pennata* served to root all the obtained trees.

The 18S sequence of *Phylo foetida* has been erroneously published as *Orbinia latreillii* by Bleidorn *et al.* (2003b).

DNA extraction, PCR amplification and sequencing

DNA extraction was performed using the Qiagen DNeasy™ Tissue Kit according to the manufacturer's instructions. PCR amplification of a ~1800bp part of the 18S rRNA gene was performed in two overlapping fragments using primer pairs F19 + R993 and F439 + R1843 (Table 2). A ~500bp part of the mitochondrial 16S rRNA gene was amplified using the primer pair 16SarL and 16SbrH (Table 1). Each amplification reaction mixture contained a 50μl volume containing 25mM Tris-HCl pH 8.0, 35 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2,5 mM Mg^{2+}, 50% glycerol, 0.5% Tween-20, 0.5% Igepal CA-630, 0.5 μM of each primer, 0.25 mM dNTP-Mix, 1U of Taq Polymerase (Eppendorf) and 1μl template DNA. All amplifications were carried out on an Eppendorf Mastercycler and Eppendorf Mastercycler gradient. The PCR temperature reaction for the 18S was 94˚C for 2 min; 34 cycles with 94˚C for 30 seconds, 56˚C for 1 min, and 72˚C for 2 min; final extension at 72˚C for 7 min. For the 16S the following file has been used: 94˚C for 3 min; 34 cycles with 94˚C for 45 seconds, 50˚C for 1 min, and 72˚C for 1 min; final extension at 72˚C for 7 min.

Table 1. Primers used for PCR and sequencing

Primer name	Sequence 5'-3'	Reference
18S		
F19	ACCTGGTTGATCCTGCCA	Turbeville *et al.* (1994)
R427	TCAGGCTCCCTCTCCGG	C. Lüter (pers. comm.)
F439 (3F)	GTTCGATTCCGGAGAGGGA	Giribet *et al.* (1996)
R993 (5R)	CTTGGCAAATGCTTTCGC	Giribet *et al.* (1996)
F1012 (5F)	GCGAAAGCATTTGCCAAGMA	Giribet *et al.* (1996)
R1372	GAGTCTCGTTCGTTATCGGA	C. Lüter (pers. comm.)
F1502	CAGGTCTGTGATGCCC	C. Lüter (pers. comm.)
R1825	CGGAAACCTTGTTACGAC	C. Lüter (pers. comm.)
R1843	GGATCCAAGCTTGATCCTTCTGCA GGTTCACCTAC	Elwood *et al.* (1985)
16S		
16SarL	CGCCTGTTTAACAAAAACAT	Palumbi (1996)
16SbrH	CCGGTCTGAACTCAGATCACGT	Palumbi (1996)

All products were purified with the Qiaquick PCR Purification Kit (Qiagen). Sequencing reactions were performed with a dye terminator procedure and loaded on capillary automatic sequencer CEQTM 8000 (Beckman Coulter, Fullerton CA, USA) according to the recommendations of the manufacturer. The primers used in the sequencing reaction are listed in table 2. All sequences (18 of the 18S rRNA gene and 22 of the 16S rRNA gene) were submitted to GenBank (for accession numbers see appendix A).

Alignment and data analysis
Sequences were aligned with CLUSTAL W (Thompson *et al.*, 1994) using the default parameters for gap opening and gap penalty and subsequently manually edited by eye using BioEdit (Hall, 1999). Gap positions and regions that could not be aligned unambiguously were excluded from the analysis. The alignment is available by emailing the author.
All phylogenetic analyses were carried out using PAUP*, version 4.0b10 (Swofford, 2001) and MrBayes 3.0B4 (Huelsenbeck & Ronquist, 2001). A chi-square test of homogeneity of base frequencies across taxa was used to estimate the frequency distribution of observed number of substitutional changes per character for each gene. An ILD test (Farris *et al.* 1995) was conducted using the partition homgeneity test in PAUP* with 1000 replicates to test the congruence between the genes.

Table 2. Models of sequence evolution use in the different analyses and the appropriate program settings

Dataset	Model	ML settings PAUP*	ML settings Mr.Bayes
18S	SYM+I+ Γ	Lset Base=equal Nst=6 Rmat=(1.2021 2.4468 1.0467 0.9498 3.9515) Rates=gamma Shape=0.5665 Pinvar=0.3038;	lset nst=6 rates=invgamma; prset Rev MatPr=dirichlet(1.0,1.0,1.0,1.0,1.0,1.0) StateFreqPr=fixed(equal) ShapePr=uniform(0.05,50.0) PinVarPr=uniform(0.0,1.0);
16S	GTR+ Γ	Lset Base=(0.3693 0.2141 0.1805) Nst=6 Rmat=(623.1858 857.2402 770.7150 17.8028 3562.4211) Rates=gamma Shape=0.3522 Pinvar=0;	lset nst=6 rates=gamma; prset RevMatPr=diri chlet(1.0,1.0,1.0,1.0,1.0,1.0) StateFreqPr=diri chlet(1,1,1,1) ShapePr=uniform(0.05,50.0);
18S+16S	GTR+I+ Γ	Lset Base=(0.2725 0.2321 0.2634) Nst=6 Rmat=(1.9304 2.7460 2.1765 0.9072 6.2102) Rates=gamma Shape=0.5783 Pinvar=0.2636;	lset nst=6 rates=invgamma; prset RevMatPr= dirichlet(1.0,1.0,1.0,1.0,1.0,1.0) StateFreqPr= dirichlet(1,1,1,1) ShapePr=uniform(0.05,50.0) PinVarPr=uniform(0.0,1.0);

Unweighted parsimony with 1,000 random addition replicates, heuristic search option with tree-bisection-reconnection (TBR) branch swapping, holding one tree per step, and keeping all most-parsimonious trees was conducted for all datasets. Clade support was assessed with nonparametric bootstrap (Felsenstein, 1985) as implemented in PAUP* (heuristic search, 500 replicates, TBR branch swapping, and simple addition sequence). For estimating the appropriate model of sequence evolution, a hierarchical likelihood ratio test (hLRT) was carried out as implemented in the program MrModeltest version 1.1b, a simplified version of Modeltest 3.06 (Posada & Crandall, 1998, 2001).

Maximum likelihood analysis was performed under the likelihood settings suggested for the given dataset by the result of the modeltest (see table 2) using the heuristic search option with TBR branch swapping and 10 random sequence addition replicates. Clade support was assessed with 500 bootstrap replicates using NNI branch swapping and simple addition sequence.

Bayesian analysis of the data set was conducted by using MrBayes 3.0B4 (Huelsenbeck & Ronquist, 2001). All priors were set according to the models as specified in table 2. Four Markov chains, three heated (mcmcp temp=0.3) and one cold, were started from a random tree and all four chains ran simultaneously for 500.000 generations, with trees being sampled every 250 generations for a total of 2,001 trees. After the likelihood of the trees of each chain converged, the first 101 trees were discarded as *burn in*. The majority-rule consensus tree containing the posterior probabilities of the phylogeny was determined from 1,900 trees.

5.3 Results

18S dataset

After the exclusion of ambiguous sites, the alignment contains 1,571 positions, of which 864 are constant, 250 are variable and 457 are parsimony informative. The chi-square test of homogeneity of base frequencies across taxa resulted in no significant *P*-values (chi-square=97.0404, df=99, *P*=0.537). It can be assumed that compositional bias has no effect on the recovery of phylogenetic signal.

Maximum Likelihood analysis and Bayesian inference revealed trees with the same topology (fig. 2). The most likely tree of the Maximum Likelihood (ML) analysis has a -ln likelihood value of 12459.92047. The chains of the Bayesian analysis reached the equilibrium at no later than 25.250 generations. Bayesian posterior probabilities for each clade were derived from the remaining 1,900 trees. Two equally parsimonious trees are recovered by the unweighted MP analysis (Tree length = 2178; CI = 0.5197). The topology (results not shown) of the strict consensus differs slightly from the ML- and
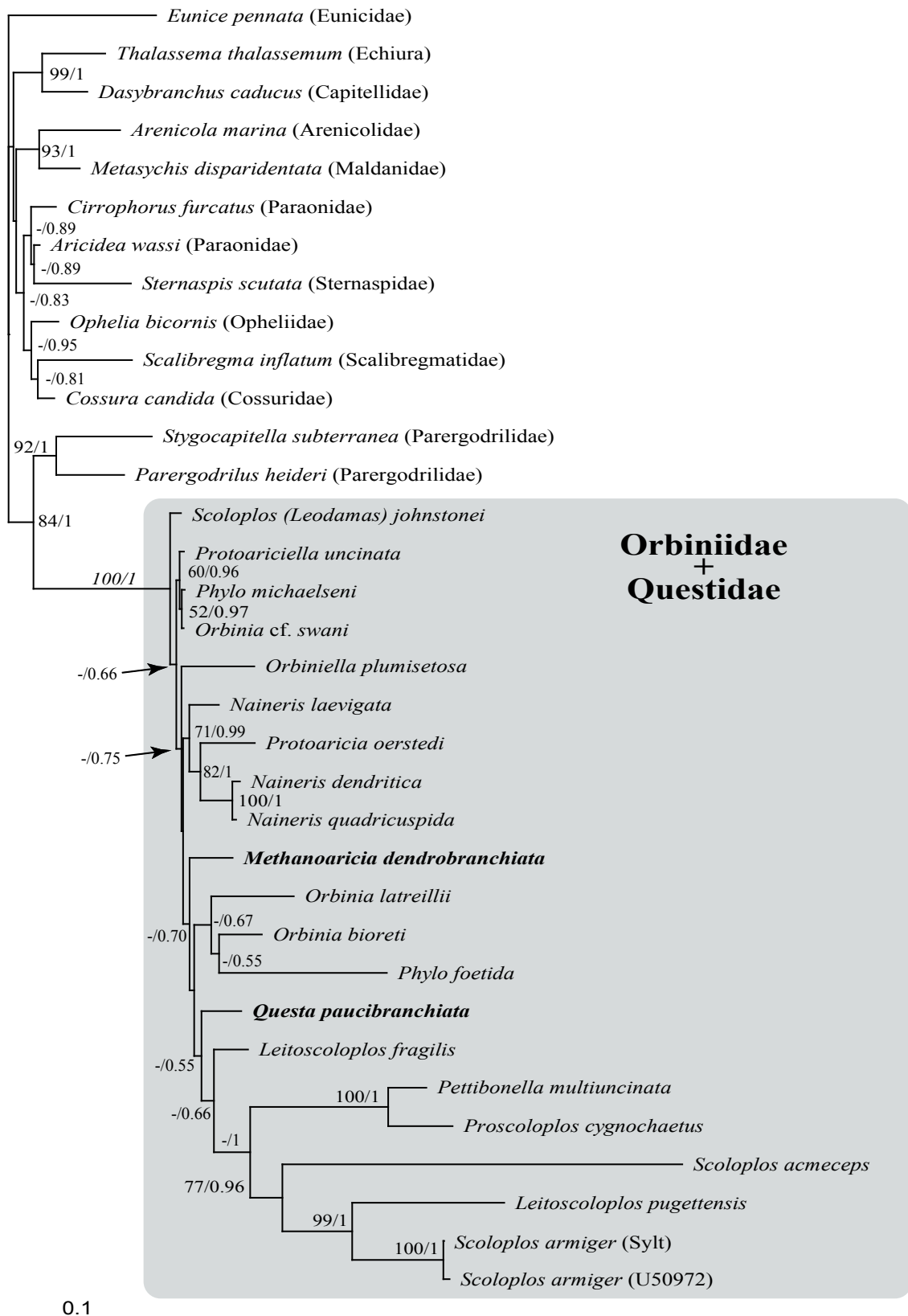
Eunice pennata (Eunicidae)

99/1 *Thalassema thalassemum* (Echiura)
Dasybranchus caducus (Capitellidae)

93/1 *Arenicola marina* (Arenicolidae)
Metasychis disparidentata (Maldanidae)

-/0.89 *Cirrophorus furcatus* (Paraonidae)
-/0.89 *Aricidea wassi* (Paraonidae)
-/0.83 *Sternaspis scutata* (Sternaspidae)

-/0.95 *Ophelia bicornis* (Opheliidae)
-/0.81 *Scalibregma inflatum* (Scalibregmatidae)
Cossura candida (Cossuridae)

92/1 *Stygocapitella subterranea* (Parergodrilidae)
Parergodrilus heideri (Parergodrilidae)

84/1

100/1 *Scoloplos (Leodamas) johnstonei*

Protoariciella uncinata
60/0.96
Phylo michaelseni
52/0.97
Orbinia cf. *swani*

-/0.66 → *Orbiniella plumisetosa*

Naineris laevigata
71/0.99
Protoaricia oerstedi
-/0.75 →
82/1 *Naineris dendritica*
100/1
Naineris quadricuspida

Methanoaricia dendrobranchiata

Orbinia latreillii
-/0.67
Orbinia bioreti
-/0.70
-/0.55
Phylo foetida

Questa paucibranchiata

Leitoscoloplos fragilis
-/0.55

-/0.66
100/1 *Pettibonella multiuncinata*
-/1
Proscoloplos cygnochaetus

Scoloplos acmeceps

77/0.96
Leitoscoloplos pugettensis
99/1
100/1 *Scoloplos armiger* (Sylt)
Scoloplos armiger (U50972)

**Orbiniidae
+
Questidae**

0.1

Figure 2. Maximum likelihood tree of the 18S rRNA gene dataset based on the SYM+I+ Γ model of sequence evolution (-log*L*=12459.92047). The first value at the node represents the ML bootstrap support, the second are bayesian posterior probabilities.

Bayes trees.

Regardless of the method used, a well supported clade (ML Bootstrap (LBT) 100%, Bayesian posterior probabilities (BPP) 1.0; MP Bootstrap (PBT) 100%) consisting of the orbiniid taxa and *Questa* is recovered. Not a close relationship to one of the other scolecid taxa, but a sistergroup relationship between Orbiniidae + *Questa* and Parergodrilidae (LBT 84%, BPP 1.0, PBT 77%) is supported. In the ML and Bayesian analysis within the outgroup, the Paraonidae appear paraphyletic in regard to *Sternaspis*. However, this is not supported in the MP analysis (PBT 83% for a monophyletic Paraonidae). A close relationship between Maldanidae and Arenicolidae (LBT 93%, BPP 1.0, PBT 85%) as well as Capitellidae and Echiura (LBT 99%, BPP 1.0, PBT 99%) receive high support. The orbiniid ingroup relationships are only poorly resolved and in most cases characterized by short branch lenghts. Clades which are well supported by all methods are *Pettibonella* + *Proscoloplos* (LBT 100%, BPP 1.0, PBT 100%), *Scoloplos acmeceps* + (*Leitoscoloplos pugettensis* + *Scoloplos armiger*) (LBT 77%, BPP 0.96, PBT 90%), and *Naineris laevigata* + (*Protoaricia oerstedii*+ (*Naineris dendritica* + *Naineris quadricuspida*) (LBT 71%, BPP 0.99, PBT 68%). All orbiniid genera which are represented by more than one taxon (*Leitoscoloplos*, *Naineris*, *Orbinia*, *Phylo*, and *Scoloplos*) appear paraphyletic in all analysis regardless of the method used. *Methanoaricia* and *Questa* are always recovered as orbiniid ingroup taxa. *Scoloplos* (*Leodamas*) *johnstonei* appears as the most basal orbiniid (BPP 0.66) but this finding is not well supported.

16S dataset

After ambiguous sites were excluded the alignment of the 16S dataset contains 382 characters, of which 149 are constant, 44 are variable and 189 are parsimony informative. The chi-square test of homogeneity of base frequencies across taxa resulted in no significant *P*-values (chi-square= 27.8839, df=66, *P*=0.999).

The ML tree (-lnL=3943.65274) is illustrated in fig. 3 and Likelihood bootstrapping values and Bayesian posterior probabilities are given at the nodes. Three equally parsimonious trees (results are not shown) are recovered by MP analysis (Tree length = 904, CI = 0.4306).

A monophyletic orbiniid clade is substantiated in all analyses (LBT 95%, BPP 1.0, PBT 95%). Well supported clades of the 18S analysis are also recovered in these analyses: *Protoaricia* + (*Naineris dendritica* + *Naineris quadricuspida*) (LBT 95%, BPP 1.0, PBT 95%), *Pettibonella* + *Proscoloplos* (LBT 80%, BPP 1.0, PBT 80%), *Scoloplos acmeceps* + (*Leitoscoloplos pugettensis* + *Scoloplos armiger*) (LBT 98%, BPP 1.0, PBT 98%). A close relationship of *Leitoscoloplos fragilis* to the latter clade receives also support, though less well (LBT 68%, BPP 0.93, PBT 68%). *Methanoaricia* appears in the ML and Bayes analysis as orbiniid ingroup taxon and the same holds true for all of the three equal
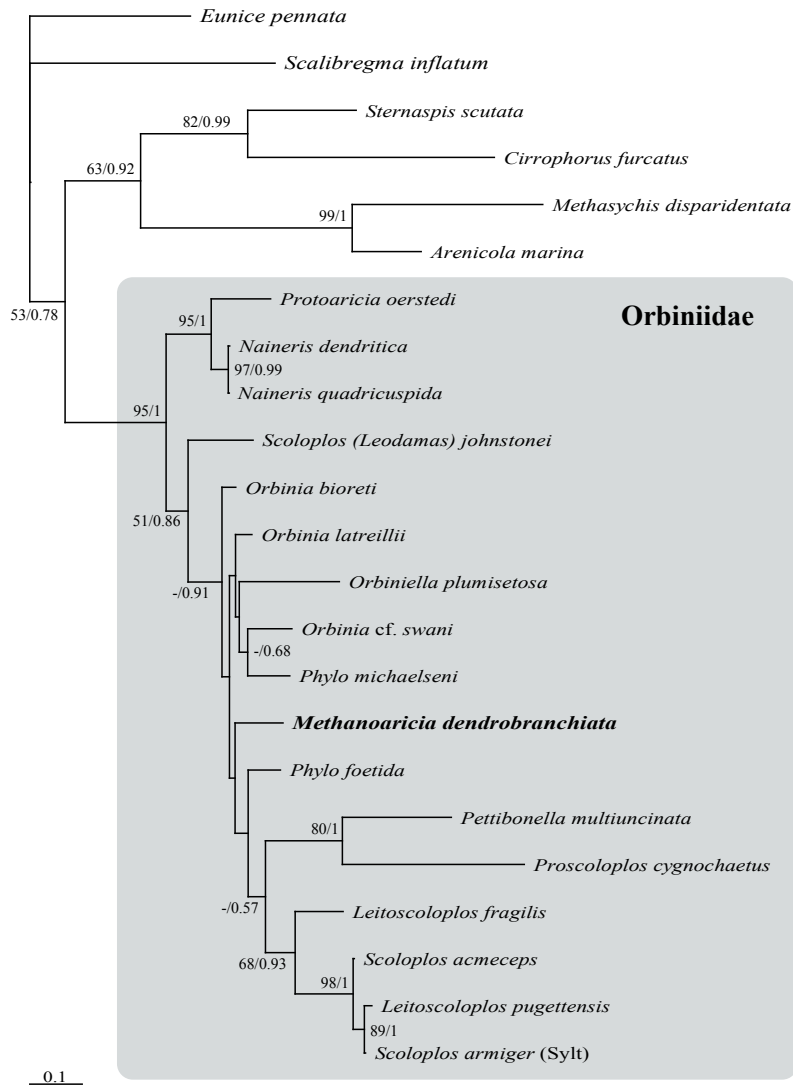
Figure 3. Maximum likelihood tree of the mt16S rRNA gene dataset based on the GTR+ Γ model of sequence evolution ($-\log L = 3943.65274$). The first value at the node represents the ML bootstrap support, the second are bayesian posterior probabilities.

most parsimonious trees.

Combined analysis

The pairwise ILD-test for the two gene was non-significant (*P*=0.233) indicating that combining the data would be meaningful. The combined data matrix includes 1,949 unambiguously aligned characters, of which 1,054 are constant, 301 are variable and 594 are parsimony informative. The chi-square test of homogeneity of base frequencies across taxa resulted in no significant *P*-values (chi-square= 59.5036, df=66, *P*= 0.7).

The resolution of the tree is clearly improved with the combination of the two datasets. Heuristic search found a single most parsimonious tree (fig. 4) in the unweighted MP analysis (Tree length = 2641, CI = 0.5388). One tree (-lnL=14756.30445) is obtained in the ML analysis (fig. 5). The chains of the Bayesian analysis reached the equilibrium at no later than 25.250 generations and the posterior probabilities derived from the remaining trees (1.900) are mapped on the ML tree (fig. 5).
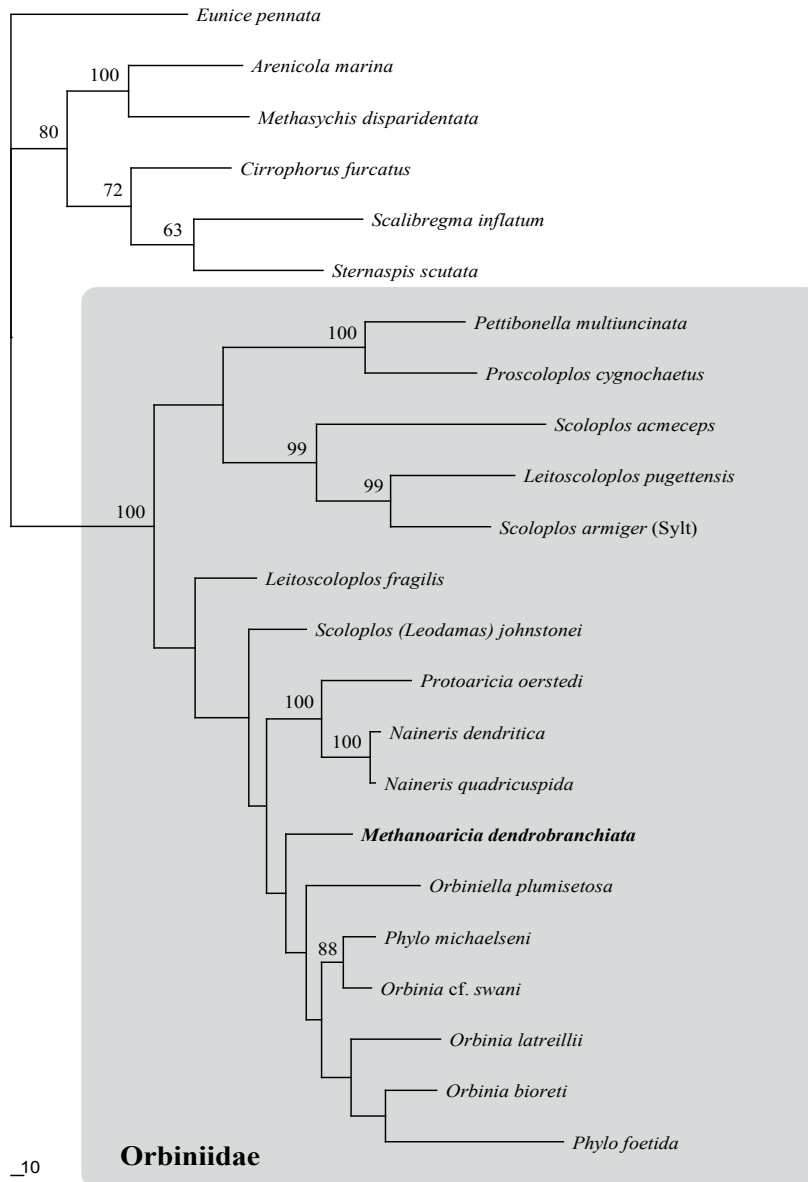
Figure 4. Most parsimonious tree (Tree length = 2641, CI = 0.5388) of the maximum parsimony analysis of the combined dataset. The values at the node represent the MP bootstrap support.

All well supported groups of the separate analyses are also well or even better supported by the combined analysis. The monophyly of the Orbiniidae is strongly supported (LBT 100%, BPP 1.0, PBT 100%) regardless of the applied method and the same holds true for clades consisting of *Protoaricia* + (*Naineris dendritica* + *Naineris quadricuspida*) (LBT 100%, BPP 1.0, PBT 100%), and *Pettibonella* + *Proscoloplos* (LBT 100%, BPP 1.0, PBT 100%), and *Scoloplos acmeceps* + (*Leitoscoloplos pugettensis* + *Scoloplos armiger*) (LBT 98%, BPP 1.0, PBT 98%). The topology of the trees obtained by ML/Bayes and MP slightly differs in the position of *Leitoscoloplos fragilis*. Whereas in the ML and Bayesian analysis a close relationship to a clade consisting of (*Pettibonella* + *Proscoloplos*) + (*Scoloplos acmeceps* + (*Leitoscoloplos pugettensis* + *Scoloplos armiger*)) is supported (LBT 56%, BPP 0.98), the MP analysis recovers a relationship to all other orbiniids.

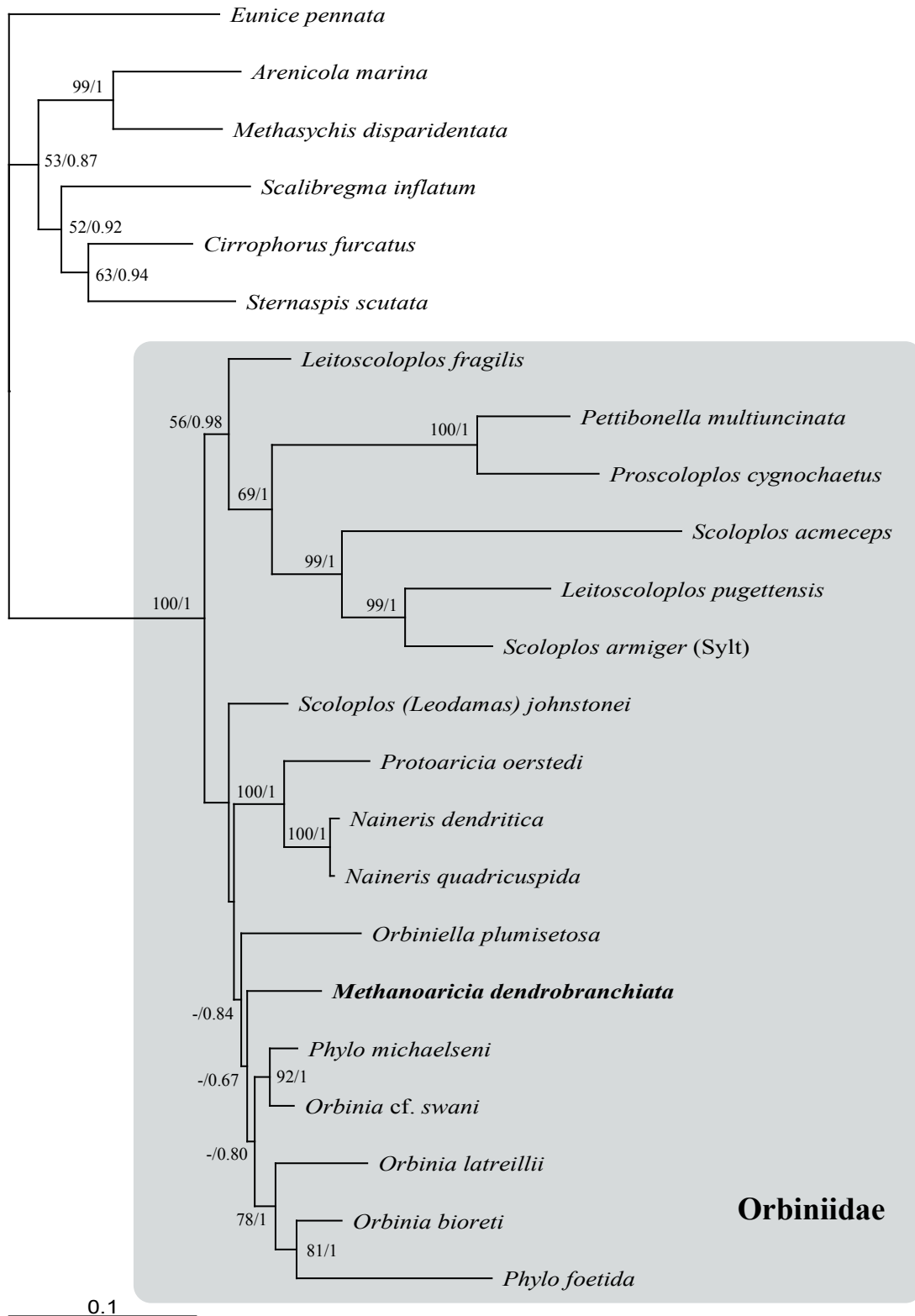A clade consisting of *Phylo michaelseni* + *Orbinia* cf. *swani* (LBT 92%, BPP 1.0, PBT

Figure 5. Maximum likelihood tree of the combined dataset based on the GTR+I+ Γ model of sequence evolution (-log*L*=14756.30445). The first value at the node represents the ML bootstrap support, the second are bayesian posterior probabilities.

88%) is recovered by all methods, whereas *Phylo foetida* clusters with *Orbinia latreillii* and *Orbinia bioreti* (LBT 78%, BPP 1.0). *Methanoaricia* appears as the sister taxon to all *Phylo* and *Orbinia* taxa in the Bayesian (BPP 0.84) and ML analysis. The position of *Scoloplos* (*Leodamas*) *johnstonei* remains uncertain, but this taxon seems to have a more basal position.

5.4 Discussion

Orbiniid monophyly and the phylogenetic position of the questids.
The analysis of the 18S dataset strongly supports the monophyly of a clade consisting of the orbiniids, *Methanoaricia* and *Questa*. The position of the enigmatic Questidae is controversially discussed since their discovery by Hartman (1966). This family comprises a group of interstitial polychaetes which superficially resemble marine oligochaetes. Like them they are annelids with gonads limited to a few body segments (Giere & Rieser, 1981) and their glandular epidermis which forms a cocoon is sometimes hypothesised as a homologous to the clitellum of the Clitellata (Almeida *et al.*, 2003). However, the presence of nuchal organs, the prostomial position of the supraoesophageal ganglia and the absence of an acrosomic tube in the spermatozoa are typical polychaete characters (Jamieson & Webb, 1984; Rouse & Fauchald, 1997; Giere & Erséus, 1998). Furthermore, phylogenetic analyses of large 18S datasets including many clitellate taxa always recover a well supported orbiniid-questid clade (Erséus, Prestegaard & Källersjö, 2000; Rota, Martin & Erséus 2001; Bleidorn *et al.*, 2003a & 2003b). The present analysis suggests that the questids are an orbiniid ingroup taxon, so that the peculiarities concerning the similarities in their reproductive biology to marine oligochaetes should be interpreted due to convergent evolution. One morphological character that is frequently stated as a possible autapomorphy for the substantiation of orbiniid monophyly is the dorsal shifting of the parapodia in the abdomen (Fauchald & Rouse, 1997). This regionalisation of the body in a dorso-ventrally compressed "thorax" and a more fragile "abdomen" arises from the general organisation of the body musculature (Glasby, 2000). While in medium to large sized taxa like *Leitoscoloplos*, *Naineris*, *Orbinia*, *Phylo* and *Scoloplos* a distinct transition between thorax and abdomen is conspicuous, the transition is only weak or not observable in small sized taxa (e.g. *Orbiniella*, *Proscoloplos*). Such a transition is also absent in *Questa* and *Methanoaricia*. The monophyly of an orbiniid-questid clade is supported by the presence of camerated (sometimes termed crenulated) chaetae (fig. 1D) on the morphological side. The formation of these characteristic crenulations is achieved by rings of microvilli (Hausam & Bartolomaeus, 2002). This type of chaetae, typical for Orbiniidae (Rouse & Pleijel, 2001) is also found in *Methanoaricia* (Blake, 2000) and

all *Questa* species (Giere & Erséus, 1998). The lack of this type of chaetae in the newly discovered *Periquesta canariensis* (Brito & Nunez, 2002) is interpreted as a derived condition. Internally chambered chaetae are present in some taxa of the Nephtyidae (Rouse & Pleijel, 2001). Own SEM investigations of *Nephtys hombergi* have shown that this chaetae lack the typical regular pattern of the camerated chaetae unique for the taxa mentioned above.

Phylogenetic position of Methanoaricia dendrobranchiata

Since the discovery of the seepworms (MacDonald *et al*., 1990) and their scientifical description as *Methanoaricia dendrobranchiata* by Blake (2000) a couple of research papers investigated the biology of this worm. Hourdez *et al*. (2001, 2002) described the functional respiratory anatomy and investigated its respiratory adaptations to the strongly hypoxic and sulfidic environment which it inhabits. Eckelbarger & Young (2002) reported about the modified sperm morphology of *Methanoaricia* and Menon *et al*. (2003) studied the epidermal ultrastructure of this worm in detail. This means that *Methanoaricia dendrobranchiata* is one of the best studied polychaete worms, but its phylogenetic position is far from being satisfactorily resolved. An unusual combination of characters led to the problem of identifying its systematic position. Although the presence of camerated chaetae and vascular branchiae are typical orbiniid characters, the nature of the prostomium, the early beginning of the branchiae, the organisation of the parapodia as well as the absence of distinct body regions distinguishes this species obviously from other large orbiniids. The analyses of the seperate 18S and 16S datasets as well as the combined dataset do not support the hypothesis that *Methanoaricia* is "a seperate and distinct sister taxon" of the orbiniids (Blake, 2000) and instead recommends an inclusion of *Methanoaricia* as an orbiniid ingroup taxon. The combined dataset suggests a close relationship between *Methanoaricia* and the *Orbinia* and *Phylo* species, which all represent orbiniids with a large body size. This means that the derived morphology of *Methanoaricia* could be interpreted as an adaptation to its unique biology and that it has evolved due to the hypoxic and sulfidic environment in which this worm live.

Ingroup relationships

One of the most noticeable results of the phylogenetic analysis of the molecular data is the paraphyly of all genera which have been included with more than one species. The genera involved (*Leitoscoloplos*, *Naineris*, *Orbinia*, *Phylo* and *Scoloplos*) are the most species rich taxa in the Orbiniidae. The genus *Leitoscoloplos* was reviewed by Mackie (1987). He distinguished five morphological groups and supposed a possible polyphyletic origin of species referred to this taxon. *Scoloplos* is usually divided into two subgenera: *Leodamas*, comprising species with an early appearance (in respect to the anterior end) of branchiae

is mainly distributed in the southern hemisphere, whereas *Scoloplos sensu stricto*, which comprises the species with a later beginning of the branchiae is more common in the northern hemisphere (Blake, 1996). The main difference between *Leitoscoloplos* and *Scoloplos* species is that only the latter bear stout, ribbed chaetae in the thoracic neurosetae. Kruse, Reusch & Schneider (2003) suggest that *Scoloplos armiger* actually represents at least two sibling species: one with a direct, holobenthic development from egg-cocoons which inhabits intertidal zones and another species with pelagic larvae preferring subtidal habitats. The *Scoloplos armiger* specimen investigated here was also collected from the intertidal and the molecular data strongly support a closer relationship to *Leitoscoloplos pugettensis,* which develops from egg cocoons as well, than to *Scoloplos acmeceps*, which produces pelagic larvae and no egg cocoons. The phylogenetic position of *Leitoscoloplos fragilis*, another species which develops from egg cocoons, depends on the choice of method and gene. It is questionable whether the characters used for species and genera diagnosis in *Scoloplos* and *Leitoscoloplos* are also informative for cladistic analysis. The investigated species of the subgenus *Leodamas* might be a basal orbiniid taxon, but this is only poorly supported by the molecular data.

Whereas the paraphyly of *Orbinia* with regard to *Phylo* has been supposed by many authors before and, consequently, the latter became a subgenus of *Orbinia* (Pettibone, 1957), the paraphyly of *Phylo* is a surprise. Species of the genus *Phylo* are unique in possessing spikelike or lanceolate spines on any posterior neuropodia and this was seen as a strong autapomophy for this taxon. The combined molecular data supports a close relationship of those *Orbinia* and *Phylo* species that occur regionally overlapping. Thus, *Orbinia latreillii*, *Orbinia bioreti* and *Phylo foetida*, each collected from the French Atlantic coast are supported as a monophyletic clade and the same applies to *Orbinia* cf. *swani* and *Phylo michaelseni*, both collected from the north american east coast. A clade consisting of all considered *Orbinia* and *Phylo* species is only poorly supported by the molecular data.

The paraphyly of *Naineris* with regard to *Protoaricia* is strongly recommended by the analysis of the 18S dataset. In several of the collected specimens of *Protoaricia oerstedi* one could see eggs through the body wall. This observation corresponds with that of Augener (1936) and clearly demonstrates that *Protoarica oerstedi* is a valid taxon and not a juvenile of *Naineris*. Instead, progenetic evolution, as supposed by Eisig (1914), seems to represent the best explanation for the similarities between *Protoaricia* and juveniles of *Naineris*.

In accordance with the results of Solis-Weiss & Fauchald (1989) all analyses of the molecular data recover a well supported *Pettibonella + Proscoloplos* clade. Both taxa are unique in possessing swan-shaped hooks. The modus of reproduction for *Pettibonella* is unknown; asexual reproduction is reckoned for *Proscoloplos* (Kelaher & Rouse, 2003),

but this has to be confirmed in further investigations.

The relationships of *Protoariciella uncinata* (only represented in the 18S dataset) and *Orbiniella plumisetosa* remain unclear. Both are small orbiniids with a rounded prostomium and two peristomal rings. Like them, *Naineris* also possesses a round prostomium and to follow the hypotheses of Blake (1996), that both taxa might represent different juvenile stages of *Naineris* species, it should be expected that these fall into a clade with *Naineris* or that their sequence data is identical with one of the investigated *Naineris* species. But this is not the case. Judging the present data, it must be concluded that all taxa of the former Protoariciinae investigated in this study (*Orbiniella plumisetosa, Pettibonella multiuncinata, Protoaricia oerstedi, Protoariciella uncinata,* and *Proscoloplos cygnochaetus*) represent valid species.

It is obvious that the results of this molecular study stand in contrast to that of the morphological cladistic analysis by Blake (2000). Support is given neither to his new combined Orbiniinae, nor to the Microrbiniinae. Looking for reasons which explain these discrepancies it must be considered that Blakes analysis was on genus level. The molecular data strongly indicate that most of the currently assigned orbiniid genera represent paraphyletic assemblages. Thus it can be reasoned that the characters which are presently used for genus diagnosis are not useful for the cladistic analysis.

Progenesis is assumed to have occurred in many annelid taxa (e.g. Westheide, 1987), but in most cases evolutionary scenarios are used as line of argument. However, a phylogenetic hypothesis of the relevant taxa is neccessary to make assumptions about heterochronic evolution (Fink, 1988) and not vice versa. This is demonstrated for *Protoaricia oerstedi*, where progenesis, the maturation at smaller size (McKinney, 1988), represents the best explanation for the presence of larval structures in the adult.

5.5 References

Almeida, W.O., Christoffersen, M.L., Amorim, D.S., Garraffoni, A.R.S., Silva, G.S., 2003. Polychaeta, Annelida, and Articulata are not monophyletic: articulating the Metameria (Metazoa, Coelomata). *Revista Brasil. Zool.* 20, 23-57.

Anderson, D.T., 1959. The embryology of the polychaete *Scoloplos armiger*. *Quart. J. Microsc. Sci.* 100, 89-166.

Anderson, D.T., 1961. The development of the polychaete *Haploscoloplos fragilis*. *Quart. J. Microsc. Sci.* 102, 257-272.

Augener, H., 1936. Zoologische Ergebnisse einer Reise nach Bonaire, Curacao und Aruba im Jahre 1930. *Zool. Jahrb. Abt. Syst. Ökol. Geo. Tiere* 67, 337-352.

Blake, J.A., 1980. The larval development of Polychaeta from the Northern California Coast. IV. *Leitoscoloplos pugettensis* and *Scoloplos acmeceps* (Family Orbiniidae). *Ophelia* 19, 1-18.

Blake, J.A., 1996. Family Orbiniidae Hartman, 1942. In: Blake, J.A., Hilbig, B. and Scott, P.H., eds. Taxonomic Atlas of the Benthic Fauna of the Santa Maria Basin and Western Santa Barbara Channel, Vol. 6: The Annelida, Part 3: Polychaeta: Orbiniidae to Cossuridae. Santa Barbara, CA: Santa Barbara Museum of Natural History, 1-26.

Blake, J.A., 2000. A new genus and species of polychaete worm (Family Orbiniidae) from methane seeps in the Gulf of Mexico, with a review of the systematics and phylogenetic interrelationships of the genera of Orbiniidae. *Cah. Biol. Marine* 41, 435-449.

Blake, J.A., Hilbig, B., 1990. Polychaeta from the vincinity of deep-sea hydrothermal vents in the Eastern Pacific. II. New species and records from the Juan de Fuca and Explorer ridge systems. *Pac. Science* 44, 219-253.

Bleidorn, C., Vogt, L., Bartolomaeus, T., 2003a. New insights into polychaete phylogeny (Annelida) inferred from 18S rDNA sequences. *Mol. Phylogenet. Evol.* 29, 279-288.

Bleidorn, C., Vogt, L., Bartolomaeus, T., 2003b. A contribution to polychaete phylogeny using 18S rDNA sequence data. *J. Zool. Syst. Evol. Res.* 41,186-195.

Borda, E., Siddall, M.E., 2004. Arhynchobdellida (Annelida: Oligochaeta: Hirudinida): phylogenetic relationships and evolution. *Mol. Phylogenet. Evol.* 30, 213-225.

Brito, M.C., Nunez, J., 2002. A new genus and species of Questidae (Annelida: Polychaeta) from the central Macaronesian region and a cladistic analysis of the family. *Sarsia* 87, 281-289.

Dahlgren, T.G., Åkesson, B., Schander, C., Halanych, K.M., Sundberg, P., 2001. Molecular phylogeny of the model annelid *Ophryotrocha*. *Biol. Bull.* 201, 193-203.

Eisig, J., 1914. Zur Systematik, Anatomie und Morphologie der Ariciiden nebst Beiträgen zur generellen Systematik. *Mitteil. Zool. Stat. Neapel* 21, 153-600.

Eckelbarger, K.J., Young, C.M., 2002. Spermiogenesis and modified sperm morphology in the "seepworm" *Methanoaricia dendrobranchiata* (Polychaeta: Orbiniidae) from a methane seep enviroment in the Gulf of Mexico: implications for fertilization biology. *Biol. Bull.* 203, 134-143.

Elwood, H.J., Olsen, G.J., Sogin, M.L., 1985. The small-subunit ribosomal RNA gene sequences from the hypotrichous ciliates *Oxytricha nova* and *Stylonchia pustulata*. *Mol. Biol. Evol.* 2, 399-410.

Erséus, C., Prestegaard, T., Källersjö, M., 2000. Phylogenetic analysis of Tubificidae (Annelida, Clitellata) based on 18S rDNA sequences. *Mol. Phylogenet. Evol.* 15, 381-389.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C., 1995. Constructing a significance test for incongruence. *Syst. Biol.* 44, 570-572.

Fauchald, K., Rouse, G.W., 1997. Polychaete systematics: past and present. *Zool. Scripta* 26, 71-138.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783-791.

Fink, W.L., 1988. Phylogenetic analysis and the detection of ontogenetic patterns. In: McKinney, M.L., ed. *Heterochrony in evolution. A multidisciplinary approach.* New York: Plenum Press, 71-91.

Giangrande, A., Petraroli, A., 1991. Reproduction, larval development and post-larval growth of *Naineris laevigata* (Polychaeta, Orbiniidae) in the Mediterrenaean Sea. *Marine Biol.* 111, 129-137.

Giere, O.W., Erséus, C., 1998. A systematic account of the Questidae (Annelida, Polychaeta), with description of new taxa. *Zool. Scripta* 27, 345-360.

Giere, O.W., Riser, N.W., 1981. Questidae – Polychaetes with oligochaetoid morphology and development. *Zool. Scripta* 10, 95-103.

Giribet, G., Carranza, S., Baguna, J., Riutort, M., Ribera, C., 1996. First molecular evidence for the existence of a Tardigrada + Arthropoda clade. *Mol. Biol. Evol.* 13, 76-84.

Glasby, C.J., 2000. Family Orbiniidae. In: Beesley, P.L., Ross, G.J.B., Glasby, C.J., eds. *Polychaetes & Allies: The Southern Synthesis. Fauna of Australia. Vol. 4A Polychaeta, Myzostomida, Pogonophora, Echiura, Sipuncula.* Melbourne: CSIRO Publishing, 79-84.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and

analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41, 95-98.

Hartman, O., 1957. Orbiniidae, Apistobranchidae, Paraonidae and Longosomidae. *Allan Hancock Pacific Expeditions* 15, 211-393.

Hartman, O., 1966. Quantitative survey of the benthos of San Pedro basin, Southern California. Part II. Final results and conclusions. *Allan Hancock Pac. Exped.* 19, 187-456.

Hausam, B., Bartolomaeus, T., 2001. Ultrastructure and development of forked and capillary setae in the polychaetes *Orbinia bioreti* and *Orbinia latreillii* (Annelida: Orbiniidae). *Invert. Biol.* 120, 13-28.

Hourdez, S., Frederick, L.A., Schernecke, A., Fisher, C.R., 2001. Functional respiratory anatomy of a deep-sea orbiniid polychaete from the brine pool NR-1 in the Gulf of Mexico. *Invert. Biol.* 120, 29-40.

Hourdez, S., Weber, R.E., Green, B.N., Kenney, J.M., Fisher, C.R., 2002. Respiratory adaptions in a deep-sea orbiniid polychaete from Gulf of Mexico brine pool NR-1: metabolic rates and hemoglobin structure/function relationships. *J. Experiment. Biol.* 205, 1669-1681.

Huelsenbeck, J.P., Ronquist, F.R., 2001., MrBayes: Bayesian inference of phylogeny. *Biometrics* 17, 754-755.

Jamieson, B.G.M., Webb, R.I., 1984. The morphology, spermatozoal ultrastructure and phylogenetic affinities of a new species of questid (Polychaeta, Annelida). In: Hutchings, P.A., ed. *Proceedings of the 1ˢᵗ International Polychaete Conference, Sydney, Australia.* Sydney: Linnean Society of New South Wales, 21-34.

Jamieson, B.G.M., Tillier, S., Tillier, A., Justine, J.L., Ling, E., James, S., McDonald, K., Hugall, A.F., 2002. Phylogeny of the Megascolecidae and Crassiclitellata (Annelida, Oligochaeta): combined versus partioned analysis using nuclear (28S) and mitochondrial (12S, 16S) rDNA. *Zoosystema* 24, 707-734.

Kelaher, B.P., Rouse, G.W., 2003. The role of colonization in determining spatial patterns of *Proscoloplos bondi* sp. nov. (Orbiniidae: Annelida) in coralline algal turf. *Mar. Biol.* 143, 909-917.

Kruse, I., Reusch, T.B.H., Schneider, M.V., 2003. Sibling species or poecilogeny in the polychaete *Scoloplos armiger*? *Mar. Biol.* 142, 937-947.

MacDonald, I.R., Reilly, II J.F., Guinasso, N.L., Brooke, J.M., Carney, R.S., Bryant, W.A., Bright, T.J., 1990. Chemosynthetic mussels at a brine-filled pockmark in the Northern Gulf of Mexico. *Science* 248: 1096-1098.

Mackie, A.S.Y., 1987. A review of species currently assigned to the genus *Leitoscoloplos* Day, 1977 (Polychaeta: Orbiniidae), with descriptions of species newly referred to *Scoloplos* Blainville, 1828. *Sarsia* 72, 1-28.

McKinney, M.L., 1988. Classifying heterochrony: Allometry, size, and time. In:

McKinney, M.L., ed. *Heterochrony in evolution. A multidisciplinary approach.* New York: Plenum Press, 17-34.

Menon, J., Willsie, J.K., Tauscher, A., Arp, A.J., 2003. Epidermal ultrastructure and implications for sulfide tolerance in six species of deep-sea polychaetes. *Invert. Biol.* 122, 334-346.

Nygren, A., Sundberg, P., 2003. Phylogeny and evolution of reproductive modes in Autolytinae (Syllidae, Annelida). *Mol. Phylogenet. Evol.* 29, 235-249.

Palumbi, S.R., 1996. Nucleic acids II: The polymerase chain reaction. In: Hillis, D.M., Moritz, C., Mable, B.K., eds. *Molecular Systematics*, second edition. Massachusetts: Sinauer, Inc, 205-247.

Pettibone, M.H., 1957. North American genera of the family Orbiniidae (Annelida: Polychaeta), with descriptions of new species. *J. Wash. Acad. Sci.* 47, 159-167.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 917-918.

Posada, D., Crandall, K.A., 2001. Selecting the best-fit model of nucleotid substitution. *Syst. Biol.* 50, 580-601.

Purschke, G., 1988. Anatomy and ultrastructure of ventral pharyngeal organs and their phylogenetic impotance in Polychaeta (Annelida). V. The pharynges of the Ctenodrilidae and Orbiniidae. *Zoomorphology* 108, 119-135.

Rota, E., Martin, P., Erseus, C., 2001. Soil-dwelling polychaetes: enigmatic as ever? Some hints on their phylogenetic relationships as suggested by a maximum parsimony analysis of 18S rRNA gene sequences. *Contr. Zool.* 70, 127-138.

Rouse, G.W., Fauchald, K., 1997. Cladistics and polychaetes. *Zool. Scr.* 26, 139-204.

Rouse, G.W., Pleijel, F., 2001. Polychaetes. Oxford: University Press.

Swofford, D.L., 2001. PAUP*. Phylogenetic analysis using parsimony, version 4.0b8. Sunderland, MA: Sinauer, INC.

Solis-Weiss, V., Fauchald, K., 1989. Orbiniidae (Annelida: Polychaeta) from mangrove root-mats in Belize, with a revision of protoariciin genera. *Proc. Biol. Soc. Wash.* 102, 772-792.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673-4680.

Turbeville, J.M., Schulz, J.R., Raff, R.A., 1994. Deuterostome phylogeny and the sister group of the chordates: evidence from molecules and morphology. *Mol. Biol. Evol.* 11, 648-655.

Westheide, W., 1987. Progenesis as a principle in meiofauna evolution. *J. Nat. Hist.* 21, 843-854.

Appendix A. List of taxa used in this study with source and GenBank Accession numbers (in bold text for newly sequenced taxa)

Taxa	Source	Accession Nos. 18S	Accession Nos. 16S
Eunice pennata (OF Müller, 1776) (Eunicidae)	Genbank	AY040684	AF321418
Scalibregma inflatum Rathke, 1843 (Scalibregmatidae)	Helgoland, Germany (coll. B. Hausam)	AF448163	**AY532331**
Cirrophorus furcatus Hartmann, 1957 (Paraonidae)	Santa Monica Bay, CA, USA (coll. C. Bleidorn)	**AY532349**	**AY532330**
Aricidea wassi Pettibone, 1965, (Paraonidae)	Santa Monica Bay, CA, USA (coll. C. Bleidorn)	**AY532351**	-
Arenicola marina (Linné, 1758) (Arenicolidae)	Arcachon, France (coll. C. Bleidorn)	AF508116	**AY532328**
Metasychis disparidentata (Moore, 1904) (Maldanidae)	Santa Monica Bay, CA, USA (coll. C. Bleidorn)	**AY532327**	**AY532352**
Dasybranchus caducus (Grube, 1846) (Capitellidae)	GenBank	AF448153	-
Thalassema thalassemum (Pallas, 1766) (Echiura)	Concarneau, France (coll. T. Bartolomaeus)	**AY532354**	-
Ophelia bicornis Savigny, 1818 (Opheliidae)	GenBank	AF508122	-
Sternaspis scutata (Ranzani, 1817) (Sternaspidae)	Adrian Sea, Croatia (coll. C. Bleidorn)	**AY532329**	**AY532353**
Cossura candida Hartman, 1955 (Cossuridae)	Santa Monica Bay, CA, USA (coll. C. Bleidorn)	**AY532350**	-
Parergodrilus heideri Reisinger, 1925 (Parergodrilidae)	GenBank	AJ310504	-
Stypocapitella subterranea Knöllner, 1934 (Parergodrilidae)	GenBank	AF412810	-

Questa paucibranchata Giere & Erséus, 1998 (Questidae)	GenBank	AF209464	-
Leitoscoloplos fragilis (Verrill, 1873)	Little Buttermilk Bay, MA, USA (coll. T. Dahlgren)	**AY532360**	**AY532341**
Leitoscoloplos pugettensis (Pettibone, 1957)	Santa Monica Bay, CA, USA (coll. C. Bleidorn)	**AY532365**	**AY532342**
Methanoaricia dendrobranchiata Blake, 2000	Brine Pool NR-1, Lousiana Slope, Gulf of Mexico, USA (coll. S. Hourdez)	**AY532333**	**AY532357**
Naineris dendritica (Kinberg, 1867)	Malibu, CA, USA (coll. C. Bleidorn)	**AY532358**	AY532345
Naineris laevigata (Grube, 1855)	GenBank	AY040696	-
Naineris quadricuspida (Fabricius, 1780)	Cattle Point, WA, USA (coll. K.M. Halanych)	**AY532361**	**AY532346**
Orbinia bioreti (Fauvel, 1919)	Concarneau, France (coll B. Hausam)	AF448158	**AY532334**
Orbinia latreilii (Audouin & Milne Edwards, 1833)	Roscoff, France (coll. C. Bleidorn)	**AY532355**	**AY532335**
Orbinia cf. *swani* Pettibone, 1963	Southern New England, MA, USA (coll. T. Dahlgren)	**AY532363**	**AY532336**
Orbiniella plumisetosa Buzhinskaja, 1992	Bering Island, Russia (coll. G. Mayer)	**AY532364**	**AY532348**
Phylo foetida (Claparède, 1870)	Arcachon, France (coll. C. Bleidorn)	**AY532356**	**AY532337**
Phylo michaelseni (Ehlers, 1897)	Southern New England, MA, USA (coll. A. Nygren)	**AY532362**	**AY532338**
Pettibonella multiuncinata Solis-Weiss & Fauchald, 1989	Twin Cayes, Belize (coll. C. Bleidorn)	**AY532359**	**AY532339**
Proscoloplos cygnochaetus Day, 1954	Roscoff, France (coll. H. Hausen)	AF448162	**AY532340**
Protoaricia oerstedi (Claparède, 1864)	Collioure, France (coll. C. Bleidorn)	AF508123	**AY532347**
Protoariciella uncinata Hartmann-Schröder, 1962	Buenos Aires, Argentinia (coll. R. Elias)	AF508124	-
Scoloplos acmeceps Chamberlin, 1919	Morro Bay, CA, USA (coll. C. Bleidorn)	**AY532366**	**AY532344**
Scoloplos armiger (O.F. Müller, 1776)	GenBank	U50972	-

Scoloplos armiger (O.F. Müller, 1776)	Sylt, Germany (coll. T. Bartolomaeus)	**AY532367**	**AY532343**
Scoloplos (Leodamas) johnstonei Day, 1934	Cape Town, South Africa (coll. B. Hausam)	AF508126	**AY532332**