

The mitochondrial DNA of *Xenoturbella bocki*: genomic architecture and phylogenetic analysis

Marleen Perseke · Thomas Hankeln · Bettina Weich ·
Guido Fritsch · Peter F. Stadler · Olle Israelsson ·
Detlef Bernhard · Martin Schlegel

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Abstract The phylogenetic position of *Xenoturbella bocki* has been a matter of controversy since its description in 1949. We sequenced a second complete mitochondrial genome of this species and performed phylogenetic analyses based on the amino acid sequences of all 13 mitochondrial protein-coding genes and on its gene order. Our

results confirm the deuterostome relationship of *Xenoturbella*. However, in contrast to a recently published study (Bourlat et al. in *Nature* 444:85–88, 2006), our data analysis suggests a more basal branching of *Xenoturbella* within the deuterostomes, rather than a sister-group relationship to the Ambulacraria (Hemichordata and Echinodermata).

M. Perseke (✉) · D. Bernhard · M. Schlegel
Institut für Zoologie, Molekulare Evolution und
Systematik der Tiere Universität Leipzig,
Talstr. 33, 04103 Leipzig, Germany
e-mail: perseke@rz.uni-leipzig.de

D. Bernhard
e-mail: bernhard@rz.uni-leipzig.de

M. Schlegel
e-mail: schlegel@rz.uni-leipzig.de

T. Hankeln · B. Weich
Institut für Molekulargenetik,
Johannes Gutenberg Universität Mainz,
J. J. Becherweg 32, 55099 Mainz, Germany
e-mail: hankeln@uni-mainz.de

B. Weich
e-mail: weich@uni-mainz.de

G. Fritsch · P. F. Stadler
Interdisziplinäres Zentrum für Bioinformatik,
Universität Leipzig, Härtelstr. 16-18, 04107 Leipzig, Germany
e-mail: fritsch@izbi.uni-leipzig.de

P. F. Stadler
Lehrstuhl für Bioinformatik, Institut für Informatik,
Universität Leipzig, Härtelstr. 16-18, 04107 Leipzig, Germany
e-mail: studla@bioinf.uni-leipzig.de

O. Israelsson
Museum of Evolution, Uppsala University,
Norbyvagen 16, 75236 Uppsala, Sweden
e-mail: olle.israelsson@bredband.net

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Introduction

Xenoturbella bocki is a small, up to 4 cm long, free-living marine animal with an irregular and flattened shape (Israelsson 1999). Its unusual morphology with an epithelial epidermis and gastrodermis, but without anus and only a “statocyst” as distinct organ (Ehlers 1991), led to highly divergent phylogenetic interpretations. Indeed, the phylogenetic position of *X. bocki* has been a matter of controversy since its description by Westblad (1949). Initially, most authors grouped *Xenoturbella* within the Protostomia. Histological data (Westblad 1949) suggested *Xenoturbella* to be a new taxon within the Acoelomorpha, a hypothesis that was later supported by ultrastructural features of the epidermal cilia (Franzèn and Afzelius 1987; Rohde et al. 1988; Rieger et al. 1991; Lundin 1998). Other morphological (Ehlers 1991), ontogenetic (Israelsson 1997, 1999; Israelsson and Budd 2005), and ultrastructural features (Raikova et al. 2000), however, casted serious doubt on the acoelomorpha hypothesis. Instead, *Xenoturbella* was variably grouped to enteropneusts or holothurians (Reisinger 1960), to bryozoans (Zrzavy et al. 1998), and to molluscs (Israelsson 1997, 1999). The immunoreactivity of *X. bocki*

nerve cells to antibodies raised against echinoderm neuropeptides suggested a close phylogenetic relationship to Ambulacraria (Stach et al. 2005). Other authors advocated a basal position within the metazoa (Jägersten 1959; Ehlers and Sopott-Ehlers 1997a; Raikova et al. 2000). With the realization that Acoela (and Nemertodermatida) are most likely basal bilaterians rather than protostomes (Ruiz-Trillo et al. 2002, 2004; Telford et al. 2003), the basal metazoa hypothesis may, however, not be as much at odds with the acoelomorpha hypothesis as previously thought.

First molecular analyses of gene sequences placed *Xenoturbella* within bivalve molluscs (Noren and Jondelius 1997), an analysis that was later recognized as flawed due to contaminations of ingested prey (Bourlat et al. 2003). Authentic nuclear SSU rDNA sequences, mitochondrial protein-coding gene sequences and expressed sequencing tags (ESTs) supported a close relationship to Deuterostomia and placed *Xenoturbella* as the sister group of Ambulacraria (Hemichordata and Echinodermata) (Bourlat et al. 2003, 2006).

In this contribution we report on the sequencing of a second mitochondrial genome of *X. bocki*, a detailed analysis of its gene order, and a re-evaluation of the phylogenetic position of *Xenoturbella* based on mitochondrial protein sequences.

Materials and methods

Specimens, sequencing, and gene identification

Large specimens of *X. bocki* were collected in Gullmarsfjorden, Bohuslän, Sweden in 2003. Total DNA was prepared from tissue of *RNAlater*-preserved specimen by standard phenol–chloroform extraction following proteinase K digestion. Long PCR primers were designed using known sequences of the mt genes *CoxI* and *CoxII* (Bourlat et al. 2003). Long PCR Enzyme Mix Kit (Fermentas) was used under recommended conditions to amplify the whole mitogenome. The amplification products were sequenced directly by primer walking using ABI Prism 3100 (Leipzig) and 3730 (GENTERprise, Mainz) automated sequencers and BigDye Termination v3.1 Cycle Sequencing Kit (Applied Biosystems) in both directions. Additional starting points for faster primer walking were obtained by preparing a “mini” DNA library using four-cutter restriction enzymes and pUC18 plasmid vector. A complete list of primer sequences can be found in the Electronic Supplement.¹

Protein and rRNA genes were identified by *blastn* (Altschul et al. 1997) database searches at NCBI. Nineteen

of 22 tRNA genes were identified by the *tRNAscan-SE* program (Lowe and Eddy 1997). The tRNA genes for leucine (UUR), serine (AGY), and alanine were identified from sequence alignments and RNA secondary prediction using the *Vienna RNApackage*, version 1.6.1 (Hofacker et al. 1994).

The complete annotated sequence of the *X. bocki* mitogenome has been deposited in GenBank (**AM296016**).

Phylogenetic analysis

We prepared three distinct data sets for phylogenetic analysis. In order to allow direct comparison and re-assessment of the results of Bourlat et al. (2006), the *X. bocki* mtDNA sequence was aligned to additional 31 complete mitochondrial genome sequences belonging to 19 deuterostomes, 9 protostomes, and 2 cnidarian species, as well as the *Xenoturbella* sequence published by Bourlat et al. (2006). This taxon selection, referred to as “Set A”, is essentially the same as the one studied by Bourlat et al. (2006).

We then expanded the data set to include additional basal metazoans, using poriferan sequences to root the tree. This “set B” also includes *Trichoplax adhaerens*. We tried to expand the representation of Protostomia to include a nematode. Even *Trichinella spiralis* (Lavrov and Brown 2001), a relatively basal nematode with normally structured tRNAs, however, exhibited an extremely long branch, suggesting its inclusion would cause long branch attraction artefacts rather than improving the phylogenetic information.

A third set C was compiled comprising only of 9 of 13 protein coding mitochondrial genes to allow the inclusion of the partial mitochondrial genome of *Paratomella rubra* (Ruiz-Trillo et al. 2004). This allowed us to test the deuterostome affinities of *Xenoturbella* in relation to a possible position as a basal bilaterian. All accession numbers of sequences used in this work are compiled in the Electronic Supplement.

All sequences of the protein coding mitochondrial genes were aligned separately using *T-Coffee* with default parameters (Notredame et al. 2000). We chose *T-Coffee*, which generates multiple alignments based on a library produced by using a mixture of local and global pair-wise alignment, because this program has been demonstrated to yield more accurate alignments in particular in difficult case with large divergences times of the taxa (Notredame et al. 2000). The color scheme and the implemented CORE index of *T-Coffee* were used as an indicator of the reliability of the alignment. Multiple alignments were then trimmed on the first and the last constant site. The concatenated matrix included the trimmed alignments of the protein coding genes with an alignment quality index over 40, a threshold that was reached by all 13 aligned proteins.

¹ <http://www.bioinf.uni-leipzig.de/Publications/SUPPLEMENTS/07-009/>

Sequence evolution models and parameters were tested separately for each protein sequence separately and for the concatenated data set using ProtTest version 1.3 (Abascal et al. 2005). The selected model was the most general, reversible Markov model of mitochondrial amino acids: MtRev model (Adachi and Hasegawa 1996) with a gamma shape (4 rate categories) parameter of $\gamma = 0.711$ and a proportion of invariable sites of 0.115. These parameters were then used for phylogenetic reconstructions. The analyses were performed with the program PAUP* (Swofford 2002) for neighbor joining and maximum parsimony, with TREE-PUZZLE, version 5.2, (Schmidt et al. 2002) for maximum likelihood and with MrBayes, version 3.1.2 (Ronquist and Huelsenbeck 2003), and PhyloBayes, version 2.1 (Lartillot and Philippe 2004, 2006) for Bayesian analysis.

To test the robustness of bifurcations, bootstrap analysis (10,000 replicates for NJ, 2,000 replicates for MP, and 1,000 puzzling steps for ML) was calculated. Bayesian analysis was run with the best-fit model as inferred by ProtTest for 1,000,000 generations, with a sampling frequency of 10 generations. From the 100,000 trees found, we determined a subset of trees for building the consensus tree by inspecting the likelihood values of trees saved by MrBayes. The burn-in was set to 25,000 trees to ensure that stable likelihood values were achieved.

For the data sets B and C we calculated the Bayesian analysis with mixture configuration of the substitution model CAT (Lartillot and Philippe 2004) with a free number of categories and continuous gamma distribution. Four chains with 1,000 sample trees (sample frequency 1) were run. The default cutoff of 0.05 was used to include only partitions with maximum probability.

Analysis of gene order

Mitochondrial gene orders (including all mRNA, rRNA and tRNA genes) were compared using circular (Fritzsche et al. 2006), which calculates distances based on a circular list alignment of the mitochondrial genome order in spite of differences and gene content. Different weights are used by this program for rearranged blocks

depending on whether they contain proteins, only rRNAs, or only tRNAs. In addition, we manually compared the gene order of *Xenoturbella* with representative basal deuterostomes.

Results

Characteristics of the *Xenoturbella* mitogenome

The *X. bocki* mt genome sequenced in our analyses (GenBank **AM296016**) spanned 15,232 bp and contained all typical metazoan mt genes: 13 proteins, 2 rRNA, and 22 tRNA genes. Most genes were encoded on the same strand, except for the ND6 gene and seven tRNA genes (Fig. 1).

Only few differences were found when comparing our data to the mtDNA sequence from *X. bocki* (15,234 bp) sequenced by Bourlat et al. (2006), Genbank accession **NC_008556**. Both nucleotide sequences revealed a compact organization of *Xenoturbella* mtDNA with only 2.4% of non-coding sequence. Most of the non-coding nucleotides resided in a single large “unassigned sequence” (UAS I, 336 bp in our sequence and 2 bp shorter in the genome sequences by Bourlat et al. 2006) flanked by tRNA^{Leu} (CUN) and ND6, and an additional smaller UAS region (UAS II, 40 bp) flanked by the tRNA^{Glu} and ND1 genes. A total of 73 nucleotide differences were observed between the two available sequences. The putative control region UAS I was found to be slightly enriched for differences between both mtDNA versions (3.3%, including two additional nucleotides in our sequence) compared to the whole sequence (0.5%). Within the protein-coding genes, we observed 39 differences (excluding sequencing ambiguities, i.e., characters such as N or Y); 15 of these substitutions were non-synonymous causing amino acid replacements.

The reading frames of all protein coding-genes were delimited by the start codons ATG or GTG and complete termination codons. However, two possible start codons were found for ND4L, CoxII and ND5.

Due to the uncertain phylogenetic position of *Xenoturbella*, different translation tables were tested to translate



Fig. 1 Linear mitochondrial genome map for *Xenoturbella bocki*. The gene order is arranged 5′–3′. Genes transcribed on the opposite strand are indicated by dark underlines. Numbers at gene boundaries indicate non-coding nucleotides between genes as inferred from sequence comparison. Negative numbers indicate gene overlaps of the corresponding size. Unassigned sequences (UAS) larger than 30 bp

are shown by shaded areas. Gene name abbreviations: CxI/CxII/CxIII cytochrome *c* oxidase subunit I/II/III, A8/A6 ATPase subunit 8/6, N1/N2/N3/N4/N4L/N5/N6 NADH dehydrogenase subunit 1/2/3/4/4L/5/6, CyB cytochrome *b*, *ssu* small subunit (12S) ribosomal RNA, and *lsu* large subunit (16S) ribosomal RNA, tRNA genes are represented by their single-letter codes

the gene sequences into amino acid sequences. All mitochondrial translation tables of deuterostomes (invertebrate, echinoderm or vertebrate code) are possible, in the sense that none of them implied a premature termination within the coding regions. Analyses using the *gendecoder* server (Abascal et al. 2006) predicted the generic invertebrate mitochondrial code, which then was used throughout the subsequent analysis. In particular, there was no evidence that *Xenoturbella* shares any one of the codon reassignments characteristic for Hemichordata (Castresana et al. 1998a) and/or Echinodermata (Himeno et al. 1987). All 15 differences between the amino acid sequences reported by Bourlat et al. (2006) and this study, respectively, were based on nucleotide differences (see above) and did not depend on the choice of translation table.

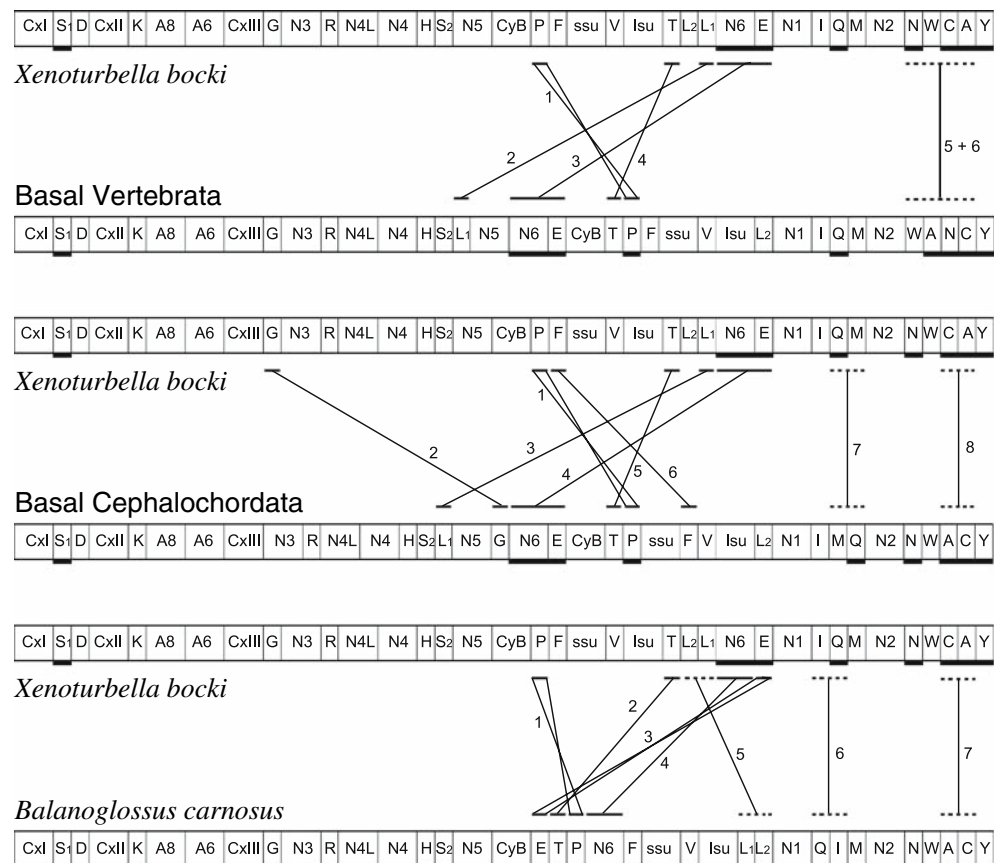
The mitochondrial genome map of *X. bocki*, (Fig. 1) was found to be largely in accordance with the gene orders of other representatives of Deuterostomia. In particular, we compared the gene order of *X. bocki* with the hemichordate *Balanoglossus carnosus* (Castresana et al. 1998b), the reconstructed basal gene order of cephalochordates derived by Nohara et al. (2005), and to the consensus gene order of vertebrates (Boore 1999) (Fig. 2). In contrast, protostome gene arrangements (Boore 1999) consistently exhibited much larger differences (data not shown).

The transformation of the gene order of *X. bocki* to the closest representative of the different deuterostome groups required the inversion of tRNA^{Pro} (Fig. 2, cross line 1) and nearly the same number of additional steps: five relocations to the consensus gene order of vertebrates [tRNA^{Leu}(CUN), ND6-tRNA^{Glu}, tRNA^{Thr}, tRNA^{Cys} and tRNA^{Asn} or tRNA^{Ala} and tRNA^{Trp}], six relocations to the gene order of *Balanoglossus carnosus* [tRNA^{Thr}, ND6, tRNA^{Glu}, tRNA^{Cys} or tRNA^{Ala}, tRNA^{Leu}(CUN) or tRNA^{Leu}(UUR), tRNA^{Ile} or tRNA^{Gln}] and seven relocations to the gene order of basal cephalochordates [tRNA^{Gly}, ND6-tRNA^{Glu}, tRNA^{Leu}(CUN), tRNA^{Phe}, tRNA^{Thr}, tRNA^{Cys} or tRNA^{Ala}, tRNA^{Met} or tRNA^{Asn}].

Hence, the gene arrangement of *X. bocki* shared features with all three groups, e.g., the fragment ND6-tRNA^{Glu} was also present in vertebrates and cephalochordates but was lacking in hemichordates. In contrast, the location of the tRNA^{Gly} in *Xenoturbella* was identical to Hemichordata and Vertebrata while the order within the fragment tRNA^{Asn}-tRNA^{Trp}-tRNA^{Cys}-tRNA^{Ala}-tRNA^{Tyr} is more similar to Hemichordata and Cephalochordata than to Vertebrata.

Not surprisingly, therefore, the analysis of the mitochondrial gene orders using *circal* (Fritzsch et al. 2006) resulted in a tree topology in which all deuterostome taxa

Fig. 2 The mitochondrial gene order of *X. bocki* compared to mitochondrial gene orders of other basal deuterostomes: consensus gene order of vertebrates (Boore 1999), hemichordate gene order represented by *Balanoglossus carnosus* (Castresana et al. 1998b), inferred ancestral gene order of cephalochordates (Nohara et al. 2005). As in Fig. 1, gene order is 5'–3'. Genes transcribed from the opposite strand are *underlined*. For gene name abbreviations see caption of Fig. 1. Translocations are marked by *straight lines*, and inversions are marked by *cross lines*. Changes of the position between joining tRNAs are shown by *interrupted lines*. See text for detailed discussion



form a common branch (Fig. 3) while the protostomes branch off in a polytomy. The first branch within the deuterostomes is formed by the echinoderms followed by an unresolved polytomy consisting of *X. bocki*, Hemichordata, Cephalochordata, and Vertebrata (Fig. 3).

A comparison of the gene order of *X. bocki* with that of the partial mitogenome of the acoel *Paratomella* did not reveal any conspicuous similarities that might place those species into closer proximity.

Lavrov and Lang (2005) reconstructed a hypothetical ancestral mitochondrial gene order of Deuterostomia and demonstrated that this arrangement was most likely very similar to that of ancestral bilaterian. If correct, their reconstruction implies that gene order rules out any affinities of *Xenoturbella* to protostomes, but at the same time makes it unlikely that gene order alone can be used to distinguish between a basal position within the deuterostomes and a basal bilaterian position.

Phylogenetic analysis of protein coding genes

We used the amino acid sequences of all 13 protein-coding genes to examine the phylogenetic relationship of *Xenoturbella*, including both our own mitogenome sequence and the sequence reported by Bourlat et al. (2006).

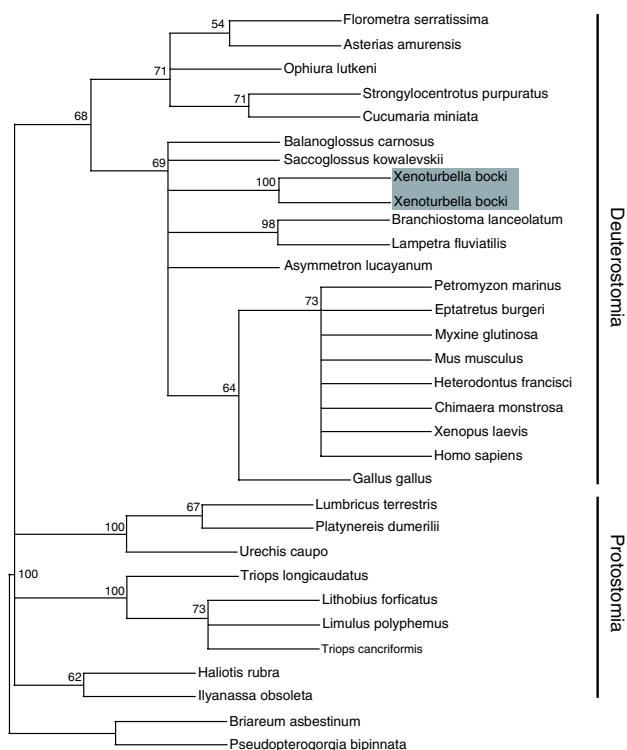


Fig. 3 Maximum Parsimony analysis of the mitochondrial gene order using the program *circular* (Fritzsche et al. 2006). Numbers at the nodes represent bootstrap values of MP analysis with heuristic search (2,000 replicates)

We analyse here three datasets. Set A was most similar to the one analyzed by Bourlat et al. (2006). Deviating from their data set, we included two representatives of diploblast metazoans as outgroups, but excluded the Urochordata (Tunicata) because our initial analyses (data not shown) always showed them basal to all other bilaterians. This exceptional result was observed repeatedly in previous studies (Yokobori et al. 2003, 2005; Bourlat et al. 2006) and can be explained by particularly rapid sequence evolution of Urochordate mt genes leading to long-branch attraction.

All phylogenetic methods yielded the same tree topology regarding the main taxa and clearly recovered Protostomia, Deuterostomia, Ambulacraria (Echinodermata, Hemichordata) and Chordata (Cephalochordata, Vertebrata) as monophyletic groups. However, we also noted that within vertebrates and echinoderms some unstable or unusual branchings occur. Our analyses always resulted in a conjoint placement of both *Xenoturbella* isolates at the base of the deuterostomes followed by a split into the Ambulacraria on the one hand, and the Cephalochordata and Vertebrata on the other hand. This basal branching of *Xenoturbella* was supported by a Bayesian posterior probability of 1.0, a quartet puzzling support value of 73% (ML), and bootstrap values of 68% (MP) and 84% (NJ). The full trees as well as support values for all nodes are compiled in the electronic supplement.

For a comparison to the analyses of Bourlat et al. (2006), we also calculated trees in which the four amino acids methionine, isoleucine, asparagine, and lysine (M, I, N, K) were converted into ‘‘missing data’’ to avoid possible influences due to codon reassignments in the Ambulacrarian mitogenomes (Himeno et al. 1987; Castresana et al. 1998b). For details on the computational procedure we refer to the work by Bourlat et al. (2006). Bayesian, NJ, and MP analyses again resulted in the same basal placement of both *Xenoturbella* isolates, while ML analyses even showed *Xenoturbella* at the base of the Metazoa (data not shown). Compared to our initial analyses, which take all amino acids into account (Fig. 4), all support values with the exception of the ML analysis were reduced in the partially converted dataset: Bayesian posterior probability = 0.89, quartet puzzling support = 89% (ML), bootstrap support values = 61% (MP) and 50% (NJ).

Set B further expanded the taxon sampling to include further outgroup species (*Trichoplax adhaerens* and two poriferans). The results were very similar to that of set A: Fig. 4 shows the tree obtained using the *PhyloBayes* program.

Set C was chosen to test possible affinities of *Xenoturbella* with basal bilaterians. Hence we included *Paratomella rubra*, the only acoelomorph for which at least a partial mitochondrial genome has been sequenced so far. In

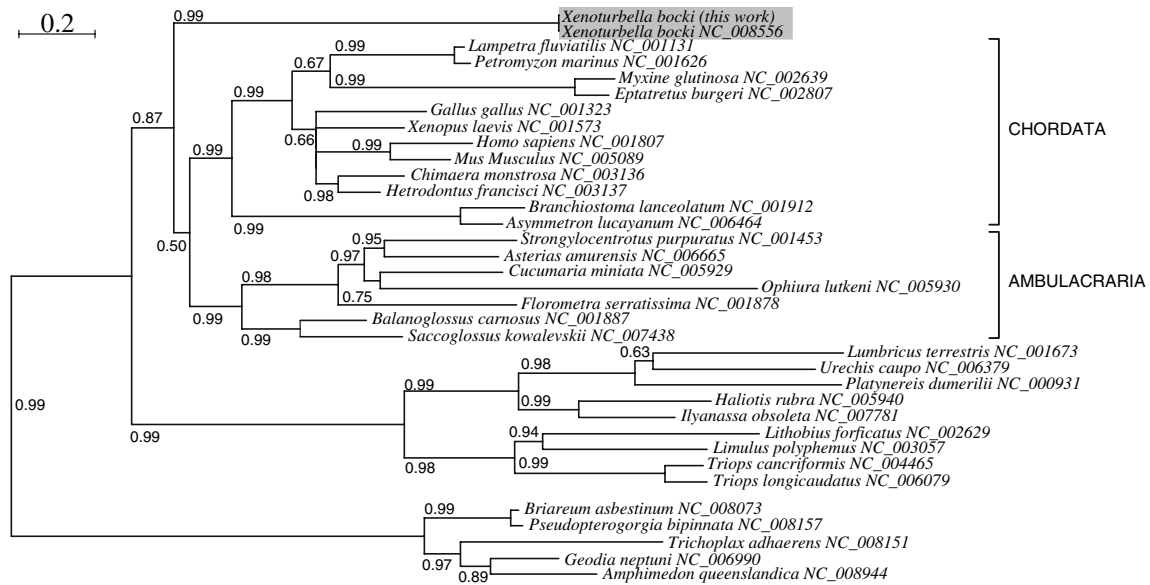


Fig. 4 Analysis of concatenated mitochondrial amino acid using PhyloBayes and the CAT model with a free number of categories and continuous gamma distribution. The tree is rooted at the two

poriferan sequences. Numbers at the nodes indicate Bayesian posterior probabilities; scale units are substitutions per site

contrast to the two other data sets, set C favored a position of *X. bocki* among basal bilaterians. However, the branch leading to *Paratomella* was unusually long (see electronic supplement for tree and support values), suggesting that the grouping of *Xenoturbella* with *Paratomella* could be a long branch attraction artifact. In contrast to the Bayesian analysis, maximum parsimony showed *Xenoturbella* again as a basal deuterostome. The results from the different data sets and methods are summarized in Fig. 5.

Discussion

The analysis of mitochondrial protein-coding genes clearly suggests that *Xenoturbella* belongs to the deuterostomes. This result is consistent with the analysis of the gene order by both visual analysis of break points and automated analysis using *circal*.

It is worth noting in this context that the mitochondrial data in work of Bourlat et al. (2006) place *Xenoturbella* as the sistergroup of Ambulacraria only when the “problematic” amino acids M, I, N, K, i.e., those that are conceivably affected by the differences in the mitochondrial genetic codes were treated as missing data, and when Urochordates were included, which then assume the basal deuterostome position. After exclusion of the Urochordate sequences, which are likely to be affected by long branch effects do to their extremely high rates of evolution, the (*Xenoturbella*, Ambulacraria) affiliation cannot be reproduced in our hands even when M, I, N, K is treated as in Bourlat et al. (2006).

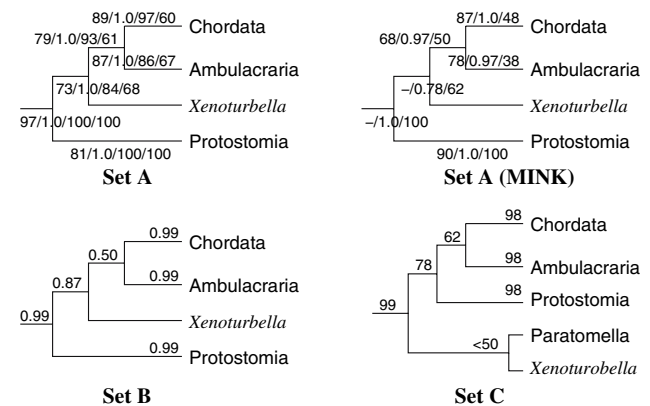


Fig. 5 Summary of phylogenetic analyses with different data sets and methods. Since the datasets are fairly similar, not all methods were applied to all data sets to save computer resources. Complete trees are provided in the electronic supplement. For set A support values are given in the format ML/bayesian/NJ/MP, with Bayesian posterior probabilities computed by MrBayes. Set A (MINK) refers to the variant of the analysis in which the amino acids M, I, N, K are treated as missing data, showing ML/Bayesian/NJ support. ML results favor *Xenoturbella* in a basal bilaterian position. For sets B and C only Bayesian posterior probabilities were computed

While the position of *Xenoturbella* within deuterostomes cannot be resolved unambiguously, the available evidence, in particular the phylogenetic analysis of the mitochondrial protein sequences, points to a basal position within deuterostomes. While the hypothesis favoured by Bourlat et al. (2006), namely a position basal to the Ambulacraria (Echinodermata and Hemichordata), cannot be strictly ruled out based on our analysis, it is not supported

by any positive evidence derivable from our data. On the contrary, a few of our analyses suggest, albeit with weak support, an even more basal position within the bilateria, possibly as the sistergroup the Eubilateria (Protostomia + Deuterostomia).

The result of our molecular study is in better agreement with most morphological studies, as it suggest a more basal position of *Xenoturbella*. Even though the morphology of *Xenoturbella* shares similarities to hemichordates, this evidence is far from unambiguous: similarities in epidermis and sub-epidermal ECM (Reisinger 1960) later have been interpreted as superficial (Pedersen and Pedersen 1986, 1988). Similarities in myocytes and their junction are described as convergences and symplesiomorphy, respectively, by Ehlers and Sopott-Ehlers (1997b).

Eggs and embryos in *Xenoturbella* (Israelsson 1997, 1999; Israelsson and Budd 2005) possesses several protostome features, in particular a non-radial cleavage, the absence of cortical reaction during fertilization, and the ciliary ultrastructure. There are, on the other hand, possible similarities in constitution to echinoderm oocytes. Israelsson and Budd (2005) therefore suggest that *Xenoturbella* may have diverged from the other deuterostomes before the deuterostomes acquired their typical deuterostome features. The nervous system is also most compatible with a basal position, even if some similarities to deuterostome exist (Raikova et al. 2000). Therefore, the plesiomorphy of the morphological similarities between *Xenoturbella* and Ambulacraria cannot be ruled out.

The difficulties and contradicting evidence regarding the phylogenetic position of *Xenoturbella* is reminiscent of the ongoing discussion about the phylogenetic position of *C. elegans*, reviewed in the Wormbook (Fitch 2005). There, the situation is far from resolved despite a finished genome and the availability of a much denser taxon sampling of Nematoda and their putative relatives compared to what we could hope for in the case of *Xenoturbella*. In addition to morphological information and the study of multi-gene sequence data—such as those from the mitogenome reported here and by Bourlat et al. (2006) and the EST-based analysis by Bourlat et al. (2006)—phylogenetic information can also be derived from complex molecular characters. The mitochondrial gene order is one such character (Sankoff et al. 1992). The hypothetical ancestral deuterostome mitogenome gene order reconstructed by Lavrov and Lang (2005) is unfortunately very similar to that of the ancestral bilaterian. *Xenoturbella* does not share characteristic features with Protostomia. A basal position within deuterostomes and a basal bilaterian position therefore cannot be distinguished on the basis of mitochondrial gene order. Furthermore, there is no significant part of the gene order shared between *Xenoturbella* and Ambulacraria that would place them together to the exclusion of Chordata.

Supplemental material

Primer sequences, accession numbers of all mitogenomes used in this study, and trees reconstructed for sets A and C are provided at <http://www.bioinf.uni-leipzig.de/Publications/SUPPLEMENTS/07-009/>.

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