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Elucidating the phylogenetic position of Gnathostomulida and first mitochondrial genomes of Gnathostomulida, Gastrotricha and Polycladida (Platyhelminthes)

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ABSTRACT

Gnathostomulida is a taxon of small marine worms, which exclusively inhabit the interstitium. The evolution of Gnathostomulida has been discussed for decades. Originally regarded as primitive animals with affinities to flatworms, the phylogenetic position of Gnathostomulida has been debated. Given the lack of an anus a close relationship to Platyhelminthes has been maintained (i.e., Plathelminthomorpha hypothesis). Alternative hypotheses proposed Gnathostomulida as being close to Gastrotricha due to the presence of a monociliary epidermis (i.e., Monokonta/Neotrichozoa hypothesis) or to Syndermata based on the complicated jaw apparatus (i.e., Gnathifera hypothesis). Molecular analyses using only few genes were inconclusive. Recent phylogenomic studies brought some progress by placing Gnathostomulida as sister to Syndermata, but support for this relationship was low and depended on the analytical strategy. Herein we present the first data of complete or nearly complete mitochondrial genomes for two gnathostomulids (*Gnathostomula paradoxa* & *G. armata*), one gastrotrich (*Lepidodermella squamata*) and one polyclad flatworm (*Stylochoplana maculata*) to address the uncertain phylogenetic affinity of Gnathostomulida. Our analyses found Gnathostomulida as sister to Syndermata (Gnathifera hypothesis). Thorough sensitivity analyses addressing taxon instability, branch length heterogeneity (also known as long branch attraction) and base composition heterogeneity showed that the position of Gnathostomulida is consistent across the different analyses and, hence, independent of potential misleading biases. Moreover, by ameliorating these different biases nodal support values could be increased to maximum values. Thus, our data support the hypothesis that the different jaw apparatuses of Syndermata and Gnathostomulida are indeed homologous structures as proposed by the Gnathifera hypothesis.

1. Introduction

The 100 species of Gnathostomulida inhabit the marine interstitium of usually detritus-rich sands (Nielsen, 2012). Hence, gnathostomulids are slender and dorsoventrally flattened animals, which range in length from 1 to 4 mm (Brusca and Brusca, 2003). Locomotion in the spaces between the sand grains is facilitated by cilia. Interestingly, the epithelial cells are only monociliated, rather than multiciliated, which is unusual for bilaterian interstitial organisms (Nielsen, 2012). A characteristic feature of Gnathostomulida is the presence of a ventral pharyngeal jaw apparatus (Herlyn and Ehlers, 1997; Kristensen and Nørrevang, 1977; Sørensen et al., 2003; Sterrer et al., 1985). Furthermore, a permanent anus is not present, but a posterior area of the ectoderm lacks a basal membrane and is in direct contact with the hindgut. Hence, this connection might function as a temporary anal opening (Knauss, 1979; Nielsen, 2012). Gnathostomulids are hermaphrodites and the cleavage follows a spiral pattern (Nielsen, 2012; Riedl, 1969).

Traditionally Gnathostomulida has been placed within Platyhelminthes or Nemathelminthes (Kristensen and Funch, 2000; Sørensen, 2002; Sterrer et al., 1985). However, based on morphological data three hypotheses dominated the recent discussion about their placement. The first hypothesis follows the traditional view by placing Gnathostomulida as sister to Platyhelminthes (Ax, 1985, 1995; Eernisse et al., 1992; Giribet et al., 2000; Meglitsch and Schram, 1991; Peterson and Eernisse, 2001; Schram and Ellis, 1994; Zrzavy et al., 1998). This hypothesis is also known as the Plathelminthomorpha hypothesis. The second

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hypothesis, the Monokonta or Neotrichozoa hypothesis, places Gnathostomulida as sister to Gastrotricha (Cavalier-Smith, 1998; Zrzavy et al., 2001) and the third one, the Gnathifera hypothesis, as closely related to Syndermata (Ahlrichs, 1997; Haszprunar, 1996; Herlyn and Ehlers, 1997; Kristensen and Funch, 2000; Melone et al., 1998; Nielsen, 2012; Sørensen et al., 2000; Zrzavy, 2003). All these hypotheses have in common that they favor a closer relationship to platyzoan taxa. Platyzoa is a taxon grouping simple-bodied bilaterians such as Platyhelminthes, Gastrotricha, Syndermata and Gnathostomulida together (e.g., Cavalier-Smith, 1998), but has recently been shown to be a paraphyletic assemblage (Struck et al., 2014).

Molecular analyses using a single or few genes were inconclusive regarding the position of Gnathostomulida. These analyses found Gnathostomulida as sister to Syndermata (e.g., Zrzavy, 2003), to Gastrotricha (e.g., Paps et al., 2009a; Zrzavy et al., 2001), within Platyzoa (e.g., Giribet et al., 2000; Todaro et al., 2006) or as sister to all other spiralian taxa (e.g., Baguñà et al., 2008; Paps et al., 2009b). However, support for any position remained low. Recently, phylogenomic analyses using hundreds of genes were able to robustly resolve problematic phylogenetic relationships in different spiralian taxa (Andrade et al., 2014; Kocot et al., 2011; Smith et al., 2011; Struck et al., 2011; Weigert et al., 2014). The first phylogenomic studies addressing bilaterian relationships including Gnathostomulida placed them either as sister to Acoela or to Gastrotricha, but support was low and Gnathostomulida was among the unstable taxa in these analyses (Dunn et al., 2008; Hejnol et al., 2009). Following phylogenomic studies, focussing on the phylogeny of platyzoan taxa, found a close relationship to Syndermata congruent with the Gnathifera hypothesis (Struck et al., 2014; Wey-Fabrizius et al., 2014; Witek et al., 2009). However, bootstrap support for this relationship was usually low and the position of Gnathostomulida depended on the analytical strategy used. Using different datasets or analytical methods alternative positions close to Platyhelminthes or Gastrotricha were found and could not be rejected with certainty (Struck et al., 2014; Wey-Fabrizius et al., 2014; Witek et al., 2009). Hence, although phylogenomic data show some support in favor of the Gnathifera hypothesis over the Monokonta or Plathelminthomorpha hypotheses, further evidence is needed for the position of Gnathostomulida (Hankeln et al., 2014).

The data of complete or nearly complete mitochondrial genomes have been successfully used to provide additional evidence for the placement of problematic spiralian taxa like Myzostomidae or Diurodrilidae using both sequence and gene order data (e.g., Bleidorn et al., 2007; Golombek et al., 2013). Therefore, to address the uncertain phylogenetic affinity of Gnathostomulida with respect to Syndermata, Platyhelminthes and Gastrotricha, we determined the first mitochondrial genomes of Gnathostomulida and Gastrotricha. Several mitochondrial genomes of Platyhelminthes are publically available, but the majority of these genomes belong to the highly derived Neodermata and genomes of turbellarian flatworms are sparse. Two complete genomes of the triclad genus *Dugesia* and a partial one of the macrostomorphan *Microstomum* were determined. Polycladida are part of the basal radiation of Platyhelminthes (Egger and Rieger, 2013) and, thus, we also characterized the mitochondrial genome of the polyclad *Stylochoplana maculata*. For Syndermata mitochondrial genomes of the different subgroups are already available. In our analyses, the mitochondrial data strongly support the Gnathifera hypothesis even using different datasets and analytical strategies. A sistergroup relationship to either Platyhelminthes or Gastrotricha was not found in any of our analyses in contrast to the previous phylogenomic studies. In the light of these findings the morphological characters have been reevaluated.

2. Materials and methods

2.1. Material

Specimens of *Gnathostomula paradoxa* Ax, 1956 and *G. armata* Riedl, 1971 (Gnathostomulida, Bursovaginoidea, location: N 55°01.508′/E 008°26.180′) were collected near List, North Sea island Sylt, Germany. Samples of *Lepidodermella squamata* (Dujardin, 1841) (Gastrotricha, Chaetonotida) were obtained from Carolina Biological Supply Company (Burlington, NC, USA). Specimens of *Stylochoplana maculata* (Quatrefages, 1845) (Platyhelminthes, Polycladida, location: N 54°11.326′/E 007°52.220′) were collected from rock pools on the North Sea island Helgoland, Germany.

Animals were preserved in RNAlater (Sigma, Hamburg, Germany) or snap frozen in liquid nitrogen and then stored at −70 °C. Genomic DNA was extracted using the DNeasy Tissue Kit (QIAGEN, Hilden, Germany) according to manufacturer's instruction.

2.2. Determination of mitochondrial genomes

The determination of mitochondrial genomes followed the protocol of Golombek et al. (2013). In specific, to increase the amount of genomic DNA, the whole genomes of *G. paradoxa*, *G. armata* as well as *L. squamata* were amplified using the illustra GenomiPhi HY DNA Amplification Kit (GE Healthcare Life Science, Freiburg, Germany). The genomic DNA of *G. paradoxa*, *G. armata*, *L. squamata* and *S. maculata* was send to Genterprise Genomics (Mainz, Germany) for genomic DNA shot-gun library paired-end sequencing on an Illumina HiSeq2000 with TruSeq v3 chemistry. 22.8–30.4 million reads with average read length of 95–97 bp after quality trimming were obtained. These libraries were assembled into contigs with CLC Genomic workbench (CLC bio, Aarhus, Denmark) using default parameters with a fragment size window of 200–600 bp (the maximum peak of the fragmented library was at 400 bp) and the scaffolding option. Using protein sequence information of the 13 protein-coding mitochondrial genes of *Platynereis dumerilii* (AF178678) as query sequences in TBLASTN searches we searched the assembled contigs for matching fragments. One large fragment of the mitochondrial genome was found in all libraries. However, due to the scaffolding option there was a stretch of undetermined nucleotides in the fragment of *G. paradoxa*. Hence, we employed two strategies to complete the mitochondrial genomes using

Table 1
Species-specific primers used to fill gaps (stretch of N′s) within *G. paradoxa* (Gpara_in_F/Gpara_in_R) and to close the gaps between assembled contigs. The primers, which were successful in both amplification and sequencing, are indicated by an asterix.

Primer	Sequence (5′ to 3′)	Direction	T_m (°C)
For *Gnathostomula paradoxa*			
*Gpara_in_F	TGGCAGAGTAAGTGCATAATATTTAG	Forward	62.0
*Gpara_in_R	AGAAGATTCCAAAAATAACACTCAAAC	Reverse	61.0
Gpara_F	GGTGTATAAAGGAGGTACTAGTGGG	Forward	61.0
Gpara_R	ACGCCGATAAAGGAGGTAGAAG	Reverse	62.0
For *Gnathostomula armata*			
*Garma_F	TTTAATACAGAGAAAGACCAAGGATAC	Forward	62.0
*Garma_R	AGGAGGTAAAATTGAAGGAAAACAG	Reverse	61.0
For *Lepidodermella squamata*			
Lepido_F	CCGCTCACGCCTTTGTAATG	Forward	60.0
Lepido_Fin	CCTGACATGGCTTTTCCTCG	Forward	60.0
Lepido_R	CTGCTCTACTAACGGCAGCA	Reverse	60.0
Lepido_Rin	AAAGCTACAAGAAGGCCCCC	Reverse	60.0
For *Stylochoplana maculata*			
*Stylo_F	CCGCTACGGCCAAAAATACC	Forward	60.0
*Stylo_R	GAATGGCATTGGGTTGTAGCC	Reverse	61.0