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Mitogenomics at the base of Metazoa

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ABSTRACT

Unraveling the base of metazoan evolution is of crucial importance for rooting the metazoan Tree of Life. This subject has attracted substantial attention for more than a century and recently fueled a burst of modern phylogenetic studies. Conflicting scenarios from different studies and incongruent results from nuclear versus mitochondrial markers challenge current molecular phylogenetic approaches. Here we analyze the presently most comprehensive data sets of mitochondrial genomes from non-bilaterian animals to illuminate the phylogenetic relationships among basal metazoan phyla. The results of our analyses illustrate the value of mitogenomics and support previously known topologies between animal phyla but also identify several problematic taxa, which are sensitive to long branch artifacts or missing data.

1. Introduction

1.1. Mitochondrial genomes in metazoan outgroups

We will certainly never be able to decipher the nature of the very first animal mitochondrial (mt) genome, but we can deduce some information from single-celled organisms. As choanoflagellata are believed to be the closest related protists to metazoans (e.g. Ruiz-Trillo et al., 2008; Torruella et al., 2012) they are generally used as a reference for early metazoan mitochondria. So far only one choanoflagellate mitochondrial genome, that of *Monosiga brevicollis*, has been sequenced (Burger et al., 2003). With more than 76 kb the *Monosiga* mitochondrial genome is roughly 5 times larger than the average animal mitochondrial genome. Such large mitochondrial (mt) genomes are normal for unicellular opisthokonts, which harbor multiple genes that are absent in Metazoa. In particular mitochondrial ribosomal proteins are missing in all animal mt genomes that have been sequenced so far. A reduction in mitochondrial genome size and gene content in the Metazoa correlates with the emergence of a multicellular body plan.

1.2. Basal metazoan relationships

Phylogenetic relationships between the four diploblastic animal phyla, Placozoa, Porifera, Cnidaria and Ctenophora, as well as class-level relationships within these phyla have been controversially discussed for more than a century. It has been widely accepted that choanoflagellates represent the closest living unicellular relatives to metazoan animals (see King et al., 2008, and references therein) but the key questions of the earliest branching metazoan lineage and the sister group to Bilateria remain problematic.

Historically, sponges (Porifera) have been seen as the most ancestral metazoan lineage. This view is based on structural similarities between sponge choanocytes and choanoflagellates and also supported by several molecular studies (e.g. Philippe et al., 2009; Pick et al., 2010). In contrast to this traditional view several alternative hypotheses on the origin of Metazoa have been discussed. The most often cited alternative scenario sees placozoans as a link between Protozoa and extant basal metazoans. A view that has traditionally been based on the morphological simplicity of placozoans, which lack an extracellular matrix, a basal lamina, any kind of defined body axis and which harbor five somatic cell types only (see Schierwater 2005, for details). This view has recently found support from a broad spectrum of molecular analyses, many of which support a sister group relationship between Bilateria and non-bilaterian animals with Placozoa as the earliest branching phylum within the diploblasts (non-bilaterians) (e.g. Dellaporta et al., 2006; Signorovitch et al., 2007; Schierwater et al., 2009a,b). However, all molecular sequence based tree topologies addressing the root of the Metazoa show problematic and weekly supported branches and are targets for critical discussions (Philippe et al., 2011; Salichos and Rokas, 2013). The rapid progress in the optimization of phylogenetic algorithms will reduce known artifacts in phylogenetic analyses

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but another problem, that of pitted data matrices (which sometimes have more holes than data) can only be overcome by gathering additional and more complete data sets.

One way to obtain a comparable and complete data set for phylogenetic analyses is to sequence small genomes with a stable set of coding genes, such as mitochondrial genomes. Recent studies trying to clarify relationships at the base of the metazoan Tree of Life using mitochondrial protein coding genes suffer from limited or no data from one or more of the key taxa. Existing analyses are also highly variable with respect to the choice of outgroups, the evolutionary model and the tree building algorithm applied. Incongruent results are no surprise and some unexpected tree topologies (e.g. the sister group relationship of Hexactinellida and Bilateria, Haen et al., 2007) seem to be typical examples for long branch artifacts. The highly contradictory results (for overview see e.g. Schierwater et al., 2010; Nosenko et al., 2013) clearly demonstrate the sensitivity of current approaches to insufficient taxon sampling, pitted data matrices and possibly inappropriate substitution models. By overcoming these issues, comparative mitogenomics promise to improve current phylogenies and to lead to a better understanding of evolutionary processes in mitochondrial genomes of early branching metazoan taxa.

1.3. Mitochondrial genomes in non-bilaterian animals

The steadily increasing number of complete mitochondrial genome data from non-bilaterian animals has revealed an unexpected heterogeneity of mitochondrial genome architecture, gene content and sequence evolution rates, compared to the relatively conserved mitochondrial genomes of bilaterians. Currently, 151 complete or nearly complete mitochondrial genomes have been sequenced from basal metazoans (Genbank as of May 2013, Benson et al., 2013; Fig. 1 and Table 1). Sponges (Phylum Porifera, 52 genomes) and Cnidaria (92 genomes) are the best-sampled non-bilaterian phyla so far (although in case of sponges complete sequence data for Hexactinellida and Calcarea are rare). Poorly studied are the Placozoa (5 genomes) and the Ctenophora (2 genomes).

The size of known circular non-bilaterian mitochondrial genomes varies between ~10 (Ctenophora) and more than 43 kb (Placozoa). The 'normal' diploblast mitochondrial genome is (like in Bilateria) a circular molecule but linear genomes (or even fragmented linear genomes) have been described for Porifera (Calcarea) and Cnidaria (Hydrozoa, Scyphozoa, Staurozoa and Cubozoa) (Bridge et al., 1992; Ender and Schierwater, 2003; Shao et al., 2006; Kayal and Lavrov, 2008; Voigt et al., 2008; Kayal et al., 2012; Zou et al., 2012; Lavrov et al., 2013). Introns are found in *cox1* and *nad5* in Placozoa, Porifera and Cnidaria (in placozoans introns are also found in the 16S rRNA) but seem to be absent in the Ctenophora. The sequence evolution rates of mitochondrial protein coding genes in Placozoa and Octocorallia (phylum Cnidaria, class Anthozoa) are relatively low compared to the accelerated sequence evolution rates observed in Hexactinellida and Calcarea (phylum Porifera) and Ctenophora (Shearer et al., 2002; Dellaporta et al., 2006; Hellberg, 2006; Haen et al., 2007; Signorovitch et al., 2007; Pett et al., 2011; Lavrov et al., 2013). The relatively slow-evolving mt genomes of the Placozoa and Octocorallia display a higher tendency for mt genome rearrangements (Signorovitch et al., 2007; Uda et al., 2011; Miyazawa et al., 2012).

The number of tRNAs in mitochondrial genomes varies largely between different diploblast phyla. A constant number of mitochondrial tRNA genes is seen in Placozoa ($N = 24$) whereas sponges encode a variable number of tRNA genes (ranging from 2 to 27). Cnidaria generally encode mostly only one or two tRNAs in their mitochondrial genome (*trnM* and *trnW*; a duplicated *trnW* was found e.g. in the genus *Seriatopora* (Chen et al., 2008b)). The ctenophore *Mnemiopsis leidyi* has lost all mitochondrial encoded tRNA genes (Pett et al., 2011). This loss of tRNA genes results in the recruitment of cytosolic tRNAs for mitochondrial translation processes. The second known ctenophore mt genome, that of *Pleurobrachia bachei*, has maintained two tRNAs. In sum, the highly reduced number of mitochondrial encoded tRNA genes seems to be a shared feature of Cnidaria and Ctenophora (the "coelenterate" clade). The presence of a control region as an initiation site for replication and/or transcription has been suggested for some cnidarian and poriferan representatives (e.g. van Oppen et al., 2002; Erpenbeck et al., 2007; Flot and Tillier, 2007; Brugler and France, 2008; Chen et al., 2008a,b; Rosengarten et al., 2008).

Comparing metazoan and protozoan mt genomes highlights some obvious phylogenetic trends. First, a general tendency within Metazoa is to reduce mitochondrial genome size and to lose non-

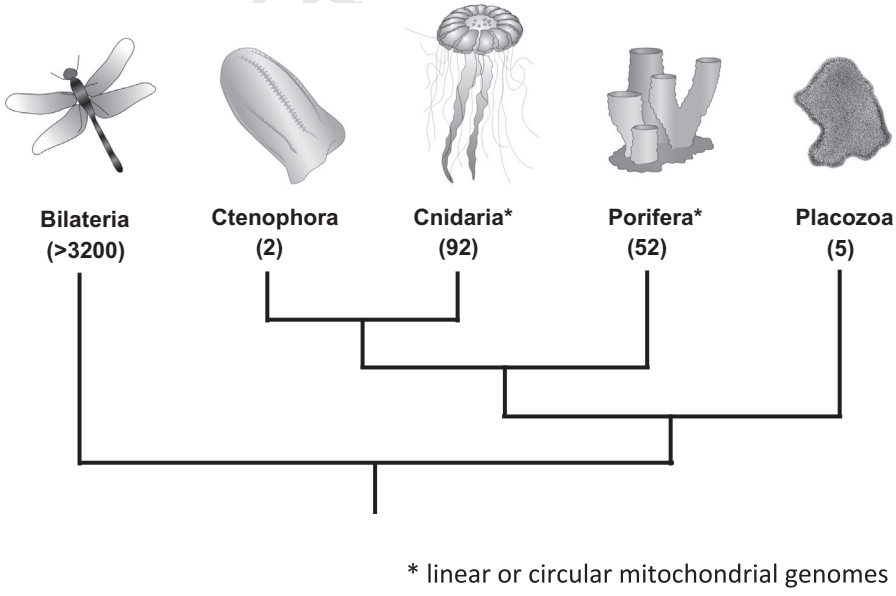

Fig. 1. Available mitochondrial genome data for Bilateria and the four diploblastic metazoan phyla. Numbers of completely sequenced mitochondrial genomes are given. A more detailed list of non-bilaterian mitochondrial genomes and properties is given in Table 1. The shown phylogenetic relationships follow molecular studies by Schierwater et al. (2009a,b). (Modified symbols of Porifera, Cnidaria, Ctenophora and Bilateria are courtesy of the Integration and Application Network (ian.umces.edu/symbols/).

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Table 1
Properties of available complete or almost complete mitochondrial genomes from Placozoa, Porifera, Cnidaria and Ctenophora.

		Accession	Genome architecture	Length	tRNA	Introns	ORFs
Placozoa							
1	Trichoplax adhaerens	NC_008151	circular	43,079	24	1 (nad5),7 (cox1),1 (16S)	8
2	Placozoan sp. 'Shirahama'	NC_015309	circular	36,676	24	7 (cox1), 1 (nad5)	3
3	Placozoan sp. BZ10101	NC_008832	circular	32,661	24	1 (nad5), 6 (cox1), 2 (16S)	2
4	Placozoan sp. BZ2423	NC_008834	circular	36,699	24	7 (cox1), 1 (16S)	2
5	Placozoan sp. BZ49	NC_008833	circular	37,194	24	7 (cox1), 1 (nad5)	3
Porifera							
Demospongiae							
1	Agelas schmidti	NC_010213	circular	20,360	24	0	0
2	Amphimedon compressa	NC_010201	circular	18,564	24	0	0
3	Amphimedon queenslandica	NC_008944	circular	19,960	17	0	0
4	Aplysina cauliformis	NC_016949	circular	19,620	25	0	0
5	Aplysina fulva	NC_010203	circular	19,620	25	0	0
6	Axinella corrugata	NC_006894	circular	25,610	25	0	0
7	Baikalospongia intermedia profundalis	NC_018343	circular	28,227	25	0	0
8	Callyspongia plicifera	NC_010206	circular	18,846	24	0	0
9	Chondrilla aff. nucula CHOND	NC_010208	circular	19,282	25	0	0
10	Cinachyrella kuekenthali	NC_010198	circular	18,089	24	0	0
11	Corvomeyenia sp. DVL-2012 (partial)	JQ302311	circular	23,958	25	0	0
12	Ectyoplasia ferox	NC_010210	circular	18,312	25	0	0
13	Ephydatia fluviatilis (partial)	JN209966	circular	26,441	25	0	0
14	Ephydatia muelleri	NC_010202	circular	23,929	25	0	0
15	Eunapius subterraneus	NC_016431	circular	24,580	25	0	0
16	Geodia neptuni	NC_006990	circular	18,020	24	0	0
17	Halisarca dujardini	NC_010212	circular	19,277	25	0	0
18	Halisarca sp. DVL-2010	NC_014876	circular	20,591	25	0	0
19	Hippospongia lachne	NC_010215	circular	16,755	2	0	0
20	Igernella notabilis	NC_010216	circular	20,310	2	0	0
21	Iotrochota birotulata	NC_010207	circular	19,112	24	0	0
22	Ircinia strobilina	NC_013662	circular	16,414	2	0	0
23	Lubomirskia baicalensis	NC_013760	circular	28,958	25	0	0
24	Negombata magnifica	NC_010171	circular	20,088	25	0	0
25	Ptilocaulis walpersi	NC_010209	circular	18,865	25	0	0
26	Rezinkovia echinata	NC_018360	circular	28,614	25	0	0
27	Suberites domuncula	NC_010496	circular	26,300	25	0	0
28	Swartschewskia papyracea (partial)	JQ302308	circular	26,518	25	0	0
29	Tethya actinia	NC_006991	circular	19,565	25	0	0
30	Topsentia ophiraphidites	NC_010204	circular	19,763	25	0	0
31	Vaceletia sp. GW948	NC_010218	circular	20,658	2	0	0
32	Xestospongia muta	NC_010211	circular	18,990	25	0	0
Calcarea							
1	Clathrina clathrus	NC_021112- NC_021118	linear	50,020	24	0	0
Hexactinellida							
1	Aphrocallistes vastus	NC_010769	circular	17,427	21	0	0
2	Iphiteon panicea (partial)	EF537576	circular	19,046	22	0	2
3	Sympagella nux (partial)	EF537577	circular	16,293	20	0	0
Homoscleromorpha							
1	Corticium candelabrum	NC_014872	circular	18,402	5	0	0
2	Oscarella carmela	NC_009090	circular	20,327	27	0	1
3	Oscarella lobularis	NC_014863	circular	20,260	27	0	1
4	Oscarella malakhovi	NC_014886	circular	20,332	26	0	1
5	Oscarella microlobata	NC_014850	circular	20,368	27	0	1
6	Oscarella tuberculata	NC_014888	circular	20,262	27	0	1
7	Oscarella viridis	NC_014856	circular	20,440	27	0	1
8	Plakina crypta	NC_014885	circular	20,069	5	1 (cox1)	0
9	Plakina jani	NC_014860	circular	18,907	5	0	0
10	Plakina monolopha	NC_014884	circular	19,371	5	0	0
11	Plakina sp. DVL-2010	NC_014892	circular	18,997	5	0	0
12	Plakina trilopha	NC_014852	circular	20,427	3	1 (cox1)	0
13	Plakinastrella cf. onkodes DVL-2011	NC_010217	circular	19,790	6	2 (cox1)	1
14	Plakortis halichondrioides	NC_014857	circular	18,385	5	0	0
15	Plakortis simplex	NC_014868	circular	18,551	5	0	0
16	Pseudocorticium jarrei	NC_014853	circular	20,472	27	0	1
Cnidaria							
Anthozoa							
Hexacorallia							
1	Acropora tenuis	NC_003522	circular	18,338	2	1 (nad5)	0
2	Agaricia humilis	NC_008160	circular	18,735	2	0	0
3	Anacropora matthai	NC_006898	circular	17,888	2	1 (nad5)	0

(*continued on next page*)

Table 1 (*continued*)

		Accession	Genome architecture	Length	tRNA	Introns	ORFs
4	Astrangia sp. JVK-2006	NC_008161	circular	14,853	2	0	0
5	Chrysopathes formosa	NC_008411	circular	18,398	2	1 (nad5)	0
6	Colpophyllia natans	NC_008162	circular	16,906	2	0	0
7	Discosoma sp. CASIZ 168915	NC_008071	circular	20,908	2	1 (cox1)	0
8	Discosoma sp. CASIZ 168916	NC_008072	circular	20,912	2	1 (cox1)	0
9	Euphyllia ancora	NC_015641	circular	18,875	2	0	0
10	Fungiacyathus stephanus	NC_015640	circular	19,381	2	0	0
11	Goniopora columna	NC_015643	circular	18,766	2	1 (nad5)	0
12	Lophelia pertusa	NC_015143	circular	16,150	2	1 (nad5)	0
13	Madracis mirabilis	NC_011160	circular	16,951	2	1 (nad5)	0
14	Madrepora oculata	NC_018364	circular	15,841	2	0	0
15	Metridium senile	NC_000933	circular	17,443	2	1 (cox1), 1 (nad5)	1
16	Montastraea annularis	NC_007224	circular	16,138	2	1 (nad5)	0
17	Montastraea faveolata	NC_007226	circular	16,138	2	1 (nad5)	0
18	Montastraea franksi	NC_007225	circular	16,137	2	1 (nad5)	0
19	Montipora cactus	NC_006902	circular	17,887	2	1 (nad5)	0
20	Mussa angulosa	NC_008163	circular	17,245	2	0	0
21	Nematostella sp. JVK-2006	NC_008164	circular	16,389	2	0	0
22	Pavona clavus	NC_008165	circular	18,315	2	0	0
23	Platygyra carnosus	JX911333	circular	16,463	2	0	0
24	Pocillopora damicornis	NC_009797	circular	17,415	2	1 (nad5)	0
25	Pocillopora eydouxi	NC_009798	circular	17,422	2	1 (nad5)	0
26	Polycyathus sp. MFL-2011	NC_015642	circular	15,357	1	0	0
27	Porites okinawensis	NC_015644	circular	18,647	1	1 (cox1)	0
28	Porites porites	NC_008166	circular	18,648	2	0	0
29	Rhodactis sp. CASIZ 171755	NC_008158	circular	20,093	2	1 (cox1)	0
30	Ricordea florida	NC_008159	circular	21,376	2	1 (cox1)	0
31	Savalia savaglia	NC_008827	circular	20,764	1	0	1
32	Seriatopora caliendrum	NC_010245	circular	17,010	3	1 (nad5)	0
33	Seriatopora hystrix	NC_010244	circular	17,059	3	1 (nad5)	0
34	Siderastrea radians	NC_008167	circular	19,387	2	0	0
35	Stichopathes lutkeni	NC_018377	circular	20,448	2	0	1
36	Stylophora pistillata	NC_011162	circular	17,177	3	1 (nad5)	0
Octocorallia							
1	Acanella eburnea	NC_011016	circular	18,616	1	0	1
2	Briareum asbestinum	NC_008073	circular	18,632	1	0	1
3	Calicogorgia granulosa	GU047880	circular	20,246	1	0	1
4	Corallium konojoi	NC_015406	circular	18,969	1	0	1
5	Dendronephthya castanea	GU047877	circular	18,907	1	0	1
6	Dendronephthya gigantea	NC_013573	circular	18,842	1	0	1
7	Dendronephthya mollis	HQ694725	circular	18,844	1	0	1
8	Dendronephthya putteri	HQ694726	circular	18,853	1	0	1
9	Dendronephthya suensoni	GU047878	circular	18,885	1	0	1
10	Echinogorgia complexa	HQ694727	circular	19,445	1	0	1
11	Euplexaura crassa	HQ694728	circular	18,674	1	0	1
12	Heliopora coerulea	KC521415	circular	18,957	1	0	1
13	Keratoisidinae sp. BAL208-1	NC_010764	circular	18,923	1	0	1
14	Paracorallium japonicum	NC_015405	circular	18,913	1	0	1
15	Paraminabea aldersladei	NC_018790	circular	19,886	1	0	1
16	Pseudopterogorgia bipinnata	NC_008157	circular	18,733	1	0	1
17	Renilla muelleri	NC_018378	circular	18,643	1	0	1
18	Sarcophyton glaucum (partial)	AF063191/AF064823	circular	18,453	1	0	1
19	Scleronephthya gracillimum	GU047879	circular	18,950	1	0	1
20	Sinularia peculiaris	NC_018379	circular	18,742	1	0	1
21	Stylatula elongata	NC_018380	circular	18,733	1	0	1
Staurozoa							
1	Craterolophus convolvulus (partial)	JN700975/JN700976	linear	12,853	1	0	0
2	Haliclystus sanjuanensis (partial)	JN700944	linear	16,472	2	0	2
3	Lucernaria janetae (partial)	JN700946	linear	14,692	2	0	1
Hydrozoa							
1	Clava multicornis	NC_016465	linear	16,898	2	0	0
2	Craspedacusta sowerbyi	NC_018537	linear	17,922	2	0	2
3	Cubaia aphrodite	NC_016467	linear	16,148	2	0	2
4	Ectopleura larynx (partial)	JN700938	linear	13,663	1	0	0
5	Hydra magnipapillata	NC_011220/NC_011221	linear	15,880	3	0	0
6	Hydra oligactis	NC_010214	linear	16,314	2	0	0
7	Hydra sinensis	JX089978	linear	16,189	2	0	0
8	Hydra vulgaris	HM369413/HM369414	linear	15,586	3	0	0
9	Laomedea flexuosa	NC_016463	linear	16,075	2	0	0
10	Millepora sp. EK-2011 (partial)	JN700943	linear	14,020	2	0	0
11	Nemopsis bachei (partial)	JN700947	linear	14,431	2	0	0
12	Obelia longissima (partial)	JN700948	linear	15,194	2	0	0
13	Pennaria disticha (partial)	JN700950	linear	14,051	2	0	0

Table 1 (*continued*)

		Accession	Genome architecture	Length	tRNA	Introns	ORFs
Scyphozoa							
1	Aurelia aurita	HQ694729	linear	16,971	2	0	2
2	Aurelia aurita	NC_008446	linear	16,937	2	0	2
3	Cassiopea andromeda (partial)	JN700934	linear	15,800	2	0	2
4	Cassiopea frondosa	NC_016466	linear	15,949	2	0	2
5	Catostylus mosaicus (partial)	JN700940	linear	14,847	2	0	1
6	Chrysaora sp. EK-2011 (partial)	JN700941	linear	14,415	2	0	0
7	Chrysaora quinquecirrha	HQ694730	linear	16,775	2	0	2
8	Cyanea capillata (partial)	JN700937	linear	16,202	2	0	2
9	Linuche unguiculata (partial)	JN700939	linear	12,129	1	0	0
10	Pelagia noctiluca (partial)	JN700949	linear	15,876	2	0	2
11	Periphylla periphylla (partial)	JN700986	linear	6815	0	0	0
12	Rhizostoma pulmo (partial)	JN700987/JN700988	linear	6400	2	0	0
Cubozoa							
1_2	Alatina moseri A/B	JN642329-JN642344	linear	28,351/28,385	3	0	2
3	Alatina moseri (partial)	JN700951-JN700958	linear	12,989	0	0	2
4	Carukia barnesi (partial)	JN700959-JN700962	linear	3370	0	0	0
5	Carybdea xaymacana (partial)	JN700977-JN700983	linear	7466	0	0	0
6	Chironex fleckeri (partial)	JN700963-JN700968	linear	6688	0	0	0
7	Chiropsalmus quadrumanus (partial)	JN700969-JN700974	linear	11,942	0	0	0
Ctenophora							
Tentaculata							
1	Mnemiopsis leidyi	NC_016117	circular	10,326	0	0	0
2	Pleurobrachia bachei	JN392469	circular	11,016	2	0	0

coding or intergenic regions. The large mitochondrial genomes of some bilaterians, e.g. molluscs (up to 40 kb) are the result of secondary extensions by repeated duplications (for details see Smith and Snyder, 2007; Stoger and Schrodl, 2012, this special issue). In diploblasts the extended overall mt genome size of more than 50 kb observed in a calcareous sponge (Lavrov et al., 2013) is probably linked to the fragmentation of the originally circular mitochondrial genome in several linear chromosomes. A second trend is the reduction of tRNA genes. While mitochondrial genomes from Cnidaria, Ctenophora and some Porifera have reduced the set of mitochondria encoded tRNA genes, the mitochondrial genomes of Placozoa harbor a complete set of tRNA genes, which is regarded as the ancestral state in Metazoa. In sharp contrast bilaterian mitochondrial genomes harbor a highly conserved set of tRNA genes indicating different evolutionary routes for these genes in Bilateria and non-bilaterians. This observation may be explained by an early split of both groups (cf. Schierwater et al., 2009a,b). A third trend is the loss of introns. Shared *cox1* and *nad5* introns are still found in protists, Placozoa, Porifera and Cnidaria. The presence of large intergenic regions and additional open-reading frames also support a close relationship of the diploblasts to a protozoan ancestor.

1.3.1. Placozoa

Several recent molecular studies suggest a high diversity within the phylum Placozoa. Currently 19 placozoan lineages (haplotypes) organized in seven distinct clades are known (Eitel et al., 2013). Based on phylogenetic studies, the only described placozoan species, *Trichoplax adhaerens* (clade I, H1), groups with H2 and H17 (clade I) and H3 (clade II) to form a monophyletic clade named 'group B'. The remaining lineages form 'group A'. The latter is subdivided into the subgroups A1 (containing four lineages in clade III) and A2 (clades IV–VII). No taxonomic system has yet been established for the Placozoa and the non-taxonomic terms 'group' and 'subgroup' only define higher taxonomic units.

With a size of ~43 kb the *Trichoplax adhaerens* mitochondrial genome is the largest so far known circular metazoan mt genome and this way links the choanoflagellate *Monosiga brevicollis* (~76 kb) (Burger et al., 2003) and the Baikal sponge *Lubomirskia baicalensis* (~29 kb; Lavrov, 2010b). The circular genome encodes a typical set of mitochondrial genes (*nad1-6*, *nad4L*, *cox1-3*, *cob*, *atp6*, *rnS and rnL*) and a full complement of 24 tRNAs (*atp8* and *atp9* are missing). In addition to large non-coding regions, it contains eight open reading frames and several group I and group II introns – very atypical features in animal mitochondria. A group I intron is found in *nad5* and a group II intron in *rnLb* (16S part b), which is split into two exons. The *cox1* gene is unusually organized in three separate segments consisting of eight exons and additional seven introns (both group I and II) encoded on both strands.

Apart from *Trichoplax adhaerens* (H1), the complete mitochondrial genomes from the placozoan haplotypes H3, H4, H8 and H15 have been sequenced (Signorovitch et al., 2007; Miyazawa et al., 2012). With sizes of ~32.6 kb (H8), ~36.6 kb (H3, H15) and ~37.2 kb (H4) these genomes are smaller than the *Trichoplax adhaerens* mt genome but still larger than all other not secondarily enlarged circular metazoan mt genomes. The overall genome structures show some differences in comparison to *Trichoplax adhaerens* e.g. in the number of open reading frames and introns (Dellaporta et al., 2006; Signorovitch et al., 2007; Burger et al., 2009; Miyazawa et al., 2012). Gene arrangements also differ between the different lineages by some remarkable features. A comparison of the mt genomes within 'group B' (H1 and H3) shows a transversion and transposition of a genome fragment that includes *nad1* and the tRNA for Valine. The *nad5* intron of *Trichoplax adhaerens* is absent in H3. Differences in the genome structure are even more pronounced between groups A and B. Comparing H8 and *Trichoplax adhaerens* mt genomes, an inversion of a ~22 kb mt genome fragment is seen as well as a reorganization of the *cox1* gene in only seven exons (implicating a loss of one *cox1* intron) and a gain of an additional *rnLb* intron. A reorganization of the *cox1* exon/intron structure is also observed in the mt genome of H4. Interestingly this placozoan representative lacks any *rnLb* introns. Even the closely related placozoan lineages H4 and H15 display different gene arrangement due to the translocation of a small genome fragment containing ORF126 and *polB*.

1.3.2. Porifera

The phylum Porifera comprises the three traditional classes Calcarea, Demospongiae and Hexactinellida and the recently sug-

gested fourth class, the Homoscleromorpha; (Gazave et al., 2010, 2012; Van Soest et al., 2012; for overview, see Worheide et al., 2012). Only one complete mitochondrial genome has yet been sequenced from a calcareous sponge, while 16 and 32 complete sequences have been published for homoscleromorphs and demosponges, respectively. Only one out of three published mt genomes from a hexactinellid sponge is complete (Haen et al., 2007; Rosengarten et al., 2008).

1.3.2.1. Homoscleromorpha. The phylogenetic position and the taxonomic rank of Homoscleromorpha within the Porifera is currently under debate (Cardenas et al., 2012; Boury-Esnault et al., 2013). Following recent molecular studies the Homoscleromorpha (termed as 'G0' in Lavrov et al., 2008) form a sister group to all other sponges (e.g. Lavrov et al., 2008; Wang and Lavrov, 2008; Burger et al., 2009). Based on the mitochondrial genome data the Homoscleromorpha can be subdivided into two distinct families: Plakinidae (Schulze, 1880) and Oscarellidae (Lendenfeld, 1887). The size of completely sequenced mt genomes ranges from 18.3 to 20.4 kb. While members of Plakinidae encode genes on one strand only, the members of Oscarellidae encode genes on both strands. The Plakinidae have a reduced set of mostly 5 tRNA genes and some representatives have introns in *cox1*. On the other hand members of the Oscarellidae have an extended set of up to 27 tRNA (with little inter-specific variation) and encode for the *tatC* gene (twin arginine translocase subunit C), which is absent in the Plakinidae (Gazave et al., 2010).

1.3.2.2. Demospongiae. The diverse group of demosponges, which comprises the majority of poriferan species (about 7000 accepted species, WoRMS, Appeltans et al., 2012), can be subdivided into four distinct subgroups based on traditional and recent molecular studies: the Keratosa (G1), the Myxospongiae (G2), marine Haplosclerida (G3) and the remaining demosponge taxa (G4) (Borchiellini et al., 2004; Lavrov et al., 2008; Wang and Lavrov, 2008; but see also Hill et al., 2013 and references therein). All demosponge mitochondrial genomes are circular and their size ranges from 16.4 to nearly 29 kb, therefore almost reaching the size of the smallest placozoan mt genomes (~32 kb). With the exception of one mt genome (see below) all mitochondrial genomes in demosponges encode 14 protein coding genes (including *atp9*, which has not been found in other meatzoan mt genomes as yet). The Keratosa (G1) encode only a reduced set of two tRNAs (in parallel evolution to Cnidaria) whereas all other demosponges encode 24–25 tRNAs. An exception is the mitochondrial genome of *Amphimedon queenslandica*, which encodes only 13 protein coding genes (no *atp9*) and 17 tRNA genes. With the exception of the genus *Aplysina* (Lavrov et al., 2008), all genes in demosponge mitochondrial genomes have an identical transcriptional orientation. Small open reading frames are at least found in *Eunapius subterraneus* (Plese et al., 2012).

1.3.2.3. Hexactinellida. The mitochondrial genome of *Aphrocallistes vastus* is so far the only completely sequenced mt genome from the Hexactinellida (Rosengarten et al., 2008). Partial mitochondrial genome data have been published for *Iphiteon panicea* and *Sympagella nux* (Haen et al., 2007). The mitochondrial genome of *Aphrocallistes vastus* has a size of 17.4 kb and encodes 13 respiratory chain subunits (including *atp9*), 2 ribosomal RNA genes, 21 tRNAs (see Lavrov, 2010a), an open reading frame of unknown function and a putative control region. All genes are encoded on one strand and several genes slightly overlap. The partially sequenced mt genomes from *Iphiteon panicea* and *Sympagella nux* have a minimum size of 19 kb and 16.3 kb, respectively. They encode for 13 and 12 respiratory chain subunits, including *atp9* but *atp8* (and *nad6* in case of *S. nux*) is missing. Two rRNA as well as 22 and 20 tRNA genes, respectively, have been identified in both genomes. Furthermore, two open-reading frames have been found in *Iphiteon panicea*. The Hexactinellida share several features with bilaterian mt genomes e.g. a derived tRNA structure and a highly accelerated sequence evolution rate (Haen et al., 2007). Although Hexactinellida group in phylogenetic analyses as a sister group to Bilateria, the shared mitochondrial characteristics seem to be a typical example of parallel evolution (Lavrov, 2010a).

1.3.2.4. Calcarea. The first complete mitochondrial genome from a calcareous sponge has recently been published in a study by Lavrov et al., 2013. Whereas all previously described poriferan mitochondrial genomes possess a circular mt genome architecture, the mitochondrial genome of the calcareous sponge *Clathrina clathrus* consists of six linear chromosomes with a size between 7.6 and 9.4 kb each. Therefore the mt genome of *C. clathrus* is the largest known non-circular animal mitochondrial genome. It encodes 13 protein coding genes, two rRNA genes and 24 tRNAs. Beside the fragmented genome architecture several remarkable features have been described: (i) a modified genetic code, (ii) posttranscriptional tRNA editing and (iii) fragmented ribosomal RNAs. Like Hexactinellida, calcareous sponge mitochondrial proteins seem to have undergone an accelerated sequence evolution rate. Enlarging and fragmentation of mitochondrial genomes has been shown for derived protists (e.g. Burger et al., 2003) and cnidarian lineages (see below, e.g. Smith et al., 2012). Similar mechanisms might be acting in the Calcarea.

1.3.3. Cnidaria

The phylum Cnidaria consists of five different classes: Anthozoa, Hydrozoa, Scyphozoa, Staurozoa and Cubozoa (following Marques and Collins, 2004). The largest number of complete (or almost complete) mt genomes is available from Anthozoa (57). Recent sequencing efforts added mt genome data from Hydrozoa (13 genomes), Scyphozoa (12 genomes), Staurozoa (three genomes) and Cubozoa (seven genomes). Recent studies on cnidarian phylogeny based on mitochondrial protein sequence data led to contradictory results with respect to the monophyly of Anthozoa and relationships within the Medusozoa (Zou et al., 2012; Kayal et al., 2013 and references therein).

1.3.3.1. Anthozoa. The class Anthozoa includes two sub-classes, the Hexacorallia and the Octocorallia, with 36 complete or partial mt genomes available from hexacorallian species. The known circular molecules of Hexacorallia have a size between 14.8 and 21.3 kb and harbor substantial intergenic regions. With only a few exceptions (i.e. a homing endonuclease found for example in several species; (e.g. Beagley et al., 1998; Sinniger et al., 2007)) they encode for a conserved set of 13 protein coding genes with group I introns found in *nad5* and *cox1* (see e.g. Emblem et al., 2011). In the hexacorallians usually two tRNAs are encoded and duplication or a loss of *trnW* have been reported (e.g. Chen et al., 2008a,b; Flot et al., 2008; Lin et al., 2011).

The mt genomes of Octocorallia most often show a size around 19 kb. In *Echinogorgia complexa* and *Calicogorgia granulosa* extended intergenic regions (and in case of *C. granulosa* also a duplicated additional ORF) lead to a slightly increased mitogenome size (20.2 kb in *C. granulosa*) (Park et al., 2011, 2012). With the exception of *C. granulosa*, known octocorallian mt genomes encode 14 protein coding genes and only one tRNA (*trnM*). Sequence evolution in Octocorallia seems to be slower than in Hexacorallia, which can mislead phylogenetic analyses (Shearer et al., 2002; Hellberg, 2006). The reduced sequence evolution is attributed to the *MutS* gene that is found uniquely in Octocorallia. *MutS* acts as a DNA mismatch repair protein (see e.g. Bilewitch and Degnan, 2011; Brockman and McFadden, 2012).

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1.3.3.2. Scyphozoa. In contrast to the ancestral circular chromosomes in Anthozoa all mitochondrial genomes of the derived Scyphozoa are linear molecules. With a size of 16.9 kb the *Aurelia aurita* mitochondrial genome has been the first completely characterized genome from this class. It encodes 15 protein coding genes (including two open reading frames) and two tRNAs (Shao et al., 2006). All genes except *cox1*, *rnL*, *ORF1 and polB* are transcribed in the same direction. The linear chromosome is terminated by inverted terminal repeats (ITR; resembling telomeres) – a shared feature among linearized mitochondrial molecules (see e.g. Burger et al., 2003; Shao et al., 2006; Kayal and Lavrov, 2008). In a study by Park and co-workers (2012) the gene arrangement of *Aurelia aurita* was confirmed by a sequence from a second *A. aurita* specimen, although the sequence divergence indicates the existence of cryptic species (cf. Schroth et al., 2002). An identical gene arrangement has also been found in *Chrysaora quinquecirrha* and seven additional discomedusan taxa characterized by Kayal and co-workers (2012) suggest a conserved gene order within the Discomedusae.

A slightly different gene arrangement has been described for the partial mt genome of *Linuche unguiculata* (Order Coronatae) (Kayal et al., 2012). While the *trnW* is located between *cox2* and *atp8* in all discomedusan Scyphozoa, it is absent in *Linuche unguiculata* (the respective region is missing in the incomplete mt genome of *Periphylla periphylla*, a second specimen of Coronatae (Kayal et al., 2012)). However, due to incomplete sequences the absence of this tRNA from both mt genomes remains uncertain.

1.3.3.3. Staurozoa. The Staurozoa has recently been erected as the fifth class within the Cnidaria (Marques and Collins, 2004) and so far three partial staurozoan mitochondrial genome sequences have been published (Kayal et al., 2012). Although these are incomplete, they still provide important insights into mt genome evolution in the Cnidaria. The observed gene arrangement is identical to that observed in Scyphozoa and therefore fits to the hypothetical ancestral medusozoan gene order (termed 'AMGO' in Kayal et al., 2012). Only few of the previously published phylogenetic analyses based on mitochondrial proteins included data from the Staurozoa (e.g. Zou et al., 2012; Kayal et al., 2013). We therefore included the new staurozoan genomes in our study to further illuminate their phylogenetic position within the Cnidaria.

1.3.3.4. Cubozoa. The first complete cubozoan mitochondrial genome of *Alatina moseri* has been published by Smith and co-workers (2012), while previous studies have used southern blot analyses to detect the fragmented mt genome architecture in *Tripedalia cystophora* and *Carybdea marsupialis* (Ender and Schierwater, 2003). The complete characterization of the *Alatina moseri* mt genome reveals a fragmentation of the mitochondrial genome in eight linear chromosomes ranging between 2.9 and 4.6 kb in size. As in Scyphozoa (and Hydrozoa; see below) all chromosomes are terminated on both ends by inverted repeats. The mt genome encodes 15 protein coding genes (including *atp8*, *polB* and ORF 314), two rRNA genes and three copies of *trnM*. Several pseudogenes (especially fragments of *rnL*) are located on different chromosomes and all chromosomes have the same transcriptional polarity. The *Alatina moseri* mt genome has also been analyzed in a study by Kayal and co-workers (2012). In this study, however, the authors failed to identify any tRNA genes in the only partially sequenced *Alatina* chromosomes as well as in partial mt genomes of four other cubozoan species. Altogether, the mitochondrial genomes of Cubozoa display highly derived features especially with respect to the observed fragmentation.

1.3.3.5. Hydrozoa. Like in all Medusozoa, linear mt genome architecture is found in Hydrozoa. With the exception of several members of the family Hydridae (including *Hydra magnipapillata*, which possess split mt genomes in two linear chromosomes (Voigt et al., 2008)) all known completely sequenced hydrozoan mt genomes are single linear molecules with a size ranging from 16 to 17.9 kb (Kayal and Lavrov, 2008; Kayal et al., 2012). Within the Hydrozoa, *Cubaia aphrodite* and *Craspedacusta sowerbyi* (Trachylina) display the identical gene content and order as the Discomedusae (Scyphozoa), probably representing the ancestral medusozoan state (Kayal et al., 2012; Zou et al., 2012). Known mitochondrial genomes from other hydrozoan taxa are missing the otherwise encoded *polB* and *orf1* genes and display slight modifications with respect to the gene order and/or direction. Inverted terminal repeats are found at all hydrozoan mt chromosome ends. In contrast to other classes, however, the ITRs are here flanked by either a functional *cox1* gene or by a duplicated *cox1* pseudogene. The only known exceptions again are *Cubaia* and *Craspedacusta*. The ITR regions present in all Medusozoa likely play an important role in chromosome replication in this group (Kayal et al., 2012).

1.3.4. Ctenophora

For a long time all efforts to characterize ctenophore mt genomes via PCR have failed. Only with next generation sequencing techniques the mt genomes of two ctenophore species could be completed (Pett et al., 2011; Kohn et al., 2012). The circular mitochondrial genome of *Mnemiopsis leidyi* displays several remarkable features highlighting the derived status of this mt genome and explaining previous difficulties in PCR amplification using standard primer sets. With a size of 10.3 kb it is the smallest animal mt genome known to date. The *atp6* gene has been transferred to the nucleus, all mitochondrial encoded tRNA genes have been lost, the mitochondrial encoded rRNA genes are reduced and their sequence is highly divergent from other animals. The high AT-content (84,3%) and the extremely evolved coding sequences underline the derived status of this mt genome and make it difficult to use the mt genome sequence data for unraveling the phylogenetic position of *Mnemiopsis* (Pett et al., 2011).

Recently, a second ctenophore mitochondrial genome from *Pleurobrachia bachei* has been published (Kohn et al., 2012). The small circular chromosome (11 kb) encodes only nine protein coding genes. Only non-functional parts of *nad2* and *nad6* have been retained and *atp6*, *apt8* and *atp9* have been entirely transferred to the nucleus. A clear homolog of the small subunit ribosomal RNA (12S) is missing and the large ribosomal RNA subunit (16S) is highly derived and very short. In contrast to *Mnemiopsis* two tRNA genes (*trn-G*, *-P*) have been identified in *Pleurobrachia*. Like in *Mnemiopsis*, the *Pleurobrachia* respiratory chain genes display a highly accelerated sequence evolution rate, which can lead to long branch attraction artifacts in phylogenetic analyses.

The steadily increasing number of available mt genome data from diploblastic animals calls for a new set of phylogenetic analyses to further illuminate diploblast relationships. We here analyze five different data sets, which provide some important insights and also underline current problems of phylogenetic analyses using mitochondrial data.

2. Material and methods

We gathered all published mitochondrial genome sequences from the NCBI genome database (RefSeq, release 41, Pruitt et al., 2007) as well as a choice of unpublished mt genomes (see Supplementary Data, Bernt et al., 2013, this special issue). From more than 2000 mitochondrial genome sequences available we selected five subsets for our phylogenetic analyses based on computational and representative taxon sampling considerations:

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(1) Data set I: 684 taxa (27 outgroups, 40 non-Bilateria (no Ctenophora), 617 Bilateria).
(2) Data set II: 143 taxa (5 outgroups, 132 non-Bilateria, 6 Bilateria).
(3) Data set III: 84 taxa (9 outgroups, 69 non-Bilateria (no Ctenophora), 6 Bilateria).
(4) Data set IV: 78 taxa (9 outgroups, 69 non-Bilateria (no Ctenophora), no Bilateria).
(5) Data set V: 114 taxa (9 outgroups, 13 non-Bilateria (no Ctenophora), 92 Bilateria).

Data set I comprises a representative choice of taxa covering all animal phyla from which mitochondrial genome data are available. A comprehensive choice of outgroups from putatively closely and distantly related taxa were added to the data set. To reduce long branch attraction artifacts the data set II comprises only protist outgroups and several moderately fast evolving bilaterian taxa. In addition, we included also closely related non-bilaterian animals (e.g. taxa from the same genus) as well as new data from the Ctenophora and Cnidaria to increase resolution within the diploblasts. The extremely fast evolving Ctenophora have been excluded in data set III together with other unstable non-bilaterian taxa. Again with the aim to reduce long-branch attraction artifacts and to test the robustness of our analysis of data set III we excluded all bilaterian taxa in data set IV. Finally, we performed Maximum Likelihood as well as Bayesian analyses on data set V to compare metazoan phylogenetic relationships inferred from different analyses methods (i.e. Maximum Likelihood and Bayesian analyses).

Taking into account inconsistencies in published mitochondrial genome annotations we have re-annotated mitochondrial genomes using the MITOS protein prediction pipeline (for details see Bernt et al., 2012, 2013). For our analyses we used amino acid sequences of 13 mitochondrial protein coding genes (*atp6*, *atp8*, *cox1-cox3*, *cob*, *nad1-nad6*, *nad4L*) and for each gene a separate alignment has been created with MAFFT version 6.716 using default settings (Katoh et al., 2002). The ends of the alignments were trimmed (criteria: less than 20% gaps in one column; max. removed columns: less or equal 100 columns) and the trimmed alignments were masked with *noisy* (release 1.5.9) with a cutoff value of 0.8 (Dress et al., 2008). The resulting protein alignments were concatenated and respective regions of regularly absent proteins (e.g. *atp8* in Placozoa) were filled with gaps. *Atp9* was not included in our data sets as only poriferan taxa possess this gene in the mitochondrial genome.

The final Maximum Likelihood analyses were performed with RAxML version 7.2.8 (Stamatakis, 2006) using the CAT + MTZOA + F model with GAMMA corrections of the final tree (for details see Bernt et al., 2013). To meet all four convergence criteria provided by RAxML (Stamatakis et al., 2008; Pattengale et al., 2009), three batches of 100 rapid bootstrap trees (or in total 400 for data set III, respectively) were generated. A best scoring ML tree search was conducted using 10 times 10 starting trees for data set I and 200 distinct starting trees for data sets II, III, IV and V, respectively. The additional Bayesian analysis with PhyloBayes-MPI version 1.3b (Lartillot et al., 2009, 2013) using the CAT MTZOA + Gamma model was only performed on data set V due to computational aspects. Six chains were run in parallel for at least 5500 iterations and the first 3000 samples were discarded as burn-in. Every tenth tree was used to compute a majority rule consensus tree and Bayesian posterior probabilities (for details, see Bernt et al., 2013).

3. Results and discussion

The main focus of our analyses was to contribute to a better understanding of both the relationships between early branching metazoan phyla, i.e. Placozoa, Porifera, Cnidaria and Ctenophora, and the overall relationships of the diploblast phyla to the Bilateria. In a first step we re-annotated published animal mitochondrial genomes with a consistent annotation pipeline to avoid wrong phylogenetic signals in our data sets due to inaccurate or inconsistent annotation of mitochondrial protein coding genes, which might have caused problems in previous phylogenetic studies (Bernt et al., 2013). In a second step the improved annotations were assembled in five separate data sets to address different phylogenetic questions at different taxonomic levels. Concerning the base of the Metazoa, the data sets I (named 'Metazoa' in Bernt et al., 2013) and II (focusing on non-bilaterian groups) (Figs. 2 and 3, respectively) led to similar tree topologies, which will be discussed in detail in the following sections. For this we compare the above trees with our additional analyses (data sets III, IV and V, Supplementary Figs. A1–A3a and b, respectively) as well as with previously published studies.

3.1. Deep metazoan relationships

Both, the data set I and II analyses, support an early split between Bilateria and non-bilaterian animals. This scenario at the base of the metazoan Tree of Life has been under strong debate and highlighted current limitations of phylogenetic analyses based on sequence data (both nuclear and mitochondrial) (e.g. Edgecombe et al., 2011; Osigus et al., 2013). Several authors have reviewed recent key studies on deep metazoan phylogeny (Pick et al., 2010; Philippe et al., 2011; Roure et al., 2013) and stressed the limits of available data sets and analyses methods to infer deep metazoan relationships. A recent comprehensive study based on the analysis of ribosomal and non-ribosomal nuclear and mitochondrial genes finds support for a new evolutionary scenario, i.e. a sister group relationship between Placozoa and Porifera, and highlights the conflicting phylogenetic signals in diverging molecular data sets (Nosenko et al., 2013). However, although only moderately supported in our analyses, the observed sister group relationship between Bilateria and non-bilaterian animals is in agreement with previous studies based on the analyses of mitochondrial data (Dellaporta et al., 2006; Signorovitch et al., 2007; Lavrov et al., 2013) and with the total evidence analyses by Schierwater and co-workers (2009a,b). In addition, the sister group relationship is seen in both, our Maximum likelihood as well as our Bayesian analyses, which is in agreement with results from a recent study by Lavrov and co-workers (2013). Despite being in conflict with the traditional view on animal evolution (i.e. with Porifera as the sister group to all other animals) the early split between Diploblasta and Triploblasta is not only supported by sequence data but also by the sum of general evolutionary tendencies in animal mitochondrial genomes. While bilaterian mitochondrial genomes are generally compact circular molecules with a conserved number of genes lacking large intergenic regions or introns, mitochondrial genomes in non-bilaterians display a large variation with respect to genome size, the number of the encoded genes and even the genome architecture (reviewed in Lavrov, 2010a). Although simple and clear synapomorphies in non-bilaterian mitochondrial genomes are missing, several separating evolutionary traits of bilaterian and non-bilaterian mt genomes are obvious and indicate an early evolutionary separation of both groups.

Focusing on the relationships within the non-bilaterian clade, both, data sets I and II, support a basal position of Placozoa within diploblasts. This topology is in agreement with previous mitochondrial analyses (e.g. Dellaporta et al., 2006; Lavrov et al., 2013). A basal position of the Placozoa within non-bilaterians is also supported by evolutionary scenarios based on the very simple placozoan bauplan (see Syed and Schierwater, 2002) and structural mitochondrial genome characteristics (reviewed in Osigus et al.,

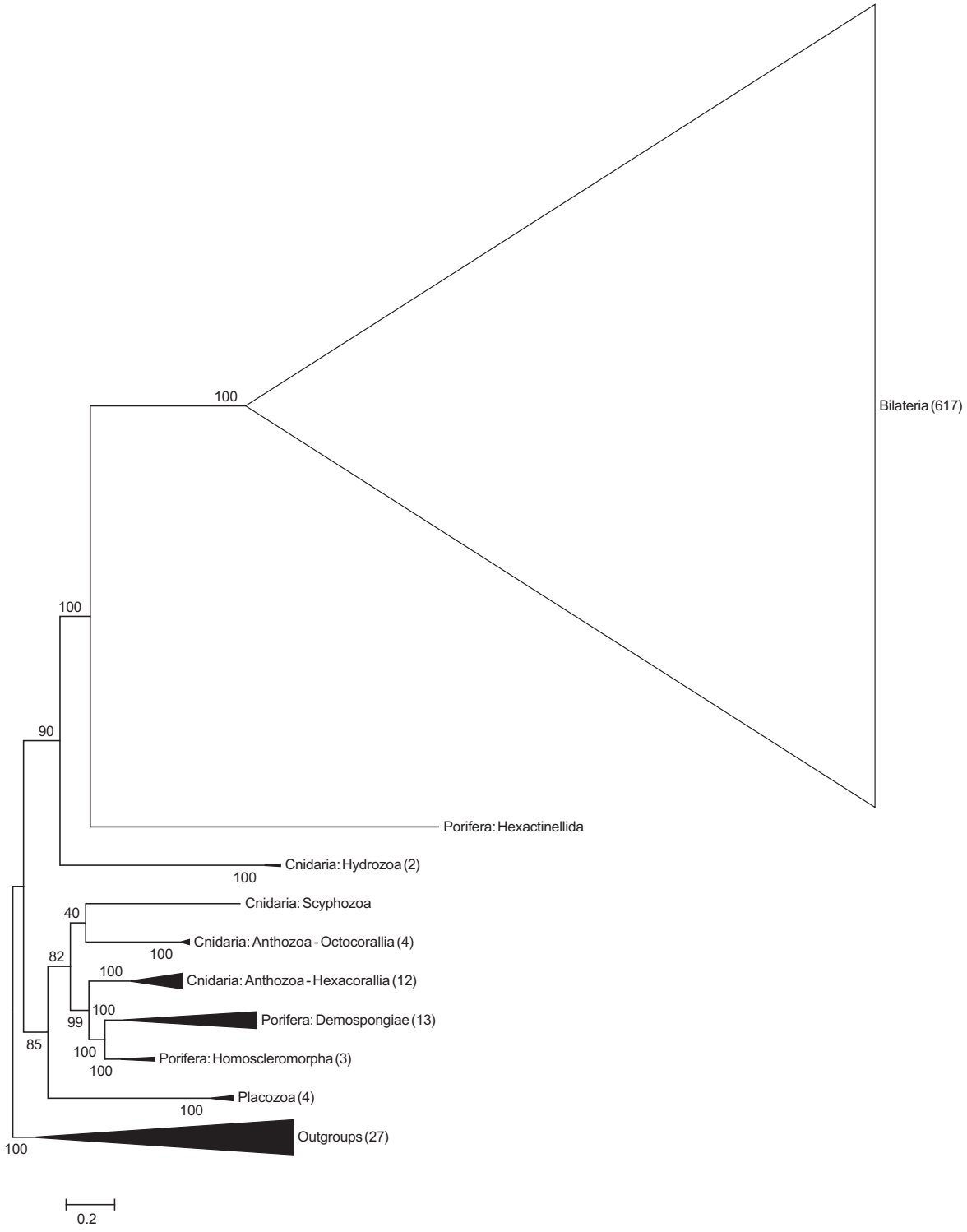

Fig. 2. Deep metazoan phylogeny based on the comprehensive mitochondrial data set I. The shown tree is based on the Maximum Likelihood analysis of 13 mitochondrial proteins (5419 aa positions) from 684 selected taxa. Phylogenetic analyses were performed under the CAT + MTZOA + F model as implemented in RAxML version 7.2.8. Bootstrap support values are shown for the major nodes and numbers of taxa within collapsed clades are given in brackets. For details within Bilateria, see Bernt et al., 2013. The fast evolving Hydrozoa (Cnidaria) and Hexactinellida (Porifera) are artificially attracted by the bilaterian clade. Placozoa are branching off first in the unaffected non-bilaterian clade. Both Cnidaria and Porifera are paraphyletic in this data set I.

2013). Interestingly, a large number of former mt genome studies did not reveal resolved topologies in this part of the tree (e.g. Burger et al., 2009; Pett et al., 2011). The placozoan mt genome size of up to 43 kb as well as the presence of introns and large intergenic regions are presumed ancestral animal mitochondrial genome features. Considering mt genome attributes of protist outgroups (i.e. choanoflagellata) an overall picture of lower animal mitochondrial genome evolution with the placozoan mt genome in an ancestral position is striking (see Osigus et al., 2013). Nevertheless, the overall inference of phylogenetic relationships

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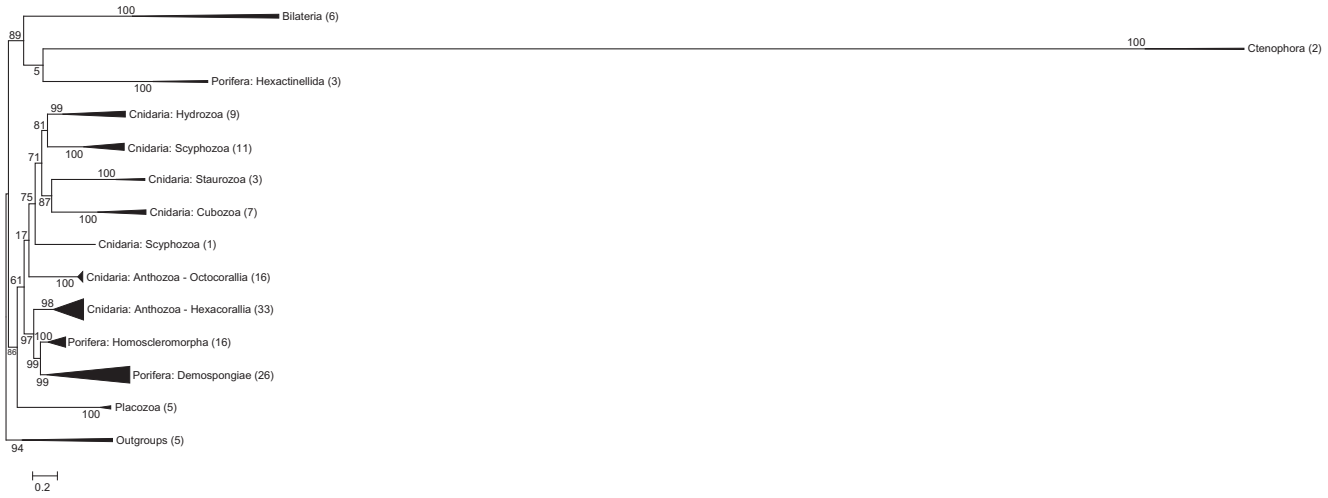

Fig. 3. Deep metazoan phylogeny based on the non-bilaterian-focused mitochondrial data set II. The shown tree is based on the Maximum Likelihood analysis of 13 mitochondrial proteins (3863 aa positions) from a total of 143 taxa. Sequence data from basal metazoan phyla and a reduced set of bilaterian taxa was included. The fast evolving Ctenophora and Hexactinellida (Porifera) are artificially attracted by the bilaterian clade. As in the comprehensive data set I, Cnidaria and Porifera come out paraphyletic. Placozoa are basal within the non-bilaterians. For further explanations see Fig. 1 and text.

between basal animal phyla based on mitochondrial genome characteristics remains problematic. Recent studies have shown the presence of fragmented linear mitochondrial genomes not only in Cnidaria but also in Porifera. Similarly, several mitochondrial genome features (e.g. a change in the genetic code) in bilaterian and hexactinellid mitochondrial genomes seem to be the result of parallel evolution. Viewing additional mitochondrial genomes from non-bilaterian animals is necessary to get a more conclusive picture of evolutionary pathways of mitochondrial genomes in non-bilaterian animals.

3.2. Problematic taxa

While the overall tree topologies of all our analyses indicate an early split between Bilateria and non-bilaterian animals the picture is blurred by several problematic taxa, which are sensitive to long branch attraction artifacts (LBA). One of these unstable taxa are the Hexactinellida (phylum Porifera). In our analyses the Hexactinellida do not group together with the other sponges included (i.e. Homoscleromorpha and Demospongiae) but instead are attracted by the Bilateria which themselves also might be attracted by the outgroups. This topology has also been observed in previous phylogenetic analyses based on mitochondrial protein sequence data (e.g. Haen et al., 2007). Even the choice of only moderately fast evolving Bilateria in the reduced data set does not help to overcome the LBA artifact. Although Hexactinellida and Bilateria share some characteristics, like a change in the mitochondrial genetic code and a comparable structure of tRNA genes, these features seem to be the result of parallel evolution (Lavrov, 2010a). The analysis of the pure non-bilaterian data set IV shows a sister group relationship of Hexactinellida and all other non-bilaterian animals, but again with paraphyletic Porifera. This topology highlights the sensitivity of Hexactinellida for attraction artifacts not only to bilaterians but also to outgroups. A close relationship between Bilateria and Hexactinellida seems highly unrealistic from a comparative zoology point of view. The non-monophyly of the Porifera (as seen in all of our analyses) has been observed in several other molecular studies based on nuclear genes, where at least the Homoscleromorpha do not group with other sponges (e.g. Hejnol et al., 2009; Sperling et al., 2009; Nosenko et al., 2013).

The mitochondrial genome data of two ctenophores have been included in the data set II. In agreement with previous observations the highly derived mitochondrial sequences in Ctenophora lead to extremely long branches in phylogenetic trees. Even the analysis of only the five most conserved mitochondrial protein coding genes does not help to overcome this problem (Pett et al., 2011). In our analysis (comprising 13 mitochondrial protein coding genes) both ctenophores group together in one clade that shows an extremely long branch. Probably due to extreme sequence evolution rates, the Ctenophora are attracted by the Bilateria which is obviously another typical case of LBA (see also Kohn et al., 2012, Supplementary Fig. 1S) and adding more sequence data from Ctenophora will likely not help to overcome this problem. At present it seems questionable whether mitochondrial protein sequence data are suitable to clarify the phylogenetic position of Ctenophora. However, the small mitogenome size and the missing mt genes clearly indicate a highly derived (and definitely not basal) position of the Ctenophora in the Metazoa (cf. Nosenko et al., 2013).

The importance of broad ingroup taxon sampling is illustrated by the varying positions of the Hydrozoa. In the data set I the included hydrozoan species (two representatives of genus *Hydra*) branch basal to a group comprising hexactinellid sponges and Bilateria. In contrast, the inclusion of eight additional hydrozoan genera in our data set II leads to monophyletic Hydrozoa deeply branching within the diploblasts. Additional taxon sampling alone cannot always help to overcome the problem of differential sequence evolution rates but it is an important option to stabilize the position of several "jumping" taxa within the ToL.

3.3. Relationships within non-bilaterian phyla

All of our analyses support the monophyly of Placozoa as well as the previously known topology within the Placozoa, i.e. a clear subdivision in two groups A and B. In agreement with previous studies based on 16S rRNA as well as mitochondrial protein coding sequences, *Trichoplax adhaerens* group together with haplotype H3 in 'group B' whereas H4, H8 and H15 form 'group A' (Signorovitch et al., 2007; Eitel and Schierwater, 2010; Eitel et al., 2013). Confirming the results of the sequence analyses the clear separation of placozoan lineages in two distinct groups is also supported by the different gene arrangement in these groups. Both, phylogenetic analyses as well as genome structure indicate the existence of higher taxonomic units.

With respect to the Porifera our analyses (data sets I and II) strongly support a sister group relationship between demosponges and the Homoscleromorpha (previously termed G0, Lavrov et al.,

2008). Additionally, our analyses recover all previously postulated demosponge groups G1–G4 (for details see Borchiellini et al., 2004; Lavrov et al., 2008), although the sister group relationship between G1 and G2 (e.g. Wang and Lavrov, 2008) is only supported by the 684 taxa analysis whereas in the 143 taxa data set the G1 clade is a sister group to all other demosponge groups (G2–G4). It should be mentioned, however, that several nodes within demosponges are only weakly supported. The slightly different topologies in both analyses might be caused by the inclusion of additional Homoscleromorpha in data set II, which probably attract members of the demosponge G1 clade.

A result observed in all analyses is the paraphyly of Cnidaria, in particular of the class Anthozoa. The subclasses Hexacorallia and Octocorallia never form a monophyletic clade. In the analysis of the large metazoan data set I, the cnidarian Hexacorallia group outside the Cnidaria and form a sister clade to the sponge classes Demospongiae and Homoscleromorpha. An inconsistent tree topology or a paraphyly of the Anthozoa has been observed in multiple previous studies based on mitochondrial protein coding data (e.g. Burger et al., 2009; Lavrov, 2010a). The reason for the latter possibly lies in the different sequence evolution rates in both groups, with the Octocorallia showing very low rates. The inclusion of a broad number of medusozoan taxa (i.e. Hydrozoa, Scyphozoa, Staurozoa and Cubozoa) in the data set II leads to a more conclusive picture with respect to cnidarian relationships. The main cnidarian clade comprises the anthozoan subclass Octocorallia as a sister group to monophyletic Medusozoa, although the Hexacorallia are grouping outside (see above).

4. Conclusions

Our analyses illustrate that mitochondrial protein sequence data have great potential to unravel phylogenetic relationships between early branching metazoan phyla as well as within these phyla. Our analyses also point at important limitations of current approaches using only mitochondrial data to infer deep metazoan relationships. Unstable taxa like Hexactinellida, Calcarea or Ctenophora are highly sensitive for long branch attraction artifacts. As seen in our analyses, the inclusion of additional taxa from previously underrepresented Hydrozoa, Scyphozoa or Cubozoa leads to improved topologies with fewer paraphyletic groups. The inclusion of additional mitochondrial genomes covering an even broader range of sequence evolution rates as well as improved models of sequence evolution seems to be a must for future studies. We also suggest to include other mt character data, namely structural information from rRNAs, tRNAs and introns, as well as gene arrangement data in future efforts to resolve relationships close to or at the base of the metazoan Tree of Life.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.07.016.

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