6. Molecular phylogeny of lugworms (Annelida, Arenicolidae) inferred from three genes

Abstract - Arenicolids comprise a group of 4 genera in which about 30 nominal species are described. Whereas the biology of many arenicolids is well known, the phylogenetic relationships of these worms are inadequately studied. A close relationship of Arenicolidae and Maldanidae is generally accepted. The phylogenetic relationships of arenicolid taxa were reconstructed based on sequence data of the mitochondrial 16S rRNA gene, the nuclear 18S rRNA gene, and a small fraction of the nuclear 28S rRNA gene. Members of all described arenicolid genera are included in the data set. Phylogenetic analyses were conducted using Maximum Likelihood, Bayesian inference, and Maximum Parsimony. The monophyly of the Maldanidae, as well as of the Arenicolidae is supported by all conducted analyses. Two well supported major clades are highest ranked sister taxa in the Arenicolidae: one containing all *Abarenicola* species and one containing *Arenicola*, *Arenicolides*, and *Branchiomaldane*. Evidence is given for a closer relationship between the two investigated *Branchiomaldane* species and *Arenicolides ecaudata* in the combined analysis. In the light of the molecular data the best explanation for structural and morphological observations is that *Branchiomaldane* evolved by progenesis.

6.1 Introduction

The polychaete taxon Arenicolidae, commonly known as lugworms, comprise a group of particle-feeding polychaetes with a worldwide occurrence (Hutchings, 2000). About 30 nominal species are described, but the status of many regionally separated subspecies remains unclear (Rouse & Pleijel, 2001). Lugworms are one of the few polychaete taxa with economic importance. Large individuals are collected for sea angling bait (McLusky *et al*., 1983) and recent studies on *Arenicola marina* have shown that its haemoglobin might be a promising blood substitute for human medicine (Zal *et al*., 2002).

Traditionally four arenicolid genera are recognized: *Arenicola*, *Abarenicola*, *Arenicolides*, and *Branchiomaldane* (Wells, 1959). *Arenicola* and *Abarenicola*, the genera which comprises the most species, are large worms which possess a long achaetous tail. They live in U-shaped burrows and prominent castings are often characteristic indicator for their appearance (Wells, 1945). Members of *Arenicolides* are only known from european waters and they lack an achaetous tail (Wells, 1950). *Branchiomaldane* species differ from all other arenicolids in their small size, a simpler structure of the branchiae, the

reduction or absence of a tail, and the presence of a beard in the hooked setae in adults (Bartolomaeus & Meyer, 1999; Nogueira & Rizzo, 2001). The type species of this enigmatic taxon was originally described as a maldanid (Langerhans, 1881) and a second species was initially considered to belong to the Capitellidae (Berkeley & Berkeley, 1932). Due to the appearance of many characters which are usually also present in other juvenile arenicolids, and due its close resemblance to post-larval *Arenicolides* species. (Fauvel, 1899; Ashworth, 1912). Bartolomaeus & Meyer (1999) proposed a progenetic evolution for *Branchiomaldane*.

Whereas the biology of many arenicolids is well known, the phylogenetic relationships of these worms are inadequately studied. A sistergroup relationship between Maldanidae and Arenicolidae seems well supported (Bartolomaeus & Meyer, 1997; Rouse & Fauchald, 1997), yet the position of *Branchiomaldane* remains doubtful (Rouse & Pleijel, 2001). Gamble & Ashworth (1912) distinguished between caudate (*Abarenicola* & *Arenicola*) and ecaudate (*Arenicolides* & *Branchiomaldane*) species. Bartolomaeus & Meyer (1999) proposed an evolutionary scenario for arenicolid ingroup relationships in which the ecaudate species represent the basal taxa and the caudate forms are seen as a derived taxon.

The aim of the present study is to test the monophyly of Maldanidae, Arenicolidae, the arenicolid genera, and to infer the phylogenetic position of *Branchiomaldane*. For this purpose three genes have been chosen (18S, 28S, 16S) which served well in previous molecular phylogenetic studies on annelid relationships (Nygren & Sundberg, 2003; Borda & Siddall, 2004).

6.2 Materials and methods

The sequences of ten lugworm taxa, representing all described genera, were analyzed (Appendix A). A sistergroup relationship between Arenicolidae and Maldanidae is predicted by morphological (Bartolomaeus & Meyer, 1997; Rouse & Fauchald, 1997), as well as molecular studies (Bleidorn *et al*., 2003) and therefore some maldanids are included as outgroup taxa (Appendix A). All obtained trees were rooted with the sequences of *Poecilochaetus serpens* (Polychaeta, Spionida).

All DNA was extracted by using the Qiagen DNeasy™ Tissue Kit according to the manufacturer's instructions. PCR amplification of a ~1800bp part of the 18S rRNA gene was carried out using primer pairs F19 + R1843 (Table 1). For the amplification of a ~350bp part of the 28S rRNA gene the primer pairs 28S-A and 28S-B (Table 1) were used and a ~500bp part of the mitochondrial 16S rRNA gene was amplified using the primer pair 16SarL and 16SbrH (Table 2). 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 28S and 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

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	GGATCCAAGCTTGATCCTTCTGCAGGTTCA CCTAC	Elwood *et al*. (1985)
28S		
28S-A	GACCCGTCTTGAAGCACG	Borda & Siddall (2004)
28S-B	TCGGAAGGAACAGCTACTA	Borda & Siddall (2004)
16S		
16SarL	CGCCTGTTTAACAAAAACAT	Palumbi (1996)
16SbrH	CCGGTCTGAACTCAGATCACGT	Palumbi (1996)

72˚C for 7 min.

All products were purified with the Qiaquick PCR Purification Kit (Qiagen). Sequencing reactions (see table 2 for additional sequencing primers) 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. All sequences were submitted to GenBank (for accession numbers see table 1).

Two different datasets were analyzed. Dataset 1 contains only 18S rRNA sequence data

and covers a wide range of maldanid taxa – this dataset is used to test the monophyly of arenicolids and maldanids. Dataset 2 contains the combined data, but less outgroup taxa and is used to infer ingroup relationships of the arenicolids.

All sequences were aligned with CLUSTAL W (Thompson *et al*., 1994) using the default parameters for gap opening and gap penalty and were 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 first 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. Data sets of the different genes were tested for heterogeneity using the partition homogeneity test (Farris *et al*. 1995), implemented in PAUP*, to assess the appropriateness of combining the data partitions. We conducted a test between each pair of gene partitions using 1.000 replicates for each test.

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 using the heuristic search option with TBR branch swapping and 10 random sequence addition replicates. The *hLRT* criterium indicates that the SYM+I+Γ (Zharkikh, 1994) represents the optimal model in respect to the 18S as well as to the combined dataset. Bootstrap values (Felsenstein, 1985) were determined from 500 replicates subject to full heuristic searches with simple addition sequence and TBR branch swapping to provide measures of relative clade support.

Bayesian analyses were conducted using MrBayes 3.0B4 (Huelsenbeck & Ronquist, 2001). All priors were set according to the chosen model (lset nst=6 rates=invgamma; prset RevMatPr=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)). 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.

Equal weighted parsimony with branch and bound search was conducted for both data sets. Clade support was assessed with nonparametric bootstrap (Felsenstein, 1985) as implemented in PAUP* (heuristic search, 500 replicates, TBR branch swapping, and

simple addition sequence).

6.3 Results

18S data set

After the exclusion of ambiguous sites, the alignment contains 1,635 positions, of which 1,226 are constant, 179 are variable and 230 are parsimony informative. Stationarity of base frequencies is an assumption of parsimony and likelihood based methods of phylogenetic reconstruction (Swofford *et al*., 1996). The chi-square test of homogeneity of base frequencies across taxa results in no significant *P*-values (chi-square=11.0645, df=60, *P*=1.0). Therefore, stationarity of base frequencies can be assumed.

Maximum likelihood (-lnL=3943.65274) and Bayesian inference resolve trees with slightly different topologies, of which the most likely tree is illustrated in fig.1. Maximum Parsimony (equal weighting) analysis results in 3 most parsimonious trees (Tree length = 904, CI = 0.4306). All inference methods results in a well supported Maldanidae + Arenicolidae clade (>90% bootstrap support). The monophyly of both families receives support independent of the chosen methods. Whereas the monophyly of the Arenicolidae gains high support (100% bootstrap support), maldanid monophyly is only weakly supported (>60% bootstrap support). Members of the Maldaninae (*Maldane sarsi*, *Metasychis disparidentata*) appear as basal most maldanids. In the *Arenicolidae*, the monophyly of *Abarenicola* and *Branchiomaldane* is well supported, but *Arenicola* appears paraphyletic with regard to *Arenicolides* in the ML analysis.

Combined data set

The pairwise ILD-tests for each pair of genes yielded non-significant *P*-values (18S-28S *P*=0.379; 18S-16S *P*=0.051; 28S-16S *P*=0.118), hence the partition homogeneity test supports the combination of the three gene partitions. The chi-square test of homogeneity of base frequencies across taxa results in no significant *P*-values (chi-square=18.4922, df=42, *P*=0.999).

Maximum likelihood (-lnL=10570.79921), bayesian inference, and maximum parsimony (MPT: Tree length = 904, CI = 0.6333) yield the same tree topology (fig. 2). In correspondence with the 18S analysis, in the combined analysis a maldanid – arenicolid clade is well supported (100% bootstrap support) and the same holds true for a monophyletic Maldanidae (100% bootstrap support) and Arenicolidae (100% bootstrap support) clade. All analyses reveal two major clades in the Arenicolidae. A clade consisting of the investigated *Abarenicola* taxa and a clade consisting of the remainder of the lugworms are well supported. In *Abarenicola* the species collect on the northern hemisphere (*A. claparedii* + *A. pacifica*) and the species from the southern hemisphere (*A. affinis* + *A. gilchristi*) are more closely related to each other. The monophyly of *Arenicola*

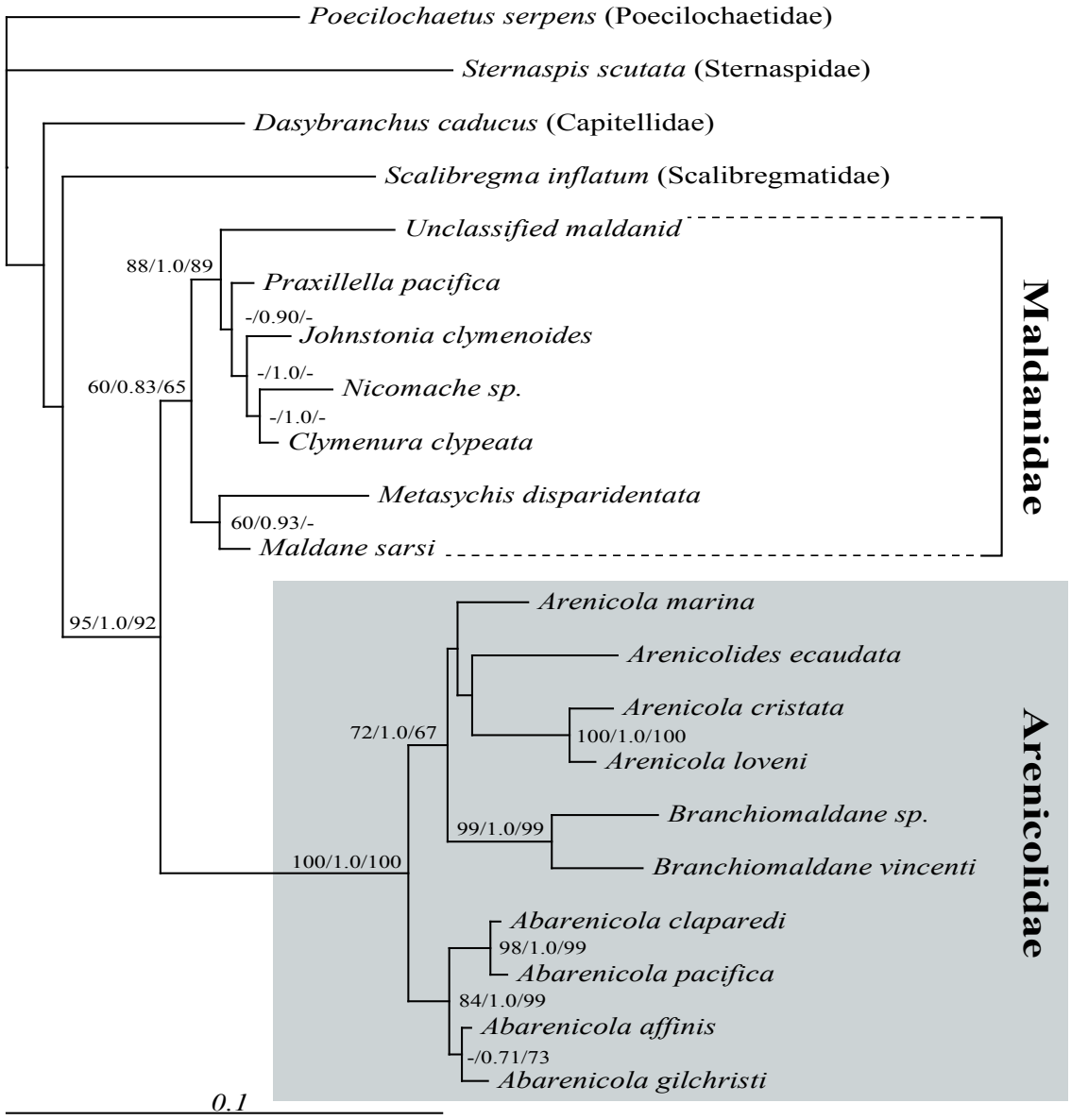

Figure 1. Maximum likelihood tree of the 18S rRNA gene dataset based on the SYM+I+Γ model of sequence evolution (-log*L*=3943.65274). The first value at the node represents the ML bootstrap support, the second are bayesian posterior probabilities, and the third represent the MP bootstrap support.

is only poorly supported by bayesian posterior probabilities (0.7) and gains no significant bootstrap support. Some support is given for a *Branchiomaldane* + *Arenicolides* clade (>70% bootstrap support), a relationship which has not been recovered by the 18S data set. Monophyly of *Branchiomaldane* gains strong support.

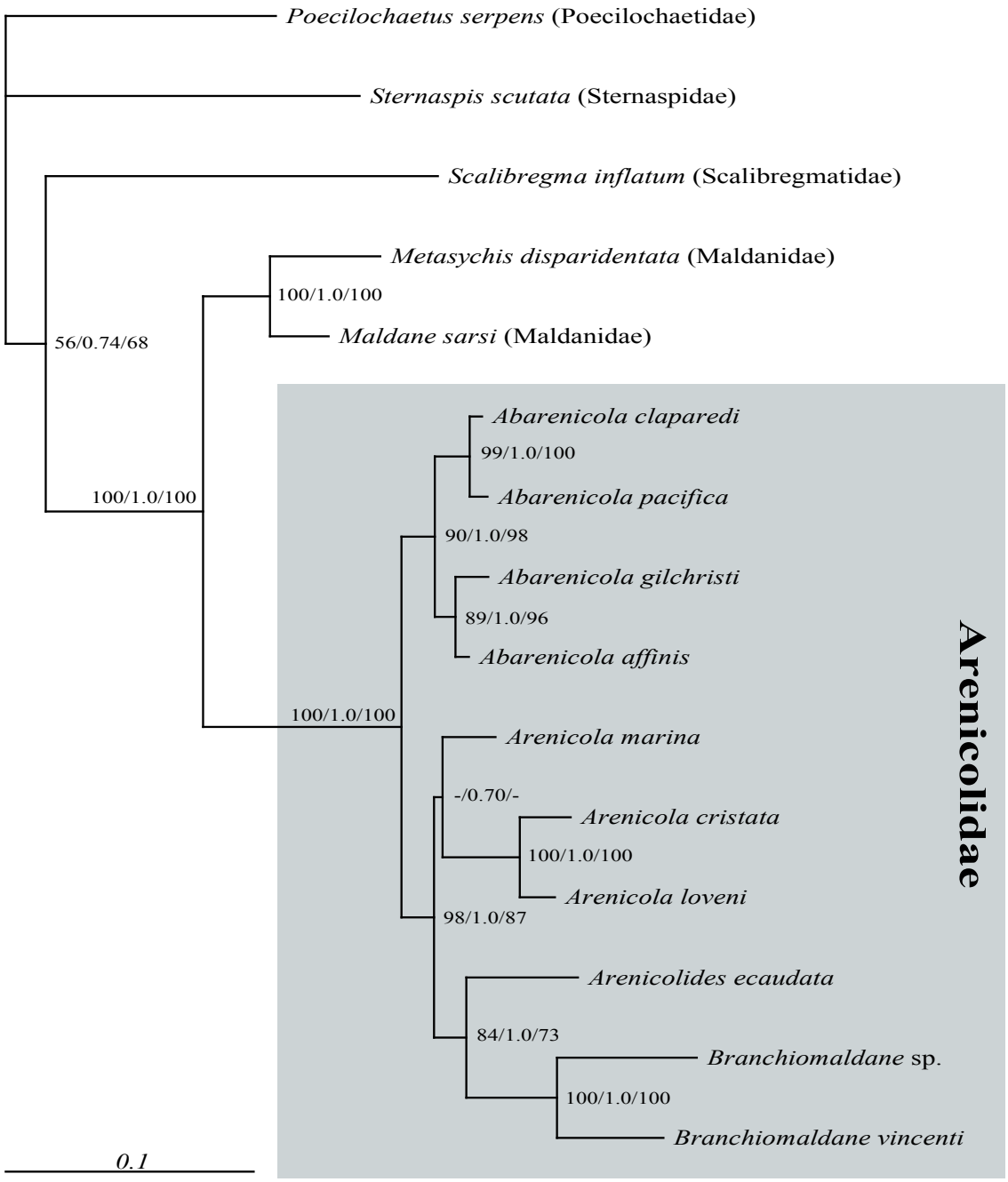

Figure 2. Maximum likelihood tree of the combined dataset based on the SYM +I+ Γ model of sequence evolution (-logL=10570.79921). The first value at the node represents the ML bootstrap support, the second are bayesian posterior probabilities, and the third represent the MP bootstrap support

6.4 Discussion

Lugworms belong to the most familiar representatives of the polychaetes and are known since pre-Linnean times (Asworth, 1912). Even though arenicolids are easily recognized, it is difficult to characterize them and their monophyly remains tentative (Fauchald & Rouse, 1997; Hutchings, 2000). The same holds true for Maldanidae, a family which is usually grouped together with Arenicolidae (Dales, 1962; Fauchald, 1977; Pettibone, 1982; Rouse & Fauchald, 1997; Bartolomaeus & Meyer, 1997). Rouse & Pleijel (2001) raised the question that one of the two families might be paraphyletic and recommended that the relationship of *Branchiomaldane* to maldanids should be investigated. *Branchiomaldane* was originally described as a maldanid (Langerhans, 1981) and later transferred to Arenicolidae (see Wells, 1959 for taxonomic history).

This study represents the first phylogenetic analysis of arenicolid relationships based on molecular sequence data. Independent of the method of phylogenetic reconstruction, all our analyses strongly supports an inclusion of *Branchiomaldane* in the Arenicolidae, as well as the monophyly of this family, whereas maldanid monophyly is only weakly supported in the analysis of the 18S data set. The monophyly of all arenicolid genera is recovered by the combined analysis. However, whereas strong support is given for the monophyly of *Branchiomaldane* and *Abarenicola*, the monophyly of *Arenicola* gains no bootstrap support in the combined analysis. *Arenicolides* is only represented by one of the two described species. Two well supported major clades are highest ranked sister taxa in the Arenicolidae: one containing all *Abarenicola* species and one containing *Arenicola*, *Arenicolides*, and *Branchiomaldane*. This means, that it is more parsimonious to assume that the caudate forms represent the plesiomorphic condition of lugworms and that a long achaetous tail can be interpreted as an autapomorphy for the Arenicolidae. These results are in contrast to the evolutionary scenario proposed by Bartolomaeus & Meyer (1999), in which the “Caudata” are interpreted as a derived group inside the Arenicolidae.

Instead, the ecaudate forms might represent a monophylum. The combined analysis gives evidence for a closer relationship between the two investigated *Branchiomaldane* species and *Arenicolides ecaudata*. The resemblance of *Branchiomaldane* to post-larval *Arenicolides* was recognized by many authors (Fauvel, 1899; Ashworth, 1912; Fournier & Barrie, 1987). On the basis of these observations Bartolomaeus & Meyer (1999) proposed a progenetic evolution of *Branchiomaldane*, a hypothesis which finds additional support in our analysis.

Morphologically the monophyly of *Branchiomaldane* is substantiated by hermaphroditism, reduction of the nephridia and an extreme elongation of the caudal nephridia. Body size of all *Branchiomaldane* species is less than one tenth of the body size of the remaining

arenicolids. As revealed by our molecular data, the next relatives of *Branchiomaldane* are large sized species, so that the small body size of *Branchiomaldane* species must be secondary. This interpretation is along with the observation that *Branchiomaldane* resembles the early postlarvae of *Arenicolides* species, i.e. they possess tiny, largely unbranched gills, but lack an achaetous, gill-less tail which is characteristic for young postlarvae of *Arenicola* and *Arenciolides* species. In the light of the molecular data the best explanation for these structural and morphological observations is that *Branchiomaldane* evolved by progenesis. This evolutionary process is characterized by an accelerated maturation of the gonads while the somatic development retains its original speed (Gould, 1977). As a result of this evolutionary process the animals show a truncated somatic development and are small compared to their closest relatives, when they are mature. Progenetic evolution is regarded as characteristic for several meiofaunal annelids (Westheide, 1984). Generally progenetic animals resemble rather an early developmental stage of their large relatives than an adult. For *Branchiomaldane*, one would expect that characters that are specific for early postlarval stages of *Arenicolides*, *Arenicola* or *Abarenicola* species. The young of all three genera possess pigmented photoreceptors, while the adults lack such organs. In *Arenicola marina* juveniles these eyes possess lenses (Bartolomaeus, unpubl.), as also has recently been described for *Branchiomaldane* sp. (Nogueira & Rizzo, 2001). Thus, persistence of a juvenile character in *Brachiomaldane* is another hint at the progenetic evolution of this taxon which is indicated by our molecular study.

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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. 28S	Accession Nos. 16S
Poecilochaetus serpens Allen, 1904 (Poecilochaetidae)	Arcachon, France (coll. H. Hausen)	**AY569652**	**AY569665**	**AY569680**
Sternaspis scutata (Ranzani, 1817) (Sternaspidae)	Adrian Sea, Croatia (coll. C. Bleidorn)	AY532329	**AY569666**	AY532353
Scalibregma inflatum Rathke, 1843 (Scalibregmatidae)	Helgoland, Germany (coll. B. Hausam)	AF448163	**AY569667**	AY532331
Dasybranchus caducus (Grube, 1846) (Capitellidae)	GenBank	AF448153	-	-
unclassified maldanid (Maldanidae)	GenBank	AY040694	-	-
Metasychis disparidentata (Moore, 1904) (Maldanidae)	Santa Monica Bay, CA, USA (coll. C. Bleidorn)	AY532327	**AY569668**	AY532352
Maldane sarsi Malmgren, 1865 (Maldanidae)	Santa Monica Bay, CAL, USA (coll. C. Bleidorn)	**AY569655**	**AY569669**	**AY569681**
Praxillella pacifica Berkeley, 1929 (Maldanidae)	Santa Monica Bay, CAL, USA (coll. C. Bleidorn)	**AY569653**	-	-
Johnstonia clymenoides (Quatrefages, 1865) (Maldanidae)	Roscoff, France (coll. C. Bleidorn)	**AY569656**	-	-
Nicomache sp. (Maldanidae)	Stykkisholmur, Iceland (coll. G. Rouse)	**AY569654**	-	-
Clymenura clypeata (Saint-Joseph, 1894) (Maldanidae)	GenBank	AF448152	-	-
Arenicolidae:				
Arenicola marina (Linné, 1758)	Arcachon, France (coll. C. Bleidorn)	AF508116	**AY569672**	AY532328
Arenicola cristata Stimpson, 1856	Newport, CAL, USA (coll. L. Vogt)	**AY569657**	**AY569670**	**AY569682**
Arenicola loveni Kinberg, 1867	Cape Town, South Africa (coll. G. Branch)	**AY569658**	**AY569671**	**AY569683**
Arenicolides ecaudata (Johnston, 1865)	Concarneau, France (coll. T. Bartolomaeus)	**AY569664**	**AY569679**	**AY569688**
Branchiomaldane vincenti Langerhans, 1881	Concarneau, France (coll. T. Bartolomaeus)	AF508117	**AY569678**	**AY569690**
Branchiomaldane sp.	Morro Bay, CAL, USA (coll. L. Vogt)	**AY569663**	**AY569677**	**AY569689**
Abarenicola claparedi (Levinsen, 1883)	False Bay, Washington, USA (coll. M. Dethier)	**AY569659**	**AY569673**	**AY569684**
Abarenicola pacifica Healy & Wells, 1959	False Bay, Washington, USA (coll. M. Dethier)	**AY569660**	**AY569674**	**AY569685**
Abarenicola gilchristi Wells, 1963	Lamberts Bay, South Africa (coll. L. Vogt)	**AY569662**	**AY569676**	**AY569686**
Abarenicola affinis (Ashworth, 1903)	Otago Harbour, New Zealand (coll. B. Paavo)	**AY569661**	**AY569675**	**AY569687**