Tierärztliche Hochschule Hannover
Institut für Tierökologie

Comparative mitochondrial genomics in basal metazoans: new phylogenetic and functional approaches

INAUGURAL - DISSERTATION
zur Erlangung des Grades eines Doktors der Naturwissenschaften
- Doctor rerum naturalium -
(Dr. rer. nat.)

vorgelegt von
Hans-Jürgen Osigus
Aurich, Deutschland

Hannover 2019

Wissenschaftliche Betreuung:	Prof. Dr. Bernd Schierwater Institut für Tierökologie, Stiftung Tierärztliche Hochschule Hannover
1. Gutachter:	Prof. Dr. Bernd Schierwater Institut für Tierökologie, Stiftung Tierärztliche Hochschule Hannover
2. Gutachter:	Prof. Dr. Dieter Steinhagen Abteilung Fischkrankheiten und Fischhaltung, Stiftung Tierärztliche Hochschule Hannover
Tag der mündlichen Prüfung:	15.05.2019

Diese Doktorarbeit wurde von der Deutschen Forschungsgemeinschaft, der Studienstiftung des Deutschen Volkes, dem Böhringer Ingelheim Fonds sowie der Stiftung Tierärztliche Hochschule Hannover unterstützt.

This thesis is dedicated to my parents

Table of contents

List of abbreviations

5`	five prime
3`	three prime
A	adenine
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartate
ATP	adenosine triphosphate
B	C or G or T (IUPAC nucleotide code)
BA	Bayesian analyses
BLAST	Basic Local Alignment Search Tool
bp	base pair
C	cytosine
cDNA	complementary deoxyribonucleic acid
CDS	coding sequence
COB	cytochrome b
COX	cytochrome c oxidase
Cys	cysteine
CYTB	cytochrome b
DNA	deoxyribonucleic acid
ds	double-stranded
e.g.	*exempli gratia* (“for example”)
EST	expressed sequence tag
et al.	*et alii*/ *et aliae* (“and others”)
Fig.	figure
frag.	fragment
G	guanine
gen.	genus
Glu	glutamate
Gln	glutamine
Gly	glycine
H	haplotype
His	histidine
i.e.	*id est* (“that is”)
Ile	isoleucine
k-mer	nucleotide string of length *k*
kb	kilobase (1000 base pairs)
LAG	LAGLIDADG homing endonuclease
LBA	long branch attraction
LCA	last common ancestor

LCMA	last common metazoan ancestor
Leu	leucine
Lys	lysine
Met	methionine
mito	mitochondrial
ML	Maximum Likelihood
mm	millimeter
mRNA	messenger ribonucleic acid
mt	mitochondrial
N	“any base” (IUPAC nucleotide code)
NAD	nicotinamide adenine dinucleotide
NCBI	National Center of Biotechnology Information
NJ	Neighbor Joining
nov.	*nova*
ORF	open reading frame
PCR	polymerase chain reaction
Phe	phenylalanine
PolB	DNA polymerase B
Pro	proline
rDNA	ribosomal deoxyribonucleic acid
Refseq	NCBI Reference Sequence Database
RNA	ribonucleic acid
RNAseq	ribonucleic acid sequencing
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
S	Svedberg unit
Ser	serine
spec.	species
sp.	species
ss	single-stranded
suppl.	supplementary
T	thymine
Thr	threonine
ToL	Tree of Life
trn	transfer ribonucleic acid
tRNA	transfer ribonucleic acid
Trp	tryptophan
Tyr	tyrosine
U	uracil
V	A or C or G (IUPAC nucleotide code)
Val	valine
vs.	*versus*

Summary

Hans-Jürgen Osigus

Comparative mitochondrial genomics in basal metazoans: new phylogenetic and functional approaches

The phylum Placozoa is crucial for understanding the early evolutionary pathways of animal mitochondrial genomes. The studies conducted in this thesis target early metazoan mitochondrial phylogenetics, placozoan mitochondrial mRNA processing and comparative placozoan mitochondrial genomics and its implications for placozoan taxonomy.

In order to test the effect of taxon sampling on metazoan mitochondrial phylogenies, five comprehensive whole mitochondrial data sets were generated and analyzed. The outcomes of the analyses support some well-known topologies, but also reveal some problematic taxa. In sum, with respect to phylogenetics, mitochondrial data sets perform best below the phylum-level, as current evolutionary models struggle with data heterogeneity and long-branch attraction artifacts.

Deep RNA sequencing revealed the existence of a single base pair *cox1* micro exon but does not support mRNA editing in Placozoa. This *cox1* micro exon is the first mitochondrial single base pair exon reported from an animal.

The comparative analyses of nine new placozoan mitochondrial genomes revealed an unexpected diversity and complex evolutionary history of mitochondrial DNA characteristics within Placozoa. Complemented by nuclear genome data, this mtDNA diversity yielded the formal description of the new placozoan genus *Polyplacotoma*. The analyses also provided new important insights into enrichment patterns of GC-rich hairpins, the evolution of gene orders and complex fragmentation patterns of placozoan mitochondrial genes.

Zusammenfassung

Hans-Jürgen Osigus

Vergleichende mitochondriale Genomanalysen in basalen Metazoen: neue phylogenetische und funktionelle Ansätze

Der Tierstamm Placozoa spielt eine Schlüsselrolle für die Rekonstruktion der frühen Evolution tierischer mitochondrialer Genome. Die vorliegende Arbeit beschäftigte sich mit mitochondrialen phylogenetischen Analysen an der Basis der Metazoa, mit mitochondrialer mRNA Prozessierung in Placozoa sowie mit vergleichenden mitochondrialen Genomanalysen und deren Einfluss auf die Taxonomie innerhalb der Placozoa.

Anhand von fünf Datensätzen wurde in dieser Arbeit der Einfluss der Taxonauswahl auf mitochondriale phylogenetische Analysen untersucht. Die Ergebnisse unterstützen einerseits bekannte Verwandtschaftsbeziehungen zwischen ursprünglichen Vielzellern und identifizieren darüber hinaus problematische Taxa. Mitochondriale Daten erscheinen insbesondere für genealogische Analysen unterhalb des Tierstamm-Niveaus hilfreich, da die aktuell verfügbaren evolutionären Modelle aufgrund von Datenheterogenität und analysebedingten Artefakten bei höheren taxonomischen Ebenen an ihre Grenzen stoßen.

Die in dieser Arbeit durchgeführte hochabdeckende RNA-Sequenzierung ergab, dass das *cox1* Gen der Placozoa anstatt einer mRNA Editierungsposition ein ultrakurzes 1-Basenpaar-Mikroexon enthält. Dieses Mikroexon ist das erste mitochondriale 1-Basenpaar-Exon, das jemals im Tierreich gefunden wurde.

Die Analyse von neun neuen mitochondrialen Genomen förderte eine unerwartete genetische Vielfalt und komplexe mtDNA Evolution innerhalb der Placozoa zutage. Unterstützt durch Kerngenomdaten führte diese mitochondriale Vielfalt zur formellen Beschreibung der neuen Placozoa-Gattung *Polyplacotoma*. Weitere mtDNA Analysen gaben darüber hinaus neue Einsichten in die Anreicherung von GC-reichen Haarnadelstrukturen, die Evolution der Genanordnung sowie die Genfragmentierung innerhalb der Placozoa.

List of previously published chapters

The following chapters have previously been published in international peer-reviewed scientific journals:

Chapter I
H.J. Osigus, M. Eitel, B. Schierwater, Chasing the urmetazoon: striking a blow for quality data?, Mol Phylogenet Evol 66(2) (2013) 551-7. doi: 10.1016/j.ympev.2012.05.028

Chapter II
H.J. Osigus, M. Eitel, M. Bernt, A. Donath, B. Schierwater, Mitogenomics at the base of Metazoa, Mol Phylogenet Evol 69(2) (2013) 339-51. doi: 10.1016/j.ympev.2013.07.016

Chapter III
M. Eitel, H.J. Osigus, R. DeSalle, B. Schierwater, Global diversity of the Placozoa, Plos One 8(4) (2013) e57131. doi: 10.1371/journal.pone.0057131

Chapter IV
H.J. Osigus, M. Eitel, B. Schierwater, Deep RNA sequencing reveals the smallest known mitochondrial micro exon in animals: The placozoan *cox1* single base pair exon, Plos One 12(5) (2017) e0177959. doi: 10.1371/journal.pone.0177959

Chapter V
K. Kamm, H.J. Osigus, P.F. Stadler, R. DeSalle, B. Schierwater, *Trichoplax* genomes reveal profound admixture and suggest stable wild populations without bisexual reproduction, Sci Rep 8(1) (2018) 11168. doi: 10.1038/s41598-018-29400-y

Chapter VI
M. Eitel, W.R. Francis, F. Varoqueaux, J. Daraspe, H.J. Osigus, S. Krebs, S. Vargas, H. Blum, G.A. Williams, B. Schierwater, G. Wörheide, Comparative genomics and the nature of placozoan species, Plos Biol 16(7) (2018) e2005359. doi: 10.1371/journal.pbio.2005359

Chapter VII
H.J. Osigus, S. Rolfes, R. Herzog, K. Kamm, B. Schierwater, *Polyplacotoma mediterranea* is a new ramified placozoan species, Curr Biol 29(5) (2019) R148-R149. doi: 10.1016/j.cub.2019.01.068

1. General introduction

The origin of Metazoa and relationships between non-bilaterian phyla

The reconstruction of the origin and the early diversification of Metazoa is one of the major challenges in the field of evolutionary biology (1). The phylogenetic relationships between the four extant non-bilaterian phyla Placozoa, Porifera, Cnidaria and Ctenophora as well as the higher systematics within these taxa are controversially discussed (2). While Choanoflagellata are generally accepted as the sister group to Metazoa (3), the question about the closest extant metazoan relative of the hypothetical last common metazoan ancestor (LCMA) is still unresolved (4). For several decades, comparative approaches to reconstruct the relationships between early branching metazoans were mainly based on morphological data (see e.g. 5,6, and references therein). Such morphology-based scenarios suggested either Placozoa or Porifera as the first branching animal phylum, while cnidarians and ctenophores have been considered as rather derived non-bilaterian animals, due to their multiple complex morphological traits like for instance a nervous system (see also 7).

Since the establishment of high-throughput DNA sequencing technologies, large multi-gene data sets have been used to address the question of the branching order of non-bilaterians. The outcome of such molecular approaches is highly inconsistent and in many respects even highly contradictory (4,8-11), and resulting tree topologies have shown to be sensitive to parameters like taxon sampling, outgroups or evolutionary models (12). The majority of recent analyses based on nuclear encoded genes support either Porifera or Ctenophora as a sister to all other animals (e.g. 8,9), while placozoans frequently come out as a sister to a clade formed by Cnidaria and Bilateria (e.g. 13,14,15). In contrast, early studies using mitochondrial data sets support a sister group relationship of bilaterian and non-bilaterian animals, with placozoans as the earliest branching phylum within the non-bilaterian clade (16,17).

The phylum Placozoa

Although the first placozoan species *Trichoplax adhaerens* has already been described in 1883 (18), the phylum Placozoa is still a scientific enigma in several

respects (19). All placozoans, which so far have been sampled, share the same general morphological bauplan, i.e. a three-layered body organization (e.g. 18,20). Complex structures like eyes or a nervous system as well as related specialized cells are missing in placozoans (21). Despite their morphological simplicity, placozoans exhibit a complex coordinated feeding behavior and the ability to perceive light or gravitation (22-24). Up to now six different morphological cell types have been described in *Trichoplax adhaerens* (25), and studies on single cell transcriptomes or cellular neuropeptide content have suggested the presence of various additional subpopulations of cell types (26,27). However, the definite cellular composition and architecture is still incompletely understood. In addition, observed ultrastructural differences between genetic lineages do not conclusively match to molecular systematics (20). Although sexual reproduction has been deduced by genetic approaches (28), observations of placozoan sexual reproduction in the field are missing. Under laboratory conditions, placozoans reproduce mostly vegetatively, i.e. by fission (29). All approaches to close the placozoan life cycle in the laboratory so far do not reach a level beyond the 128-cell-stage of embryos (30). Microscopic and genetic studies have identified bacterial endosymbionts in placozoans, which are transmitted via oocytes (30,31, and references therein). Little is known about the interaction of placozoans with other organisms in the field, as well as their precise ecological niches (reviewed in 29). Nevertheless, different gastropod species belonging to the genus *Rhodope* have been observed while feeding on placozoans (32, and references therein).

Despite the macroscopic morphological uniformity of placozoans, several recent genetic studies have revealed a remarkable genetic diversity, indicating the existence of various cryptic species (33-35). The molecular systematic within the phylum Placozoa is primarily based on the mitochondrial 16S rDNA (33). Sampling efforts in tropical and subtropical oceans worldwide so far have led to 16 genetic lineages described until 2010, although a much higher number of different placozoan lineages in the field is to be expected (34). Based on mitochondrial 16S rDNA phylogenies, known placozoan lineages are currently subdivided into two groups A and B, with group A again being subdivided into two subgroups A1 and A2, respectively (34). The groups harbor different genetic

clades, which unify different 16S rDNA lineages (34). Although the preliminary classification of placozoans into different groups and clades is a practical solution, an accepted Linnean systematic is still missing (19). Therefore, all placozoans except *Trichoplax adhaerens* H1 (18) are currently provisionally called "haplotype/lineage", with newly identified lineages assigned to a consecutive "H"-number (e.g. H2)(33).

Mitochondrial genomics in early diverging metazoans

Mitochondria are small eukaryotic cell organelles, which play a fundamental role in cellular processes like oxidative phosphorylation or apoptosis (36). As a relict of their alpha-proteobacterial origin, mitochondria retained their own genome, although most mitochondrial genes have been transferred into the nuclear genome (37,38). Since the characterization of the first complete mitochondrial genome (i.e. that of *Homo sapiens*) in 1981 (39), ongoing sequencing efforts have yielded a total number of more than 8,100 complete metazoan mtDNA sequences (Refseq, 01/2019). The most informative characters for comparative whole mitochondrial genome analyses are genome size, genome architecture, nucleotide composition, gene content, gene order and presence/absence of introns or open reading frames of unknown function, respectively (40).

Mitogenomic data from Choanoflagellata (the often assumed sister group of Metazoa, see above) are of outstanding importance to understand the early evolution of metazoan mitochondrial genomes (41). Several choanoflagellate genome sequencing projects are currently in progress, but the only available complete choanoflagellate mitogenome still is that of *Monosiga brevicollis* (42). In contrast to most animal mitochondrial genomes, the circular mitogenome of *Monosiga brevicollis* is a large molecule, which codes for several open reading frames (ORFs) of unknown function. In addition, introns can be found in the *Monosiga cox1* and *nad5* gene, respectively. The most remarkable deviation from animal mitochondrial genomes, however, is the presence of mitochondrial encoded ribosomal proteins (42). Although the precise characteristics of the hypothetical urmetazoan mitochondrial genome are unknown, it is reasonable to suppose that the urmetazoan mtDNA shares several traits with extant choanoflagellate mitogenomes (41,42).

Early comparative studies on animal mitochondrial genome evolution suffer from data scarcity from non-bilaterian phyla (43). The resulting strong bias towards bilaterian mitogenomes has misled several authors to prematurely postulate that animal mitochondrial genomes in general are highly uniform, compact molecules (43). This picture, however, has been refuted since more non-bilaterian mitogenome sequences have become available (40). Given the actual mitogenome size variation between and within non-bilaterian phyla, an overall size specification is problematic. However, the majority of non-bilaterian mitogenomes is in a range of 15-23 kb (44). While most non-bilaterian mitochondria possess a circular chromosome, multipartite linear mitogenomes have nevertheless been reported from some sponges and cnidarians, respectively (reviewed in 40). Non-bilaterian mtDNAs have a GC-content below 40%, with few exceptions found in Placozoa as well as some sponge and cnidarian species (NCBI, Organelle Genome Resources, 01/2019). The typical mitochondrial encoded gene set in non-Bilateria comprises 14 protein coding genes (*cox1-3*, *cob*, *nad1-6*, *nad4L*, *atp6*, *atp8*, *atp9*), 2 rRNAs (12S and 16S) as well as approximately 24 tRNAs (40). However, the number of mitochondrial encoded tRNA genes can be dramatically reduced, as occasionally seen in all non-bilaterian phyla except Placozoa (44). Independent intron/ORF gain or loss events have likewise been reported, further highlighting the high dynamic of mitogenome evolution at the base of Metazoa (40). This dynamic is also reflected by sequence evolution rates, which can substantially differ even between closely related non-bilaterian taxa (e.g. 45). Finally, unusual molecular mechanisms like tRNA/mRNA editing and translational frameshifting emphasize the outstanding role of non-bilaterians as model systems for studies on complex molecular processes in Metazoa (46,47).

Placozoan mitogenomics

The very first Genbank entry of a placozoan mitochondrial DNA sequence, i.e. a partial *Trichoplax adhaerens* H1 16S rDNA sequence, backdates to the year 2003 (48). In 2006, the first complete placozoan mitochondrial genome of *Trichoplax adhaerens* H1 has been published (16), followed by in sum four additional placozoan mitochondrial genomes, which have been described afterwards in

2007 and 2012, respectively (17,49). The mitochondrial genomes of *Trichoplax adhaerens* H1 (clade I), haplotype H3 (clade II), haplotype H4 (clade V), haplotype H8 (clade III) and haplotype H15 (clade V) share several general characteristics (17,49). All these placozoan mitochondrial genomes have a size above 30 kb, large intergenic spacer regions and an uneven distribution of genes on both strands. Furthermore, they contain several introns as well as open reading frames of unknown function. Other shared characteristics are the absence of *atp8*/*atp9* and the presence of a conserved set of 24 tRNAs. A single control region, which is known e.g. from bilaterian mitochondrial genomes (50), could not be identified yet, as multiple large non-coding candidate regions are present in placozoan mitogenomes. Despite overall similarities, the order of typical mitochondrial genes is different in each of the so far characterized placozoan 16S clades (17). These high rearrangement dynamics are exceptional among animals and make complete placozoan mitogenomes an ideal data set not only to further illuminate placozoan relationships, but also to better understand the genetic radiation of fast evolving metazoan mtDNAs (17). However, given the small number of available complete mitogenome sequences, the ancestral mitochondrial gene order in placozoans could not be reliably reconstructed until today. Furthermore, the molecular mechanisms underlying the observed rearrangements are unknown(17).

Another remarkable placozoan feature is the unusual *cox1* gene structure. In detail, the splitting of the *cox1* gene into up to eight exons (some of them encoded on different strands) requires trans-splicing to generate a consecutive mRNA transcript (16,17,49). This *cox1* gene fragmentation combined with trans-splicing is exceptional among animals (51). In this context, a remarkable hypothesis postulated by Burger and co-workers in 2009 (51) even suggests mitochondrial mRNA editing in the placozoan *cox1* gene. In this scenario, the *cox1* mRNA is edited at a specific position from “U” to “C” to maintain an evolutionary conserved histidine. The origin and mechanisms of the placozoan *cox1* mRNA editing, however, are still awaiting further investigation (51).

Aims and scope

Non-bilaterian mitogenomics is a rapidly growing research field, since modern high-throughput sequencing technologies allow the fast and cost-efficient generation of large molecular data sets, even for non-model organisms. In the course of this thesis, intensive mitochondrial genome sequencing efforts of other research groups already covered a broad range of taxa from Porifera, Cnidaria and Ctenophora. The main focus of this thesis is therefore on mitochondrial genomes from the fourth non-bilaterian phylum, i.e. the Placozoa.

Resolving placozoan mitogenomics is crucial to a broad spectrum of questions: **1)** Mitochondrial genetic markers are commonly used in studies on placozoan biodiversity and inner systematics. **2)** Mitochondrial protein coding genes are an important resource for deep metazoan phylogenetic approaches targeting the base of the metazoan tree of life. **3)** The unusual and incompletely understood placozoan mitochondrial *cox1* mRNA editing mechanism calls for further investigation using state-of-the-art RNA sequencing technologies. **4)** Additional placozoan mtDNA sequence data are essential to further understand the complex history of placozoan mitogenome evolution and to improve scenarios on the early evolution of metazoan mitogenomes. **5)** Mitochondrial genome data are an important complement for comparative studies on placozoan nuclear genomes to minimize errors caused by potential genome-related analyses artifacts.

The aim of my thesis is to address the above-mentioned topics and this way to contribute to a better understanding of mitochondrial genome evolution pathways at the base of Metazoa.

Thesis outline and author contributions

This cumulative thesis is subdivided into nine different chapters I-IX. Chapters I-VII have already been published in peer-reviewed scientific journals. Chapters VIII and IX likewise are going to be submitted in the present or slightly modified versions to peer-reviewed scientific journals. The contributions of the authors to the respective chapters are described in the following section:

Chapter I

H.J. Osigus, M. Eitel, B. Schierwater, Chasing the urmetazoon: striking a blow for quality data?, Mol Phylogenet Evol 66(2) (2013) 551-7.

Conceptualization: HJO ME BS.
Visualization: HJO ME BS.
Writing: HJO ME BS.
Funding acquisition: BS.

Chapter II

H.J. Osigus, M. Eitel, M. Bernt, A. Donath, B. Schierwater, Mitogenomics at the base of Metazoa, Mol Phylogenet Evol 69(2) (2013) 339-51.

Conceptualization: HJO ME BS.
Data curation: HJO ME MB AD.
Formal analysis: HJO ME MB AD.
Funding acquisition: BS.
Investigation: HJO ME MB AD BS.
Methodology: HJO ME MB AD BS.
Project administration: BS.
Resources: MB AD BS.
Supervision: BS.
Validation: HJO ME MB AD BS.
Visualization: HJO.
Writing – original draft: HJO ME BS.
Writing – review & editing: HJO ME MB AD BS.

Chapter III

M. Eitel, H.J. Osigus, R. DeSalle, B. Schierwater, Global diversity of the Placozoa, Plos One 8(4) (2013) e57131.

Conceived and designed the experiments: ME HJO BS.
Performed the experiments: ME HJO.
Analyzed the data: ME HJO RD BS.
Contributed reagents/materials/analysis tools: BS.
Wrote the paper: ME HJO RD BS.

Chapter IV

H.J. Osigus, M. Eitel, B. Schierwater, Deep RNA sequencing reveals the smallest known mitochondrial micro exon in animals: The placozoan *cox1* single base pair exon, Plos One 12(5) (2017) e0177959.

Data curation: HJO BS.
Formal analysis: HJO ME.
Funding acquisition: BS.
Investigation: HJO.
Methodology: HJO ME BS.

Project administration: BS.
Resources: BS.
Supervision: BS.
Validation: HJO ME BS.
Visualization: HJO.
Writing – original draft: HJO.
Writing – review & editing: HJO ME BS.

Chapter V

K. Kamm, H.J. Osigus, P.F. Stadler, R. DeSalle, B. Schierwater, *Trichoplax* genomes reveal profound admixture and suggest stable wild populations without bisexual reproduction, Sci Rep 8(1) (2018) 11168.

KK coordinated the project, assembled the genome and the transcriptomes, analyzed the data and wrote the manuscript; BS Initiated, funded and coordinated the project and wrote the manuscript; RD wrote the manuscript; HJO coordinated animal material and Illumina sequencing of the genome and the transcriptomes and provided general expertise regarding placozoans; PFS provided computational resources and data curation. All authors reviewed, discussed and approved the final version of the manuscript.

Chapter VI

M. Eitel, W.R. Francis, F. Varoqueaux, J. Daraspe, H.J. Osigus, S. Krebs, S. Vargas, H. Blum, G.A. Williams, B. Schierwater, G. Wörheide, Comparative genomics and the nature of placozoan species, Plos Biol 16(7) (2018) e2005359.

Conceptualization: ME WRF SV BS GW.
Data curation: ME SK SV HB GW.
Formal analysis: ME WRF SV.
Funding acquisition: ME GAW BS GW.
Investigation: ME WRF FV JD HJO SK SV HB BS GW.
Methodology: ME WRF SV HB GAW.
Project administration: ME GW.
Resources: ME HB GAW BS GW.
Software: WRF.
Supervision: SV BS GW.
Validation: ME WRF SV GW.
Visualization: ME WRF FV JD SK SV.
Writing – original draft: ME WRF GW.
Writing – review & editing: ME WRF HJO SV GAW BS GW.

Chapter VII

H.J. Osigus, S. Rolfes, R. Herzog, K. Kamm, B. Schierwater, *Polyplacotoma mediterranea* is a new ramified placozoan species, Curr Biol 29(5) (2019) R148-R149.

Conceptualization: HJO BS.
Data curation: HJO KK BS.
Formal analysis: HJO KK BS.
Investigation: HJO SR RH KK BS.
Field work: SR RH BS.
Resources: BS.
Funding acquisition: BS.
Writing: HJO SR RH KK BS.
Visualization: HJO KK BS.
Supervision: BS.
Project administration: BS.

Chapter VIII

H.J. Osigus, M. Eitel, K. Kamm, S. Rolfes, M. Tessler, A. Narechania, R. DeSalle, B. Schierwater, Accumulation of GC-rich hairpins in large placozoan mitochondrial genomes, unpublished.

Conceptualization: HJO BS.
Data curation: HJO ME MT AN RD BS.
Formal analysis: HJO.
Funding acquisition: RD BS.
Investigation: HJO ME KK SR BS.
Methodology: HJO ME BS.
Project administration: RD BS.
Resources: RD BS.
Supervision: BS.
Validation: HJO ME KK SR.
Visualization: HJO.
Writing – original draft: HJO BS.

Chapter IX

H.J. Osigus, M. Eitel, S. Rolfes, K. Kamm, M. Tessler, J.S. Neumann, R. DeSalle, B. Schierwater, New insights into complex mitochondrial genome evolution within the Hoilungia-group (phylum Placozoa), unpublished.

Conceptualization: HJO BS.
Data curation: HJO MT JSN RD BS.
Formal analysis: HJO.
Funding acquisition: RD BS.
Investigation: HJO ME KK SR BS.
Methodology: HJO ME BS.
Project administration: RD BS.
Resources: RD BS.
Supervision: BS.
Validation: HJO KK SR.

Visualization: HJO.
Writing – original draft: HJO BS.

References

1. Schierwater, B. and DeSalle, R. (2007) Can we ever identify the Urmetazoan? *Integr Comp Biol*, **47**, 670–676.
2. Schierwater, B., Holland, P.W.H., Miller, D.J., Stadler, P.F., Wiegmann, B.M., Wörheide, G., Wray, G.A. and DeSalle, R. (2016) Never Ending Analysis of a Century Old Evolutionary Debate: "Unringing" the Urmetazoon Bell. *Frontiers in Ecology and Evolution*, **4**.
3. King, N. and Rokas, A. (2017) Embracing Uncertainty in Reconstructing Early Animal Evolution. *Current Biology*, **27**, R1081-R1088.
4. Schierwater, B., Eitel, M., Jakob, W., Osigus, H.J., Hadrys, H., Dellaporta, S.L., Kolokotronis, S.O. and Desalle, R. (2009) Concatenated analysis sheds light on early metazoan evolution and fuels a modern "urmetazoon" hypothesis. *PLoS Biol*, **7**, e20.
5. Ax, P. (1996) *Multicellular Animals. A new approach to the Phylogenetic Order in Nature*. Springer-Verlag, Berlin, Heidelberg.
6. Gruner, H. (1993) *Einführung, Protozoa, Placozoa, Porifera.*, Jena.
7. Syed, T. and Schierwater, B. (2002) The Evolution of the Placozoa: A new morphological model. *Senckenbergiana lethaea*, **82**, 315-324.
8. Dunn, C.W., Hejnol, A., Matus, D.Q., Pang, K., Browne, W.E., Smith, S.A., Seaver, E., Rouse, G.W., Obst, M., Edgecombe, G.D. *et al.* (2008) Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature*, **452**, 745-749.
9. Hejnol, A., Obst, M., Stamatakis, A., Ott, M., Rouse, G.W., Edgecombe, G.D., Martinez, P., Baguna, J., Bailly, X., Jondelius, U. *et al.* (2009) Assessing the root of bilaterian animals with scalable phylogenomic methods. *Proceedings*, **276**, 4261-4270.
10. Philippe, H., Derelle, R., Lopez, P., Pick, K., Borchiellini, C., Boury-Esnault, N., Vacelet, J., Renard, E., Houliston, E., Queinnec, E. *et al.* (2009) Phylogenomics Revives Traditional Views on Deep Animal Relationships. *Current Biology*, **19**, 706-712.
11. Nosenko, T., Schreiber, F., Adamska, M., Adamski, M., Eitel, M., Hammel, J., Maldonado, M., Muller, W.E.G., Nickel, M., Schierwater, B. *et al.* (2013) Deep metazoan phylogeny: When different genes tell different stories. *Molecular Phylogenetics and Evolution*, **67**, 223-233.
12. Philippe, H., Brinkmann, H., Lavrov, D.V., Littlewood, D.T., Manuel, M., Worheide, G. and Baurain, D. (2011) Resolving difficult phylogenetic questions: why more sequences are not enough. *PLoS Biol*, **9**, e1000602.
13. Pisani, D., Pett, W., Dohrmann, M., Feuda, R., Rota-Stabelli, O., Philippe, H., Lartillot, N. and Worheide, G. (2015) Genomic data do not support comb jellies as the sister group to all other animals. *P Natl Acad Sci USA*, **112**, 15402-15407.

14. Feuda, R., Dohrmann, M., Pett, W., Philippe, H., Rota-Stabelli, O., Lartillot, N., Worheide, G. and Pisani, D. (2017) Improved Modeling of Compositional Heterogeneity Supports Sponges as Sister to All Other Animals. *Current Biology*, **27**, 3864-+.
15. Simion, P., Philippe, H., Baurain, D., Jager, M., Richter, D.J., Di Franco, A., Roure, B., Satoh, N., Queinnec, E., Ereskovsky, A. *et al.* (2017) A Large and Consistent Phylogenomic Dataset Supports Sponges as the Sister Group to All Other Animals. *Current Biology*, **27**, 958-967.
16. Dellaporta, S.L., Xu, A., Sagasser, S., Jakob, W., Moreno, M.A., Buss, L.W. and Schierwater, B. (2006) Mitochondrial genome of Trichoplax adhaerens supports placozoa as the basal lower metazoan phylum. *Proc Natl Acad Sci U S A*, **103**, 8751-8756.
17. Signorovitch, A.Y., Buss, L.W. and Dellaporta, S.L. (2007) Comparative genomics of large mitochondria in placozoans. *PLoS Genetics*, **3**, e13.
18. Schulze, F.E. (1883) Trichoplax adhaerens, nov. gen., nov. spec. *Zoologischer Anzeiger*, **6**, 92-97.
19. Schierwater, B. (2005) My favorite animal, Trichoplax adhaerens. *Bioessays*, **27**, 1294-1302.
20. Guidi, L., Eitel, M., Cesarini, E., Schierwater, B. and Balsamo, M. (2011) Ultrastructural analyses support different morphological lineages in the phylum Placozoa Grell, 1971. *J Morphol*, **272**, 371-378.
21. Schierwater, B. and DeSalle, R. (2018) Placozoa. *Curr Biol*, **28**, R97-R98.
22. Mayorova, T.D., Smith, C.L., Hammar, K., Winters, C.A., Pivovarova, N.B., Aronova, M.A., Leapman, R.D. and Reese, T.S. (2018) Cells containing aragonite crystals mediate responses to gravity in Trichoplax adhaerens (Placozoa), an animal lacking neurons and synapses. *PLoS One*, **13**, e0190905.
23. Smith, C.L., Pivovarova, N. and Reese, T.S. (2015) Coordinated Feeding Behavior in Trichoplax, an Animal without Synapses. *Plos One*, **10**.
24. Srivastava, M., Begovic, E., Chapman, J., Putnam, N.H., Hellsten, U., Kawashima, T., Kuo, A., Mitros, T., Salamov, A., Carpenter, M.L. *et al.* (2008) The Trichoplax genome and the nature of placozoans. *Nature*, **454**, 955-960.
25. Smith, C.L., Varoqueaux, F., Kittelmann, M., Azzam, R.N., Cooper, B., Winters, C.A., Eitel, M., Fasshauer, D. and Reese, T.S. (2014) Novel Cell Types, Neurosecretory Cells, and Body Plan of the Early-Diverging Metazoan Trichoplax adhaerens. *Current Biology*, **24**, 1565-1572.
26. Varoqueaux, F., Williams, E.A., Grandemange, S., Truscello, L., Kamm, K., Schierwater, B., Jekely, G. and Fasshauer, D. (2018) High Cell Diversity and Complex Peptidergic Signaling Underlie Placozoan Behavior. *Curr Biol*, **28**, 3495-3501 e3492.
27. Sebe-Pedros, A., Chomsky, E., Pang, K., Lara-Astiaso, D., Gaiti, F., Mukamel, Z., Amit, I., Hejnol, A., Degnan, B.M. and Tanay, A. (2018) Early metazoan cell type diversity and the evolution of multicellular gene regulation. *Nat Ecol Evol*, **2**, 1176-1188.
28. Signorovitch, A.Y., Dellaporta, S.L. and Buss, L.W. (2005) Molecular signatures for sex in the Placozoa. *Proceedings of the National Academy of Sciences USA*, **102**, 15518-15522.

29. Voigt, O. and Eitel, M. (2018) In Schmidt-Rhaesa, A. (ed.), *Miscellaneous Invertebrates*. De Gruyter, Berlin, Boston, pp. 41-54.
30. Eitel, M., Guidi, L., Hadrys, H., Balsamo, M. and Schierwater, B. (2011) New insights into placozoan sexual reproduction and development. *PLoS One*, **6**, e19639.
31. Driscoll, T., Gillespie, J.J., Nordberg, E.K., Azad, A.F. and Sobral, B.W. (2013) Bacterial DNA Sifted from the Trichoplax adhaerens (Animalia: Placozoa) Genome Project Reveals a Putative Rickettsial Endosymbiont. *Genome Biol Evol*, **5**, 621-645.
32. Cuervo-González, R. (2017) Rhodope placozophagus (Heterobranchia) a new species of turbellarian-like Gastropoda that preys on placozoans. *Zoologischer Anzeiger - A Journal of Comparative Zoology*, **270**, 43-48.
33. Voigt, O., Collins, A.G., Pearse, V.B., Pearse, J.S., Ender, A., Hadrys, H. and Schierwater, B. (2004) Placozoa -- no longer a phylum of one. *Curr Biol*, **14**, R944-945.
34. Eitel, M. and Schierwater, B. (2010) The phylogeography of the Placozoa suggests a taxon-rich phylum in tropical and subtropical waters. *Mol Ecol*, **19**, 2315-2327.
35. Signorovitch, A.Y., Dellaporta, S.L. and Buss, L.W. (2006) Caribbean placozoan phylogeography. *Biological Bulletin*, **211**, 149-156.
36. Tao, M., You, C.P., Zhao, R.R., Liu, S.J., Zhang, Z.H., Zhang, C. and Liu, Y. (2014) Animal mitochondria: evolution, function, and disease. *Curr Mol Med*, **14**, 115-124.
37. Martijn, J., Vosseberg, J., Guy, L., Offre, P. and Ettema, T.J.G. (2018) Deep mitochondrial origin outside the sampled alphaproteobacteria. *Nature*, **557**, 101-+.
38. Roger, A.J., Munoz-Gomez, S.A. and Kamikawa, R. (2017) The Origin and Diversification of Mitochondria. *Current Biology*, **27**, R1177-R1192.
39. Anderson, S., Bankier, A.T., Barrell, B.G., Debruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F. *et al.* (1981) Sequence and Organization of the Human Mitochondrial Genome. *Nature*, **290**, 457-465.
40. Lavrov, D.V. and Pett, W. (2016) Animal Mitochondrial DNA as We Do Not Know It: mt-Genome Organization and Evolution in Nonbilaterian Lineages. *Genome Biol Evol*, **8**, 2896-2913.
41. Lavrov, D.V. (2010) In DeSalle, R. and Schierwater, B. (eds.), *Key transitions in animal evolution*. CRC Press, pp. 34-53.
42. Burger, G., Forget, L., Zhu, Y., Gray, M.W. and Lang, B.F. (2003) Unique mitochondrial genome architecture in unicellular relatives of animals. *Proc Natl Acad Sci U S A*, **100**, 892-897.
43. Lavrov, D.V. (2007) Key transitions in animal evolution: a mitochondrial DNA perspective. *Integr Comp Biol*, **47**, 734-743.
44. Desalle, R., Schierwater, B. and Hadrys, H. (2017) MtDNA: The small workhorse of evolutionary studies. *Front Biosci (Landmark Ed)*, **22**, 873-887.
45. Stampar, S.N., Maronna, M.M., Kitahara, M.V., Reimer, J.D. and Morandini, A.C. (2014) Fast-evolving mitochondrial DNA in Ceriantharia: a reflection of hexacorallia paraphyly? *PLoS One*, **9**, e86612.

46. Lavrov, D.V., Adamski, M., Chevaldonne, P. and Adamska, M. (2016) Extensive Mitochondrial mRNA Editing and Unusual Mitochondrial Genome Organization in Calcaronean Sponges. *Curr Biol*, **26**, 86-92.
47. Haen, K.M., Pett, W. and Lavrov, D.V. (2014) Eight new mtDNA sequences of glass sponges reveal an extensive usage of +1 frameshifting in mitochondrial translation. *Gene*, **535**, 336-344.
48. Ender, A. and Schierwater, B. (2003) Placozoa are not derived cnidarians: evidence from molecular morphology. *Mol Biol Evol*, **20**, 130-134.
49. Miyazawa, H., Yoshida, M.A., Tsuneki, K. and Furuya, H. (2012) Mitochondrial genome of a Japanese placozoan. *Zoolog Sci*, **29**, 223-228.
50. Bernt, M., Braband, A., Schierwater, B. and Stadler, P.F. (2013) Genetic aspects of mitochondrial genome evolution. *Mol Phylogenet Evol*, **69**, 328-338.
51. Burger, G., Yan, Y., Javadi, P. and Lang, B.F. (2009) Group I-intron trans-splicing and mRNA editing in the mitochondria of placozoan animals. *Trends Genet*, **25**, 381-386.

2. Chapters

Chapter I

H.J. Osigus, M. Eitel, B. Schierwater, Chasing the urmetazoon: striking a blow for quality data?, Mol Phylogenet Evol 66(2) (2013) 551-7.

https://www.sciencedirect.com/science/article/pii/S1055790312002060?via%3Dihub
doi: 10.1016/j.ympev.2012.05.028

Abstract

The ever-lingering question: "What did the urmetazoan look like?" has not lost its charm, appeal or elusiveness for one and a half centuries. A solid amount of organismal data give what some feel is a clear answer (e.g. Placozoa are at the base of the metazoan tree of life (ToL)), but a diversity of modern molecular data gives almost as many answers as there are exemplars, and even the largest molecular data sets could not solve the question and sometimes even suggest obvious zoological nonsense. Since the problems involved in this phylogenetic conundrum encompass a wide array of analytical freedom and uncertainty it seems questionable whether a further increase in molecular data (quantity) can solve this classical deep phylogeny problem. This review thus strikes a blow for evaluating quality data (including morphological, molecule morphologies, gene arrangement, and gene loss versus gene gain data) in an appropriate manner.

Chapter II

H.J. Osigus, M. Eitel, M. Bernt, A. Donath, B. Schierwater, Mitogenomics at the base of Metazoa, Mol Phylogenet Evol 69(2) (2013) 339-51.

https://www.sciencedirect.com/science/article/pii/S1055790313002935?via%3Dihub
doi: 10.1016/j.ympev.2013.07.016

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 early branching 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.

Chapter III

M. Eitel, H.J. Osigus, R. DeSalle, B. Schierwater, Global diversity of the Placozoa, Plos One 8(4) (2013) e57131.

https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0057131
doi: 10.1371/journal.pone.0057131

Abstract

The enigmatic animal phylum Placozoa holds a key position in the metazoan Tree of Life. A simple bauplan makes it appear to be the most basal metazoan known and genetic evidence also points to a position close to the last common metazoan ancestor. *Trichoplax adhaerens* is the only formally described species in the phylum to date, making the Placozoa the only monotypic phylum in the animal kingdom. However, recent molecular genetic as well as morphological studies have identified a high level of diversity, and hence a potential high level of taxonomic diversity, within this phylum. Different taxa, possibly at different taxonomic levels, are awaiting description. In this review we firstly summarize knowledge on the morphology, phylogenetic position and ecology of the Placozoa. Secondly, we give an overview of placozoan morphological and genetic diversity and finally present an updated distribution of placozoan populations. We conclude that there is great potential and need to erect new taxa and to establish a firm system for this taxonomic tabula rasa.

Chapter IV

H.J. Osigus, M. Eitel, B. Schierwater, Deep RNA sequencing reveals the smallest known mitochondrial micro exon in animals: The placozoan *cox1* single base pair exon, Plos One 12(5) (2017) e0177959.

https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0177959
doi: 10.1371/journal.pone.0177959

Abstract

The phylum Placozoa holds a key position for our understanding of the evolution of mitochondrial genomes in Metazoa. Placozoans possess large mitochondrial genomes which harbor several remarkable characteristics such as a fragmented cox1 gene and trans-splicing cox1 introns. A previous study also suggested the existence of *cox1* mRNA editing in *Trichoplax adhaerens*, yet the only formally described species in the phylum Placozoa. We have analyzed RNA-seq data of the undescribed sister species, Placozoa sp. H2 ("Panama" clone), with special focus on the mitochondrial mRNA. While we did not find support for a previously postulated *cox1* mRNA editing mechanism, we surprisingly found two independent transcripts representing intermediate *cox1* mRNA splicing stages. Both transcripts consist of partial cox1 exon as well as overlapping intron fragments. The data suggest that the cox1 gene harbors a single base pair (cytosine) micro exon. Furthermore, conserved group I intron structures flank this unique micro exon also in other placozoans. We discuss the evolutionary origin of this micro exon in the context of a self-splicing intron gain in the cox1 gene of the last common ancestor of extant placozoans.

Chapter V

K. Kamm, H.J. Osigus, P.F. Stadler, R. DeSalle, B. Schierwater, *Trichoplax* genomes reveal profound admixture and suggest stable wild populations without bisexual reproduction, Sci Rep 8(1) (2018) 11168.

https://www.nature.com/articles/s41598-018-29400-y
doi: 10.1038/s41598-018-29400-y

Abstract

The phylum Placozoa officially consists of only a single described species, *Trichoplax adhaerens*, although several lineages can be separated by molecular markers, geographical distributions and environmental demands. The placozoan 16S haplotype H2 (*Trichoplax* sp. H2) is the most robust and cosmopolitan lineage of placozoans found to date. In this study, its genome was found to be distinct but highly related to the *Trichoplax adhaerens* reference genome, for remarkably unique reasons. The pattern of variation and allele distribution between the two lineages suggests that both originate from a single interbreeding event in the wild, dating back at least several decades ago, and both seem not to have engaged in sexual reproduction since. We conclude that populations of certain placozoan haplotypes remain stable for long periods without bisexual reproduction. Furthermore, allelic variation within and between the two *Trichoplax* lineages indicates that successful bisexual reproduction between related placozoan lineages might serve to either counter accumulated negative somatic mutations or to cope with changing environmental conditions. On the other hand, enrichment of neutral or beneficial somatic mutations by vegetative reproduction, combined with rare sexual reproduction, could instantaneously boost genetic variation, generating novel ecotypes and eventually species.

Chapter VI

M. Eitel, W.R. Francis, F. Varoqueaux, J. Daraspe, H.J. Osigus, S. Krebs, S. Vargas, H. Blum, G.A. Williams, B. Schierwater, G. Wörheide, Comparative genomics and the nature of placozoan species, Plos Biol 16(7) (2018) e2005359.

https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.2005359
doi: 10.1371/journal.pbio.2005359

Abstract

Placozoans are a phylum of nonbilaterian marine animals currently represented by a single described species, *Trichoplax adhaerens*, Schulze 1883. Placozoans arguably show the simplest animal morphology, which is identical among isolates collected worldwide, despite an apparently sizeable genetic diversity within the phylum. Here, we use a comparative genomics approach for a deeper appreciation of the structure and causes of the deeply diverging lineages in the Placozoa. We generated a high-quality draft genome of the genetic lineage H13 isolated from Hong Kong and compared it to the distantly related *T. adhaerens*. We uncovered substantial structural differences between the two genomes that point to a deep genomic separation and provide support that adaptation by gene duplication is likely a crucial mechanism in placozoan speciation. We further provide genetic evidence for reproductively isolated species and suggest a genus-level difference of H13 to *T. adhaerens*, justifying the designation of H13 as a new species, *Hoilungia hongkongensis* nov. gen., nov. spec., now the second described placozoan species and the first in a new genus. Our multilevel comparative genomics approach is, therefore, likely to prove valuable for species distinctions in other cryptic microscopic animal groups that lack diagnostic morphological characters, such as some nematodes, copepods, rotifers, or mites.

Chapter VII

H.J. Osigus, S. Rolfes, R. Herzog, K. Kamm, B. Schierwater, *Polyplacotoma mediterranea* is a new ramified placozoan species, Curr Biol 29(5) (2019) R148-R149.

https://www.cell.com/current-biology/fulltext/S0960-9822(19)30097-1
doi: 10.1016/j.cub.2019.01.068

Abstract

The enigmatic phylum Placozoa is harboring an unknown number of cryptic species and has become a challenge for modern systematics. Only recently, a second species has been described [1], while the presence of more than a hundred additional species has been suggested [2]. The original placozoan species *Trichoplax adhaerens* [3], the second species *Hoilungia hongkongensis* [1] and all yet undescribed species are morphologically indistinguishable (i.e. no species diagnostic characters are available [4]). Here, we report on a new placozoan species, *Polyplacotoma mediterranea* gen. nov., spec. nov., which differs from other placozoans in its completely different morphological habitus, including long polytomous body branches and a maximum body length of more than 10 mm. *Polyplacotoma mediterranea* also necessitates a different view of placozoan mitochondrial genetics. *P. mediterranea* harbors a highly compact mitochondrial genome with overlapping mitochondrial tRNA and protein coding genes. Furthermore, the new species lacks typical placozoan features, including the *cox1* micro exon and *cox1* barcode intron. As phylogenetic analyses suggest a sister group relationship of *P. mediterranea* to all other placozoans, this new species may also be relevant for studies addressing the relationships at the base of the metazoan tree of life.

Chapter VIII

H.J. Osigus[1], M. Eitel[3], K. Kamm[1], S. Rolfes[1], M. Tessler[2], A. Narechania[2], R. DeSalle[2], B. Schierwater[1], Accumulation of GC-rich hairpins in large placozoan mitochondrial genomes, unpublished

[1] Stiftung Tierärztliche Hochschule Hannover, ITZ, Ecology & Evolution, Bünteweg 17d, 30559 Hannover, Germany.
[2] American Museum of Natural History, Sackler Institute for Comparative Genomics and Division of Invertebrate Zoology, New York, NY, USA.
[3] Ludwig-Maximilians-Universität München, Department of Earth and Environmental Sciences, Paleontology and Geobiology, München, Germany

Abstract

Placozoans are an important model system to understand the evolution of mitochondrial genomes in early branching metazoans. The size of placozoan mitochondrial genomes reaches 40 kb and more, mostly due to the presence of introns, large intergenic spacers and open reading frames of unknown function. We here present complete mitochondrial genomes from two members of the *Trichoplax* 16S clade I, namely haplotype H17 "Keio" and haplotype H2 "Panama", which are sister lineages to *Trichoplax adhaerens* H1 "Grell". With a size of 43.183 bp the H17 mitochondrial genome is slightly larger than its counterpart in *T. adhaerens* (43.079 bp). The H2 mtDNA (44.210 bp) represents the largest known placozoan mitochondrial genome. While the overall gene order is identical in all three lineages, major differences are seen with respect to the number of predicted open reading frames. A most striking feature shared by all three genomes is the unusual high abundance of hairpins belonging to the 5'-GGVBCC-$(N)_3$-GGVBCC-3' hairpin family, which are present even within protein coding genes. Hairpins located in intergenic regions are also discussed in the evolutionary context of having played a potential role in mitogenome rearrangements. The enrichment of derived characteristics in clade I mtDNAs also provides new evidences for secondary mitogenome size amplification in Placozoa.

Introduction

Placozoans are among the earliest branching metazoans and are of crucial importance for understanding the early evolution of animal mitochondrial genomes (1-4). The phylum Placozoa so far consists of only three described species (*Trichoplax adhaerens* H1 (5), *Hoilungia hongkongensis* H13 (6) and *Polyplacotoma mediterranea* H0 (7)). However, numerous studies have revealed a remarkably higher diversity in this phylum, comprising at least 17 additional genetic lineages (for overview, see e.g. 8).

Complete mitochondrial genomes have been described for *P. mediterranea* H0, *T. adhaerens* H1, *H. hongkongensis* H13 as well as haplotypes H3, H4, H8 and H15 (6,7,9-11). The mitochondrial genome of *P. mediterranea* H0 is a highly compact molecule (23.5 kb), which derives from a different evolutionary path than the *Trichoplax* and *Hoilungia* mitogenomes (7). We here mainly focus on mitochondrial genomes from the former placozoan groups A (now *Hoilungia*-group) and B (now *Trichoplax*-group) (12). With a size of up to more than 40 kb, mitochondrial genomes from these groups are among the largest known circular mtDNAs from non-bilaterian animals (3). They encode 12 protein coding genes (*nad1-nad6, nad4L, cob, cox1-3, atp6*), 2 ribosomal RNAs (12S and 16S), 24 tRNAs, and also harbor remarkable features like numerous introns, a *cox1* micro exon, large intergenic regions and variable numbers of open reading frames of unknown function (for overview, see e.g. 13). While the protein coding sequence evolution rate is low (compared to e.g. Ctenophora or Bilateria (13)), there is a high tendency for mt genome rearrangements (11). The molecular mechanisms underlying such rearrangements are unknown for placozoans, but studies from distant eukaryotic taxa have suggested that hairpin forming elements might be involved in such processes (see e.g. 14,15). In non-bilaterian animals, hairpin forming elements have been reported e.g. from sponge mitochondrial genomes (16,17), and also have been mentioned in studies on placozoans (see 11,12,18). However, in case of Placozoa, these mitochondrial hairpins have not yet been analyzed in an evolutionary context.

In order to better understand the evolution and maintenance of large mitochondrial genomes in placozoans, we analyzed the complete mitogenomes of haplotypes H17 “Keio” and H2 “Panama” (19), both from the *Trichoplax* 16S

clade I, respectively (8). Haplotype H17 has originally been described based on a sample from Monterey Bay, California, USA (20), and afterwards has been reported from the coast of Japan, indicating a broad distribution of H17 in the Pacific Ocean (21). Members of haplotype H2 are abundant in all tropical and sub-tropical marine waters, but can also be found in temperate marine ecosystems (8). So far, the mitochondrial lineage H2 has been the most frequently sampled placozoan lineage, which also shows the broadest latitudinal distribution (2,8).

Material and Methods

Animal material

The H17 "Keio" clone has been collected in 2010 by Dr. Hiroyuki Kaneko and Dr. Ritsu Kuraishi in Japan and a clonal lineage has been maintained in Hannover since 2014 as already described (12). The genetic lineage H2 'Panama` has likewise been cultured in Hannover as previously described (12,22). For both lineages total DNA was extracted from clonal animals using standard protocols (23).

DNA sequencing, data processing and sequence analyses

Sequencing of total DNA from haplotype H17 "Keio" was performed on an Illumina HiSeq2500 machine (2x125 bp) at the New York Genome Center. In total, 147.067.540 paired-end reads were generated. The H17 "Keio" mitochondrial genome was assembled in Geneious version 8.x (24) using an iterative mapping approach (see e.g. 25) with the 16S rDNA sequence of H17 (20) as "seed".

The H2 "Panama" total DNA was sequenced on (A) an Illumina GAIIx sequencer (72 bp fragments) and (B) an Illumina HiSeq 2500 system (151 bp fragments). The two sequencing approaches resulted in a total of 85.390.360 and 56.428.444 paired-end reads, respectively (22). Draft versions of the mitochondrial genome of H2 "Panama were independently generated from the two data sets using the following pipelines: A) Error correction of the 72 bp reads was conducted with the stand alone error correction script of ALLPATHS-LG (26,27). The assembly of the 72 bp reads was afterwards done using the

ABySS assembler (28) with subsequent reassembly of reads using CAP3 (29). B) The de novo assembly of the 151 bp reads was performed as already described (22). C) The 151 bp reads were mapped on the 16S rDNA sequence of H2 'Panama' (19) with subsequent bidirectional extension in multiple iteration steps. Resulting draft mitochondrial genome sequences were inspected, and poorly resolved GC-rich repetitive regions were reanalyzed and approved by mapping of reads against respective regions and subsequent manual corrections in Geneious (24).

Mitochondrial protein coding and ribosomal RNA genes were annotated using available placozoan mitochondrial genomes as reference (9,11). Predicted gene boundaries were subsequently confirmed via blast-search (30). In addition, mitochondrial tRNA genes were predicted using tRNAscan-SE (31). The whole mitochondrial genome sequences were screened for additional open reading frames via ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) using stringent parameters (minimal ORF length >300 bp, only "ATG" start codon permitted). K-mer searches were performed using wordcount (http://www.bioinformatics.nl/cgi-bin/emboss/wordcount). Direct repeat searches and screening for intact and rudimental GC-rich hairpins were afterwards conducted in Geneious (24).

For pairwise genetic distance calculations, single gene nucleotide and amino acid alignments were generated with MAFFT (32) as implemented in Geneious (24). In detail, nucleotide and amino acid sequences have been extracted from the already published mitochondrial genome of *Trichoplax adhaerens* H1 (NC_008151) (9) and afterwards added to the data sets which already comprise nucleotide and amino acid sequences from H2 "Panama" and H17 "Keio", respectively. Genetic distances were afterwards calculated in Geneious (24). For overall sequence similarity calculations, single gene alignments have been concatenated and likewise analyzed accordingly.

Nucleotide sequences of the mitochondrial trnSer (uga) gene have been extracted from previously published mitochondrial genomes (9-11) and have been added to trnSer (uga) gene sequences from the new placozoan mitochondrial genomes (Chapters VI, VII, VIII and IX). The nucleotide sequence

alignment has likewise been generated in MAFFT (32) as implemented in Geneious (24).

Results and Discussion

General characteristics of H2 and H17 mitochondrial genomes

The mitochondrial genome of H2 'Panama' is a circular molecule with a size of 44.210 bp, thus so far being the largest known placozoan mitochondrial genome. It is more than 1 kb larger than the mitochondrial genome of *T. adhaerens* H1 (43.079 bp) (9) and even almost 12 kb larger than the mitochondrial genome of haplotype H8 (32.661 bp) (11). The circular mitochondrial genome of H17 is, at a size of 43.183 bp, just slightly larger than the mitochondrial genome of *T. adhaerens* H1. In general, a mitogenome size of >40 kb is a shared feature between H1, H2 and H17, and therefore likely a synapomorphy of clade I.

The observed gene order in H2 and H17 is overall identical compared to the reference mitochondrial genome of *T. adhaerens* H1 (9), but small sequence insertions/deletions are distributed across all clade I mt genomes. The most notable difference in H2 relates to the intergenic region between trnP (ugg) and *cox2*, which is substantially larger than seen in H1 and H17, respectively. This variable intergenic spacer shows at least very low sequence similarity to the haplotype H4 (clade V) mitogenome (11). Like all placozoan mitochondrial genomes, the H2 and H17 mitogenomes encode 12 mitochondrial respiratory chain genes (*nad1-nad6, nad4L, cob, cox1-3* and *atp6*) and lack genes coding for a*tp8* and *atp9*. In addition to 2 ribosomal RNA genes (12S and 16S), a set of 24 mt tRNA genes is seen in H2 and H17, respectively. The exon/intron structure of *cox1*, *nad5* and 16S in H2/H17 is identical to the corresponding gene structures in *T. adhaerens* H1 (including the *cox1* micro exon; (33)). All protein coding genes of H2 and H17 (except *nad4L,* which has a GTG start codon) posses an ATG start codon and complete stop codons (TAA or TAG, respectively).

The analysis of mitochondrial protein coding genes revealed an overall high sequence similarity (on both, the nucleotide (nt) and the amino acid (aa) level) between the three clade I placozoans (Figure 1). The protein coding gene similarity values between *T. adhaerens* H1 and H17 generally match the similarity value of the diagnostic 16S rDNA fragment (19) (99,6%), although the

cox3 gene is an outlier in this comparison. On the opposite site, the 16S fragment similarity values between *T. adhaerens* H1 vs. H2 (96,6%) and H17 vs. H2 (96,3%) generally overestimate the actual sequence divergences on the protein coding sequence level (with the major exception of the *nad2* gene).

A conservative prediction of mitochondrial open reading frames in introns/intergenic regions using the NCBI ORFfinder reveals a higher than expected number of open reading frames in the *Trichoplax* clade I (see also Chapter IX in this thesis and Digital appendix). While previous studies predicted up to eight open reading frames in the *T. adhaerens* H1 mitochondrial genome (9), our reanalysis revealed up to 13 predicted open reading frames. In comparison, the closely related H17 mitogenome possesses 15 predicted open reading frames, and even 18 predicted open reading frames are found in the H2 mitogenome. It should be highlighted that the total number of predicted ORFs in all mitogenomes could be even higher if less stringent search parameters would be applied. However, except for a putative reverse transcriptase and a homing endonuclease, none of the stringently predicted open reading frames yield any reliable blast hit in the database. Therefore, the functionality of any other hypothetical ORF, which would have been predicted under more relaxed parameters, would be even more speculative. The deviant number of ORFs in closely related placozoan lineages, however, indicates a high sequence evolution rate in intron/intergenic spacer regions in clade I mitogenomes (11). Potentially, the differences in mitochondrial ORF content might somehow relate to speciation processes in placozoans (11).

GC-content and sequence motifs

In order to better understand the evolutionary pathways of large placozoan mitochondrial genomes, we compared available mt genomes with respect to nucleotide composition and overrepresented sequence motifs (see Digital appendix). With a GC-content of 48% the H2 mitochondrial genome possesses the highest known GC-content among placozoans. Compared to the mitochondrial genome of *P. mediterranea* (32,9% GC-content), the GG-content in H2 is substantially (i.e. 15%) higher. The lowest GC-content outside *Polyplacotoma* can be found in H8 (39.2%) (11), which is 9% below the value

observed in H2, but still more than 6% higher than in H0. With 47,1%, the mitochondrial genome of H17 has an almost identical GC-content like the mitogenome of *T. adhaerens* H1 (47%). Similarly GC-rich mitochondrial genomes have mostly been known from higher metazoans (e.g. birds and fishes, Organelle Genome Resources, 2019, https://www.ncbi.nlm.nih.gov/genome/organelle/) and only as sporadic exceptions from some Cnidaria and Porifera (e.g. 17,34). Thus we hypothesize that the high GC-content (especially observed in *T. adhaerens* H1, H2 and H17, respectively) might be a potentially derived character and a synapomorphy of the placozoan clade I.

The systematic screening for overrepresented sequence motifs (k-mers) revealed a differential distribution of specific hexamer sequences between placozoan clades (see Digital appendix). In clade V mitochondrial genomes, mononucleotide motifs (poly-“A”, -“T”, -“G” or -“C”) are particularly frequent among hexamer sequences. In clade III, poly-“G” and poly-“C” stretches are less abundant among the ten most frequent hexamer motifs, although clade III and clade V are closely related (8). Possible explanations for the lower abundance of poly-“G” and poly-“C” stretches in clade III are either a higher sequence motif heterogeneity among GC-rich hexamers and/or the overall lower GC-content in the mitogenome. In the *Trichoplax*-group, the picture is likewise complex. The H3 mitochondrial genome (clade II) (11) also mainly possesses AT-rich hexamer motifs (similar to clade III and V), while poly-“G” and poly-“C” stretches are less abundant (as seen in clade III, but in contrast to clade V mitogenomes). However, two complex GC-rich motifs still can be found in H3 among the ten most frequent hexamers. In contrast to the clade II/III/V mitogenomes, the three clade I mitochondrial genomes (i.e. from *T. adhaerens* H1, H2 and H17) show a clear overrepresentation of GC-rich complex hexamers. Among the ten most frequent hexamers in these three placozoans, there are three motifs (i.e. 5’-GGCGCC-3’, 5’-GGATCC-3’, and 5’-GGGCCC-3’), which potentially form hairpin structures if a second copy of this motif is located nearby on the same strand as a direct repeat. These three specific motifs can likewise be found in all other placozoan mitochondrial genomes (except for 5’-GGCGCC-3’ in H0), albeit with a far lower abundance.

GC-rich hairpin structures

A global screening for the distribution of the three candidate hairpin motifs 5'-GGCGCC-3', 5'-GGATCC-3', and 5'-GGGCCC-3' in placozoan mitochondrial genomes reveals that these motifs frequently occur as direct repeats on the same DNA strand. Despite some deviations, the majority of these direct repeats comprise a 3 bp spacer (see Digital appendix). The resulting 15-mers (i.e. 5'-GGCGCC-$(N)_3$-GGCGCC-3', 5'-GGATCC-$(N)_3$-GGATCC-3', and 5'-GGGCCC-$(N)_3$-GGGCCC-3') therefore depict a multiple of a triplet. These 15-mer hairpins even frequently occur within protein coding genes (Figure 2). Exceptionally occurring hairpin length deviations within protein coding sequences (i.e. not a multiple of a triplet) are mostly found in anyway variable regions, which finally preserve the open reading frame.

The strong numerical bias towards 15-mer hairpins even outside protein coding sequences may indicate a global selective pressure on these 15-mer hairpins due to functional constraints. Nevertheless, motif and/or secondary structure disrupting point mutations can be found, indicating simultaneously acting hairpin-loss processes. Surprisingly, different hairpins located at the same homologous mitogenome locus can occur scattered for instance in *T. adhaerens* H1, H2 and H17, indicating sporadic transformations of hairpins. Together with the striking sequence similarity, this observation supports the hypothesis that all three hairpins belong to the same hairpin family, i.e. the placozoan 5'-GGVBCC-$(N)_3$-GGVBCC-3' hairpin family, which we herewith define in this study.

The evolutionary origin of placozoan hairpin structures is unclear. Hairpin structures have previously been reported e.g. in sponge mitochondrial genomes (16,17), but their diverging sequences do not indicate a common origin with the hairpins found in placozoans. As placozoan mitochondrial hairpins are GC-rich, it generally does not seem likely that they are of metazoan mitochondrial origin, but rather are mobile non-metazoan genetic elements (see also discussion in 16). However, it remains speculative if they originally have been integrated into the mitogenome as isolated hairpins, or together with an intron or open reading frame.

Like the unknown origin of GC-rich hairpins, the point of emergence of these hairpins in placozoan mitogenomes remains elusive. The mere absence of

intact 5'-GGVBCC-$(N)_3$-GGVBCC-3' hairpins in *P. mediterranea* H0 (7) suggests on the first sight that these hairpins might have been introduced into placozoan mitogenomes after the split-off of *P. mediterranea*. However, a detailed analysis of the trnSer (uga) gene predates this scenario: A shared feature of all placozoan mitochondrial trnSer (uga) genes is the presence of an additional variable stem-loop, which is not conserved among metazoans (see e.g. 35). In clade V and VII placozoans, this variable loop consists of a perfect 5'-GGCGCC-$(N)_3$-GGCGCC-3' hairpin. Over clade IV, III, II and I up to *P. mediterranea*, this stem-loop region shows decreasing sequence similarity to the original hairpin, although the respective stem-loop sequences remain GC-rich (Figure 3). As multiple independent invasions seem unlikely, we instead deduce a single evolutionary origin of this specific tRNA stem-loop. In detail, we hypothesize that a perfect 5'-GGCGCC-$(N)_3$-GGCGCC-3' hairpin has been integrated into the trnSer (uga) gene of the last common ancestor of all extant placozoans and that this hairpin sequence subsequently evolved differentially in different placozoan taxa. However, it remains unknown if the trnSer (uga) hairpin has been introduced from outside the mitogenome (maybe even as the original "founder" hairpin) or if this specific hairpin integration was the result of an intra-mitogenomic hairpin proliferation event (implying that the hairpin itself was already established at another locus in the mitogenome).

The molecular mechanisms, which either influence the grade of hairpin proliferation or loss, respectively, are unclear. The mere presence of hairpins in mitochondrial genomes itself does not seem to lead to an excessive enrichment of these structures. The enrichment of hairpins potentially might be linked to specific mitochondrial open reading frames, which are (or temporary were) present in some placozoan mitochondrial genomes. Another future approach should also focus on nuclear encoded mitochondrial genes which are related for instance to mitochondrial DNA replication processes. Especially the nuclear genome of haplotype H3 might be a valuable resource for such a comparative approach, as H3 is the sister to the hairpin-rich placozoan clade I (8,12), but itself does not possess an increased mitochondrial hairpin content.

Concerning their potential function, hairpin structures generally have been assigned to the regulation of replication and/or transcription processes in

mitochondrial genomes, especially in the mitochondrial D-loop/control region (36). However, there are several aspects, which contradict this hypothesis for placozoans: 1. The complete absence of intact 5'-GGVBCC-$(N)_3$-GGVBCC-3' hairpins in *P. mediterranea*; 2. An overrepresentation of hairpins in clade I placozoans; 3. The integration of hairpin structures even within protein coding sequences. On the opposite site, the obvious strong numerical bias on intact 15-mer hairpin structures even outside protein coding sequences indicates a still acting selective pressure and functional constraints on these specific hairpin secondary structures.

It has also previously been assumed that DNA secondary structures like (double) hairpins might favor mitochondrial genome rearrangements (e.g. 14,15). We found evidence for this scenario in placozoans for instance in the placozoan clade V mitogenomes. The major difference between clade V haplotype H4 and H15 mitogenomes is the translocation of a fragment containing the *PolB* gene+ORF126 (10). Remarkably, one excision site boundary and one insertion site boundary in H15, respectively, still possesses hairpin structures and/or imperfect hairpin motifs. These molecular signatures might represent relicts of the previous rearrangement event, which potentially was catalyzed by these hairpins. We therefore postulate the hypothesis, that the long-term establishment of CG-rich hairpins was a starting point for rearrangement events in placozoan mitochondrial genomes. However, it is likely that additional molecular mechanisms are involved in rearrangement/insertion/deletion events and therefore have likewise shaped placozoan mitogenomes.

<u>An updated working hypothesis on mitogenome evolution in early diverging metazoans</u>

If compared to other placozoan mitochondrial genomes (i.e. from the *Polyplacotoma*-, the *Hoilungia*-, and even to clade II from the *Trichoplax*-group), the insights gained from clade I mitogenomes call for a revised scenario of placozoan mtDNA evolution. In order to establish an improved evolutionary working hypothesis, three informative characteristics, which are most prominent in clade I mitogenomes, should be highlighted. First, a remarkably high degree of gene fragmentation (illustrated in extreme by the intron gain

which leads to the isolation of the *cox1* micro exon); second, an exceptionally high frequency of (either intact or secondarily disrupted) ORFs and third, an accumulation of hairpin structures. All of these derived characteristics support a scenario of secondary mtDNA size amplification in clade I. As the three previously mentioned characteristics are less pronounced in clade II-VII, mitogenomes from these clades can be seen as intermediate size stages, while the highly compact *Polyplacotoma* mtDNA maintained most of the presumably ancestral characteristics. A summary of deduced overall evolutionary pathways of derived mitogenomes in the four non-bilaterian phyla is illustrated in Figure 4, but see also related scenarios discussed for instance in (3,37,38). With special focus on Placozoa, it should be highlighted, however, that the inferred general tendency for size amplification in placozoan mtDNAs is sometimes at least partially compensated by sporadic losses of introns and/or ORFs. Furthermore, all current approaches to infer mitogenome characteristics of the last common metazoan ancestor still suffer from limited data availability from closely related non-metazoan outgroups (i.e. especially from choanoflagellates (39)), and therefore should be taken with caution.

Implications on systematics and taxonomy within the *Trichoplax*-group

The new mitochondrial genomes from clade I stimulate further discussions on the taxonomic status within the *Trichoplax*-group. A previous comparative study on the nuclear genomes of *Trichoplax adhaerens* H1 "Grell" and *Trichoplax* sp. H2 "Panama" revealed a high degree of allele sharing between both lineages (22). A high degree of allele sharing between different H1 and H2 lineages was likewise reported in an independent study restricted on nuclear-encoded ribosomal proteins (6). The mitochondrial and endosymbiont (Kamm et al., in prep.) genomes from *Trichoplax adhaerens* H1 "Grell" and *Trichoplax* sp. H2 "Panama" are overall similar as well, but nevertheless show substantial differences beyond the single nucleotide polymorphism level. In sum, both placozoan lineages show clear signatures for an ongoing diversification process, a picture, which is likewise supported by differences in the temperature/pH sensitivity (40) as well as their differential global abundance in the field (2). However, it still remains

unclear if H1 and H2 are only two different subspecies of *Trichoplax adhaerens*, or alternatively, if H2 already represents a new *Trichoplax* species.

In contrast to *Trichoplax* sp. H2 "Panama", the analyses of whole nuclear as well as endosymbiont genome data from the lineage H17 "Keio" are still in progress. Nevertheless, the analysis of the complete mitochondrial genome of H17 revealed an exceptional high degree of sequence similarity to the complete mitochondrial genome of *Trichoplax adhaerens* H1 "Grell" (i.e. 96,5%). As the similarity of concatenated protein coding nucleotide sequences from both lineages is even higher (i.e. 99,6%), it is reasonable to assume that H17 "Keio" is just a second haplotype of the formally described species *Trichoplax adhaerens* H1 "Grell", and not a distinct placozoan species.

The taxonomic status of the second 16S clade within the *Trichoplax*-group (i.e. clade II) remains likewise uncertain. However, following the concept applied to clade I, the only haplotype within clade II (i.e. H3) might represents at least a different *Trichoplax* species. Future whole nuclear genome approaches are nevertheless needed to gain further support for this hypothesis.

Conclusions

The two mitochondrial genomes of haplotype H2 and H17 provide important insights into the evolution of large placozoan mitogenomes. The comparative mitogenome analyses with special focus on derived characteristics like hairpins provide evidence for secondary size amplification in *Trichoplax* clade I mitochondrial genomes. Additional molecular data, however, are needed to further understand the underlying mechanisms. It is furthermore suggestive and quite intriguing to discuss the maintaining of costly large mt genomes in the context of ecological radiation, since haplotype H2 (which possesses the largest mitochondrial genomes and the highest number of GC-rich hairpins) is by far the most frequently sampled lineage in the field and apparently inhabits the broadest ecological niche among all known placozoans.

Acknowledgements

HJO acknowledges a doctoral fellowship of the Studienstiftung des Deutschen Volkes. We acknowledge support from the German Science Foundation (DFG Schi-277/26, Schi-277/27, Schi-277/29). We thank Dr. Hiroyuki Kaneko and Dr. Ritsu Kuraishi (Research and Education Center for Natural Sciences, Keio University, 4-1-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8521, Japan) for providing live animals of haplotype H17 “Keio”.

Digital appendix

Further information related to this chapter can be found in the Digital appendix of this thesis.

A)

Nucleotide identity (%)			
	H1 vs. H17	H1 vs. H2	H17 vs. H2
atp6	99,5	97,4	97,4
cox1	99,9	99,7	99,6
cox2	99,7	99,2	99,2
cox3	98,4	99,1	97,7
cob	100	99	99
nad1	99,9	98,6	98,7
nad2	99,2	94,6	94,3
nad3	99,7	99,7	100
nad4	99,9	98,3	98,4
nad4L	100	100	100
nad5	99,8	98,3	98,5
nad6	99,3	98	97,6
concat	99,6	98,1	98,0

B)

Protein identity (%)			
	H1 vs. H17	H1 vs. H2	H17 vs. H2
atp6	99,6	96,5	96,9
cox1	100	100	100
cox2	99,6	99,2	98,8
cox3	98,4	99	97,4
cob	100	99	99
nad1	100	98,3	98,3
nad2	99,1	92	91,8
nad3	100	100	100
nad4	99,8	97,8	97,8
nad4L	100	100	100
nad5	100	98,4	98,4
nad6	99,3	97,5	96,9
concat	99,6	97,6	97,4

C)

Nucleotide identity (%)			
	H1 vs. H17	H1 vs. H2	H17 vs. H2
16S frag.	99,6	96,6	96,3

Figure 1

Mitochondrial genetic similarities between *Trichoplax adhaerens* H1, *Trichoplax* sp. H2 and haplotype H17.

Pairwise nucleotide (A) and amino acid (B) similarity values of mitochondrial protein coding genes for the three clade I placozoan lineages. The respective lowest similarity value is highlighted in each pairwise comparison. The similarity values for concatenated gene sequences are given at the bottom of the respective table. The pairwise nucleotide similarity values of the diagnostic 16S rDNA fragment for the three clade I placozoan lineages are given in (C).

A)

Size		GGCGCCNNNGGCGCC	GGATCCNNNGGATCC	GGGCCCNNNGGGCCC	Total number of mt hairpins
43.079	H1	122	56	20	**198**
43.183	H17	126	48	17	**191**
44.210	H2	163	56	20	**239**
36.699	H3	15	6	5	**26**
32.661	H8	5	26	1	**32**
37.194	H4	31	27	1	**59**
36.676	H15	27	24	1	**52**
36.537	H13	33	25	1	**59**

B)

Size		GGCGCCNNNGGCGCC	GGATCCNNNGGATCC	GGGCCCNNNGGGCCC	Total number of mt hairpins in CDS
43.079	H1	nad2(4), nad5(2),nad6(4)	atp6 (1), nad1 (1), nad2 (2), nad5 (1)	nad2 (1)	**16**
43.183	H17	nad2(3), nad5(2),nad6(4)	atp6 (1), nad1 (1), nad2 (2), nad5 (1)	nad2 (1)	**15**
44.210	H2	atp6(1), nad1(1), nad2(4),nad4(1), nad5(2), nad6(3)	nad2 (2), nad5 (1)	nad2 (1)	**16**
36.699	H3	nad1(1)	nad2 (1)	nad2 (1), nad5 (1)	**4**
32.661	H8	0	cox2 (2), cox3 (1), nad1 (1), nad2 (4), nad4 (1)	0	**9**
37.194	H4	nad6(1)	nad1 (1), nad2 (1), nad5 (2)	0	**5**
36.676	H15	nad6(1)	nad1 (1), nad2 (1), nad5 (1)	0	**4**
36.537	H13	nad6(1)	nad1 (1), nad2 (1), nad5 (1)	0	**4**

Figure 2

Frequency of occurrence of the three 5'-GGVBCC-$(N)_3$-GGVBCC-3' hairpins in placozoan mitogenomes. Placozoan lineages which belong to the same 16S clade are highlighted by identical colors.

A) Total numbers of intact 5'-GGVBCC-$(N)_3$-GGVBCC-3' hairpins in the mtDNAs of different placozoan lineages.

B) Numbers of intact 5'-GGVBCC-$(N)_3$-GGVBCC-3' hairpins in mitochondrial protein coding sequences of respective placozoan lineages. The number of respective hairpins in specific protein coding genes is given in brackets.

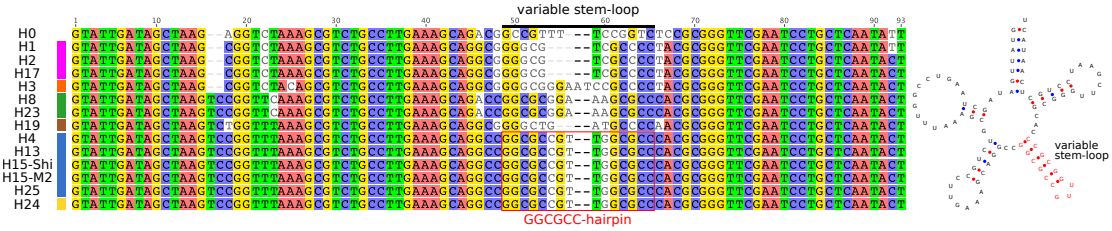

Figure 3

Nucleotide comparison of the placozoan mitochondrial tRNA-serine (uga).

The nucleotide sequences of the mitochondrial trnSer (uga) from 14 highly diverse placozoan lineages have been aligned. The vertical color bars next to the haplotype numbers are corresponding to the respective 16S clade. Colored nucleotides within the alignment are in agreement with the 50%-majority consensus sequence. The variable stem-loop region (position 49-65) is indicated by a black bar on top of the alignment. The intact 5'-GGCGCC-$(N)_3$-GGCGCC-3' hairpin in H4, H13, H15, H24 and H25, respectively, is highlighted by a red box within the alignment. The tRNA secondary structure (as predicted by tRNAscan-

SE) on the right side corresponds to the trnSer (uga) in *Hoilungia hongkongensis* H13. The variable stem-loop (i.e. an intact 5'-GGCGCC-$(N)_3$-GGCGCC-3' hairpin) is highlighted by red nucleotides.

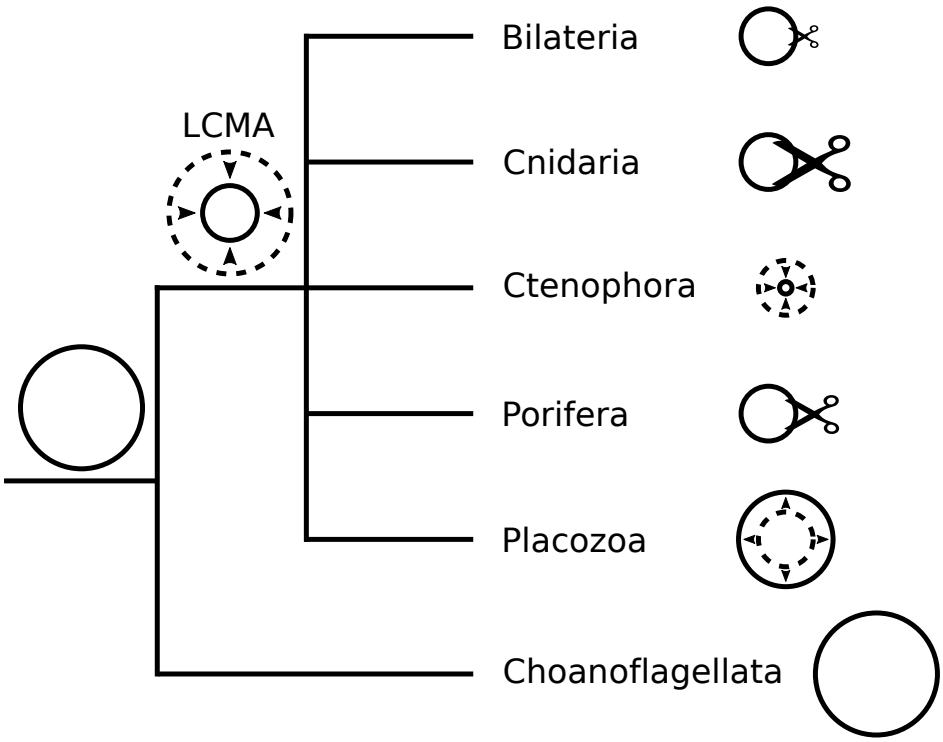

Figure 4

Working hypothesis on the evolution of mitochondrial genomes in early diverging metazoans.

In this scenario, a compaction of the mitochondrial genome has occurred along the stem leading from the hypothetical urmetazoon to the last common metazoan ancestor (LCMA), mostly due to the transfer of mitochondrial ribosomal proteins into the nuclear genome. A shared compact state of the mtDNA was afterwards the starting point for independent (but sometimes parallel) evolution of mitogenomes in non-Bilateria. The sizes of circular mitochondrial chromosomes are only relative approximations and are not directly to scale. The size of scissors corresponds to the overall frequency of mitogenome fragmentation in respective taxa. Please note that fragmentations might imply (multipartite) linear or multipartite circular mitochondrial chromosomes, respectively.

References

1. Schierwater, B. and DeSalle, R. (2018) Placozoa. *Curr Biol*, **28**, R97-R98.
2. Voigt, O. and Eitel, M. (2018) In Schmidt-Rhaesa, A. (ed.), *Miscellaneous Invertebrates*. De Gruyter, Berlin, Boston, pp. 41-54.
3. Lavrov, D.V. and Pett, W. (2016) Animal Mitochondrial DNA as We Do Not Know It: mt-Genome Organization and Evolution in Nonbilaterian Lineages. *Genome Biol Evol*, **8**, 2896-2913.
4. DeSalle, R., Schierwater, B. and Hadrys, H. (2017) MtDNA: The small workhorse of evolutionary studies. *Front Biosci-Landmrk*, **22**, 873-887.
5. Schulze, F.E. (1883) Trichoplax adhaerens, nov. gen., nov. spec. *Zoologischer Anzeiger*, **6**, 92-97.
6. Eitel, M., Francis, W.R., Varoqueaux, F., Daraspe, J., Osigus, H.J., Krebs, S., Vargas, S., Blum, H., Williams, G.A., Schierwater, B. *et al.* (2018) Comparative genomics and the nature of placozoan species. *PLoS Biol*, **16**, e2005359.
7. Osigus, H.J., Rolfes, S., Herzog, R., Kamm, K. and Schierwater, B. (2019) Polyplacotoma mediterranea is a new ramified placozoan species. *Curr Biol*, **29**, R148-R149.
8. Eitel, M., Osigus, H.J., DeSalle, R. and Schierwater, B. (2013) Global diversity of the Placozoa. *PLoS One*, **8**, e57131.
9. Dellaporta, S.L., Xu, A., Sagasser, S., Jakob, W., Moreno, M.A., Buss, L.W. and Schierwater, B. (2006) Mitochondrial genome of Trichoplax adhaerens supports Placozoa as the basal lower metazoan phylum. *Proceedings of the National Academy of Sciences USA*, **103**, 8751-8756.
10. Miyazawa, H., Yoshida, M.A., Tsuneki, K. and Furuya, H. (2012) Mitochondrial genome of a Japanese placozoan. *Zoolog Sci*, **29**, 223-228.
11. Signorovitch, A.Y., Buss, L.W. and Dellaporta, S.L. (2007) Comparative genomics of large mitochondria in placozoans. *PLoS Genetics*, **3**, e13.
12. Eitel, M. and Schierwater, B. (2010) The phylogeography of the Placozoa suggests a taxon-rich phylum in tropical and subtropical waters. *Mol Ecol*, **19**, 2315-2327.
13. Osigus, H.J., Eitel, M., Bernt, M., Donath, A. and Schierwater, B. (2013) Mitogenomics at the base of Metazoa. *Mol Phylogenet Evol*, **69**, 339-351.
14. Paquin, B., Laforest, M.J. and Lang, B.F. (2000) Double-hairpin elements in the mitochondrial DNA of allomyces: evidence for mobility. *Mol Biol Evol*, **17**, 1760-1768.
15. Kim, J.I., Yoon, H.S., Yi, G., Shin, W. and Archibald, J.M. (2018) Comparative mitochondrial genomics of cryptophyte algae: gene shuffling and dynamic mobile genetic elements. *BMC Genomics*, **19**, 275.
16. Erpenbeck, D., Voigt, O., Worheide, G. and Lavrov, D.V. (2009) The mitochondrial genomes of sponges provide evidence for multiple invasions by Repetitive Hairpin-forming Elements (RHE). *BMC Genomics*, **10**, 591.
17. Lavrov, D.V. (2010) Rapid proliferation of repetitive palindromic elements in mtDNA of the endemic Baikalian sponge Lubomirskia baicalensis. *Mol Biol Evol*, **27**, 757-760.

18. Signorovitch, A.Y., Dellaporta, S.L. and Buss, L.W. (2006) Caribbean placozoan phylogeography. *Biological Bulletin*, **211**, 149-156.
19. Voigt, O., Collins, A.G., Pearse, V.B., Pearse, J.S., Ender, A., Hadrys, H. and Schierwater, B. (2004) Placozoa -- no longer a phylum of one. *Curr Biol*, **14**, R944-945.
20. Pearse, V.B. and Voigt, O. (2007) Field biology of placozoans (Trichoplax): distribution, diversity, biotic interactions. *Integr Comp Biol*, **47**, 677-692.
21. Miyazawa, H. and Nakano, H. (2018) Multiple surveys employing a new sample-processing protocol reveal the genetic diversity of placozoans in Japan. *Ecol Evol*, **8**, 2407-2417.
22. Kamm, K., Osigus, H.J., Stadler, P.F., DeSalle, R. and Schierwater, B. (2018) Trichoplax genomes reveal profound admixture and suggest stable wild populations without bisexual reproduction. *Sci Rep*, **8**, 11168.
23. Ender, A. and Schierwater, B. (2003) Placozoa are not derived cnidarians: evidence from molecular morphology. *Mol Biol Evol*, **20**, 130-134.
24. Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C. *et al.* (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, **28**, 1647-1649.
25. Herzog, R., Osigus, H.J., Feindt, W., Schierwater, B. and Hadrys, H. (2016) The complete mitochondrial genome of the emperor dragonfly Anax imperator LEACH, 1815 (Odonata : Aeshnidae) via NGS sequencing. *Mitochondrial DNA Part B*, **1**, 783-786.
26. Gnerre, S., Maccallum, I., Przybylski, D., Ribeiro, F.J., Burton, J.N., Walker, B.J., Sharpe, T., Hall, G., Shea, T.P., Sykes, S. *et al.* (2011) High-quality draft assemblies of mammalian genomes from massively parallel sequence data. *Proc Natl Acad Sci U S A*, **108**, 1513-1518.
27. Ribeiro, F.J., Przybylski, D., Yin, S., Sharpe, T., Gnerre, S., Abouelleil, A., Berlin, A.M., Montmayeur, A., Shea, T.P., Walker, B.J. *et al.* (2012) Finished bacterial genomes from shotgun sequence data. *Genome Res*, **22**, 2270-2277.
28. Simpson, J.T., Wong, K., Jackman, S.D., Schein, J.E., Jones, S.J. and Birol, I. (2009) ABySS: a parallel assembler for short read sequence data. *Genome Res*, **19**, 1117-1123.
29. Huang, X. and Madan, A. (1999) CAP3: A DNA sequence assembly program. *Genome Res*, **9**, 868-877.
30. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol*, **215**, 403-410.
31. Lowe, T.M. and Chan, P.P. (2016) tRNAscan-SE On-line: integrating search and context for analysis of transfer RNA genes. *Nucleic Acids Res*, **44**, W54-57.
32. Katoh, K. and Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol*, **30**, 772-780.
33. Osigus, H.J., Eitel, M. and Schierwater, B. (2017) Deep RNA sequencing reveals the smallest known mitochondrial micro exon in animals: The placozoan cox1 single base pair exon. *Plos One*, **12**.

34. Chi, S.I. and Johansen, S.D. (2017) Zoantharian mitochondrial genomes contain unique complex group I introns and highly conserved intergenic regions. *Gene*, **628**, 24-31.
35. Haen, K.M., Lang, B.F., Pomponi, S.A. and Lavrov, D.V. (2007) Glass sponges and bilaterian animals share derived mitochondrial genomic features: a common ancestry or parallel evolution? *Mol Biol Evol*, **24**, 1518-1527.
36. Sbisa, E., Tanzariello, F., Reyes, A., Pesole, G. and Saccone, C. (1997) Mammalian mitochondrial D-loop region structural analysis: identification of new conserved sequences and their functional and evolutionary implications. *Gene*, **205**, 125-140.
37. Lavrov, D.V. (2007) Key transitions in animal evolution: a mitochondrial DNA perspective. *Integr Comp Biol*, **47**, 734-743.
38. Lavrov, D.V. (2010) In DeSalle, R. and Schierwater, B. (eds.), *Key transitions in animal evolution*. CRC Press, pp. 34-53.
39. Burger, G., Forget, L., Zhu, Y., Gray, M.W. and Lang, B.F. (2003) Unique mitochondrial genome architecture in unicellular relatives of animals. *Proc Natl Acad Sci U S A*, **100**, 892-897.
40. Schleicherova, D., Dulias, K., Osigus, H.J., Paknia, O., Hadrys, H. and Schierwater, B. (2017) The most primitive metazoan animals, the placozoans, show high sensitivity to increasing ocean temperatures and acidities. *Ecology and Evolution*, **7**, 895-904.

Chapter IX

H.J. Osigus[1], M. Eitel[4], S. Rolfes[1], K. Kamm[1], M. Tessler[2], J.S. Neumann[2,3], R. DeSalle[2], B. Schierwater[1], New insights into complex mitochondrial genome evolution within the *Hoilungia*-group (phylum Placozoa), unpublished

[1] Stiftung Tierärztliche Hochschule Hannover, ITZ, Ecology & Evolution, Bünteweg 17d, 30559 Hannover, Germany.
[2] American Museum of Natural History, Sackler Institute for Comparative Genomics and Division of Invertebrate Zoology, New York, NY, USA.
[3] American Museum of Natural History, Richard Gilder Graduate School, New York, NY, USA.
[4] Ludwig-Maximilians-Universität München, Department of Earth and Environmental Sciences, Paleontology and Geobiology, München, Germany

Abstract

The lineage-rich *Hoilungia*-group is one of the three major branches in the phylum Placozoa. Despite previous efforts, the evolution of mitogenomes within this group is still poorly understood. We here describe five new mitogenomes, covering four different 16S clades. With only 31,8 kb, the H19 mitogenome is the smallest *Hoilungia*-group mtDNA reported until today. The order of typical mitochondrial genes is surprisingly conserved within the *Hoilungia*-group, although the number of encoded ORFs is variable. However, one major rearrangement of an mtDNA fragment spanning half of the entire mitogenome of H25 challenges our understanding of mitogenome evolution in closely related placozoan lineages. We also discuss independent intron loss scenarios for the placozoan *cox1*, *nad5* and 16S rDNA genes in distantly related lineages. In sum, our data reveal complex patterns of mitogenome evolution within the *Hoilungia*-group and support the hypothesis of fast genetic radiation of particular placozoan clades.

Introduction

Placozoans are an ideal model system to study complex mitogenome evolution processes at the non-bilaterian phylum level (1-3). The phylum Placozoa currently consists of 20 haplotypes, which can be arranged into the *Polyplacotoma*-group (H0) (4), the *Trichoplax*-group (H1-H3, H17) and the *Hoilungia*-group (H4-H16, H18-H19), respectively (5).

The mitochondrial genomes from the *Polyplacotoma*-group and the *Trichoplax* clade seem to follow remarkably different evolutionary pathways than mitogenomes from clade II, III and V (see 4,6,7,8, and Chapter VIII in this thesis). The evolutionary pathways of mitogenomes in the *Hoilungia*-group are poorly understood. Only 4 out of 15 mitogenomes (i.e. from H4, H8, *Hoilungia hongkongensis* H13 and H15) have so far been characterized in this group (5,6,8). These mitogenomes belong to members of only two out of five known clades (i.e. clade III and clade V) (9). As the taxonomy within the *Hoilungia*-group is likewise controversial (5), whole mitochondrial genome analyses might also be helpful for a better systematic understanding.

Since *Hoilungia hongkongensis* H13 (clade V, (5)) is the only described species in the *Hoilungia*-group, we use its mitogenome as a reference genome. The overall gene order in the *Hoilungia hongkongensis* H13 mitogenome is similar to the gene order as observed in haplotype H15 "Shirahama" (clade V) (8). However, despite their close relationship, an inversion of an approx. 840 bp long non-coding region is the major difference between the two lineages. The major differences between the H13 mitogenome and the mitogenome of haplotype H4 (likewise clade V) are the translocation of the same non-coding region as seen between H13 and H15 "Shirahama", as well as the translocation+inversion of the *PolB* open reading frame (5,6,8). Taken together, all three clade V lineages are separated from each other by at least one mitogenome rearrangement event. The major difference between the *Hoilungia hongkongensis* H13 (clade V) and the haplotype H8 (clade III) mitogenome (6) is the inversion of a fragment comprising two tRNA genes, as well as the absence of the *PolB* open reading frame in H8.

To test the idea of a high dynamic of open reading frame evolution in the *Hoilungia*-group, we here isolate and analyze complete mitochondrial genomes of haplotypes H15 (clade V), H19 (clade IV), H23 (clade III), H24 (clade VII) and H25 (clade V). H23, H24 and H25 represent previously unknown 16S rDNA haplotypes.

Material and Methods

Animal material

The clonal strain "M2/11" (haplotype H15, clade V) originates from Hong Kong (22.352728N 114.251733E) and has been sampled by Michael Eitel in 2012. The clonal strain "Big Mama" (haplotype H19, clade IV) has been published formerly (9). In 2015, the clonal strain H23 "Oberjatzas - OJ Gamma" (clade III) has been provided by Ulrike and Günter Oberjatzas (Hannover, Germany) from their private seawater aquarium, which contains marine samples of unknown geographic origin. The total DNA sample of haplotype H24 "Aq2-1" (clade VII) originates from the DNA collection of the Institute of Animal Ecology (TiHo Hannover). The geographic origin of haplotype H24 "Aq2-1" is unknown as it originates from an aquarium, which contains multiple seawater samples of unknown origin. The clonal strain H25 "Cuba" (clade V) has been extracted from rock samples from Cuba by Sacha Hanig in 2012. All clonal strains have been cultured in the Institute of Animal Ecology under standard laboratory conditions as previously described (10). For H15, H19, H23 and H25, total DNA was extracted from these clonal animal cultures using standard phenol-chloroform protocols (11) for subsequent sequencing. The total DNA from haplotype H24 has been amplified before sequencing using the REPLI-g Mini Kit (Qiagen) following the manufacturer's recommendations.

DNA sequencing, data processing and sequence analyses

The sequencing of total DNA from H23 and H24 was conducted as previously described (4). The paired-end library preparation (TruSeq, PCR-free, 450 bp insert size) and sequencing of total DNA from H15, H19 and H25 was likewise conducted on an Illumina HiSeq2500 (2x125, High Output) at the New York Genome Center. The sequencing resulted in the following total numbers paired-end reads: 159.778.438 (H15), 147.087.050 (H19), 150.218.874 (H23), 142.156.322 (H24) and 149.611.550 (H25).

The complete mitochondrial genomes were assembled from these paired-end read data sets in Geneious version 8.x (12) using an iterative mapping approach using previously extracted 16S rDNA sequences as starting point (see e.g. 13). The quality of draft mitochondrial genomes has afterwards been

improved by mapping back the entire paired-end data sets to the respective mitogenome sequences using Geneious.

Mitochondrial ribosomal RNA genes as well as protein coding genes were annotated using previously published placozoan mitochondrial genomes as guidance (6). Gene boundaries have afterwards been verified via Blast search (14). Mitochondrial encoded tRNA genes were predicted with tRNAscan-SE (15). ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) has been used to screen all new complete placozoan mitochondrial genomes characterized in the course of this thesis for additional open reading frames using stringent parameters (minimal ORF length >300 bp, only "ATG" start codon permitted). The hypothetical functions of respective ORFs have been predicted via protein Blast searches. Previously published placozoan mitochondrial genomes (6-8) have accordingly been re-analyzed with respect to their open reading frame content (see Digital appendix).

The new haplotypes H23, H24 and H25 have been assigned to existing *Hoilungia*-group clades based on the analysis of their diagnostic 16S rDNA fragment (16). In detail, 16S rDNA fragments of all published *Hoilungia*-group lineages (see 9) have been aligned with MAFFT v7.017 (17) as implemented in Geneious using the E-INS-i algorithm. Subsequent phylogenetic analyses were conducted with FastTree 2.1.5 (18) (likewise implemented in Geneious) under default settings. The resulting tree has afterwards been modified in INKSCAPE (19).

For the analyses of *cox1*, *nad5* and 16S gene structures, single gene alignments have been generated in MAFFT (17) as implemented in Geneious (12), using gene sequences from previously published placozoan mitochondrial genomes (6-8) as well as from all new placozoan mitochondrial genomes characterized in the course of this thesis.

Results and Discussion

The initial characterization of the diagnostic 16S rDNA fragments revealed that two out of five analyzed clonal lineages belong to previously published placozoan haplotypes (i.e. H15 and H19, respectively) (see 9,10). In contrast, three analyzed clonal lineages possess unique 16S sequences, which do not

match to any described placozoan haplotype. As the haplotype numbers H20-H22 have already been assigned to other lineages (Michael Eitel, pers. communication), we here assign the haplotype numbers H23 (clade III), H24 (clade VII) and H25 (clade V) to the new placozoan lineages (Figure 1).

The five new mitochondrial genomes of H15, H19, H23, H24 and H25 share a series of characteristics with previously sequenced *Hoilungia*-group mitogenomes (5,6,8). Shared features are a mitogenome size between 30 kb – 40 kb, the missing *atp8*/*atp9* genes, a conserved set of 24 tRNAs, fragmented 16S/ *cox1*/*nad5* genes, and a *cox1* micro exon. Furthermore, all new *Hoilungia*-group mitogenomes possess only a moderate GC-content. The clade-specific characteristics are discussed in detail below. The open reading frame content of respective placozoan mitogenomes is summarized in Figure 2.

Clade III

The complete mitochondrial genome of haplotype H23 is a circular molecule with a size of 32,980 bp. 16S rDNA sequences clearly identify H23 as a member of clade III (see Figure 1). Its mitochondrial gene order is identical to that of haplotype H8 (same clade) (6). When compared to the reference genome from *Hoilungia hongkongensis* (H13), H8 as well as H23 deviate by an inverted mitogenome fragment coding for trnT+trnK (see Figure 3). However, considering the identical gene orders in the *Trichoplax*-group (6), the gene order of this specific tRNA-locus in H8/H23 can be seen as the ancestral state within the *Hoilungia*-group. Therefore, the inversion of the trnT+trnK locus is a synapomorphy of the *Hoilungia*-subgroup A2 (see 10), as suggested in a previous study by Signorovitch and co-workers (6). The trnT+trnK locus seems to be a hotspot for changes in the mitochondrial gene order (see section on clade V mitogenomes, below). With respect to additional open reading frames, both clade III mitogenomes share a placozoan-specific LAGLIDADG-endonuclease (6) located in a *cox1* intron, while H23 possesses an additional LAGLIDADG-endonuclease in another *cox1* intron. Remarkably, both clade III mitogenomes lack a reverse transcriptase known from other placozoans (Figure 2). The potential function of the remaining predicted ORFs remains unclear.

Clade IV

The mitochondrial genome of haplotype H19 represents the first mitochondrial chromosome, which has been characterized in clade IV. With a size of only 31,792 bp it is even smaller than the so far smallest *Hoilungia*-group mitogenome (i.e. that of H8, clade III, 32,661 bp, (6)) and therefore now marks the lower size boundary of *Hoilungia*-group mitogenomes. This observed mitogenome size difference between H8 and H19 is mostly caused by the absence of one *cox1* intron in H19, which is likely the result of a secondary intron loss (see below). However, there still remains a size gap between the smallest *Hoilungia*-group mitogenome and the largest circular mitogenome from sponges (*Lubomirskia baicalensis*, 29 kb) (20).

The gene order of conserved mitochondrial genes in H19 is identical to the reference mitogenome of *Hoilungia hongkongensis* H13. The H19 mitogenome lacks a *PolB* open reading frame known from clade V (6), while it possesses one LAGLIDADG-endonuclease as well as a reverse transcriptase in different *cox1* introns. Again, the potential function of the remaining predicted ORFs remains unclear (Figure 2).

Clade VII

The first complete mitogenome from clade VII (represented by haplotype H24) has a size of 33,532 bp. The relative gene order of typical mitochondrial genes is identical to the gene order in the reference *Hoilungia hongkongensis* H13 mitogenome (5). This implies that identical orders of typical mitochondrial genes can be found in different lineages from three out of four analyzed *Hoilungia*-group clades. With respect to additional open reading frames, the H24 mitogenome harbors a reverse transcriptase as well as two LAGLIDADG-endonuclease genes. The occurrence of a second LAGLIDADG-endonuclease now in three out of four analyzed clades (see also H25 in clade V, below) suggests that this specific endonuclease originally was present in all *Hoilungia*-group clades and has been lost independently in various distantly related placozoan lineages. Interestingly, only one additional open reading frame of unknown function has been predicted in H24 (Figure 2).

Clade V

The mtDNA analyses of haplotype H15 "M2/11" and haplotype H25 revealed a complex history of mitogenome evolution within clade V. While H25 represents a previously unknown haplotype, the H15 "M2/11" mitogenome is of particular interest as it is the second mitogenome from this specific haplotype (8), allowing for the first time to study the mtDNA evolution in two different populations of the same haplotype.

The H15 "M2/11" mitogenome (36,521 bp) is highly similar to the reference mitochondrial genome of *Hoilungia hongkongensis* H13. Surprisingly, the inversion of a specific non-coding region, which is one of the major structural differences between H15 "Shirahama" (36,676 bp) (8) and *Hoilungia hongkongensis* H13, cannot be found in the H15 "M2/11" mtDNA. This inversion therefore is not a synapomorphy of the haplotype H15, but rather indicates that mitogenome rearrangements can independently occur in different populations of H15. This case study also points to current limitations of the commonly used 16S fragment, which seems to fail to resolve such structural changes at the population level. While *Hoilungia hongkongensis* H13 potentially is endemic in Hong Kong (5), the placozoan haplotype H15 has been reported not only from Hong Kong (clone "M2/11", this study) and Japan (clone "Shirahama" (8)), but also from the Philippines (10). The complete mitogenome sequence of the Philippine H15 population therefore would help to reconstruct the dispersal history of H15 in the Pacific. However, based on available mitogenome data, any predictions on the succession of emergence of haplotypes H13 and H15 in clade V remain speculative at this point. With respect to predicted open reading frames, both H15 mitogenomes share a reverse transcriptase, a LAGLIDADG-endonuclease and a *PolB* DNA polymerase (Figure 2). Nevertheless, the number of additional ORFs of unknown function differs between the two different H15 mitogenomes.

The mitogenome of H25 (35,364 bp) reveals a remarkable case of a mitogenome rearrangement within clade V (Figure 3). While the relative gene order of typical mitochondrial genes (which omits additional ORFs of unknown function or proteins which are not related to the respiratory chain) is identical in H4, H13 and both H15 mitogenomes, the gene order in H25 differs from these

haplotypes by the inversion of a fragment, which spans half of the entire mitogenome. This major mitogenome rearrangement is almost in a range as observed for instance between members of different placozoan groups (e.g. *Trichoplax adhaerens* H1 vs. haplotype H8, (6)). Remarkably, the boundaries of the inverted mitogenome fragment in H25 (i.e. trnT-*nad1* and trnK-*nad4L*, see Figure 3) exactly match the position of the *PolB* open reading frame in haplotypes H4 and H13/H15, respectively. This indicates that the loci flanking *PolB* in H4 (*nad1*-*PolB*-*nad4L*) and H13/H15 (trnT-*PolB*-trnK) may be hot spots for rearrangement events (see also the section on clade III, above). The *PolB* open reading frame itself, however, is absent in H25, whereas a reverse transcriptase, two LAGLIDADG-endonuclease genes and two additional ORFs of unknown function can be found (Figure 2).

General aspects

The comprehensive analyses of complete mitogenomes from the *Hoilungia*-group revealed a high conservation of gene orders within this group (Figure 3). In detail, the gene orders in clade IV, V (except for H25) and VII are overall identical when focused on typical mitochondrial genes. The only exception in this group is clade III, which possesses a slightly deviating gene order within a tRNA-rich mitogenome locus. This different (possibly more ancestral) gene order in clade III, however, matches previous 16S phylogenies, which support a sister group relationship of clade III to the subgroup A2 (comprising clade IV, V, VI and VII) (9). With respect to the overall evolutionary history of mitochondrial gene orders within the *Hoilungia*-group, it seems that the radiation of subgroup A2 into clades IV-VII is characterized by a long period, in which no mitogenome rearrangements have occurred. This period, however, ended with the emergence of H25 in clade V.

Previous studies suggested an overall correlation between sequence divergence and frequency/fragment size of rearrangement events at the group- and clade-level, respectively (6,8). The mitochondrial genome of H25 (clade V) depicts a remarkable deviation from this general picture. In this case, the order within a clade is more variable than between clades, although the deviating gene order originates from just a single rearrangement event. Nevertheless, this

observation may indicate an accelerated structural evolution rate (i.e. a higher tendency for rearrangements) in clade V, which has to be confirmed by additional mitogenome data from this clade.

Cox1 evolution

The five new placozoan mitogenomes allow us to further reconstruct the evolutionary history of placozoan *cox1* gene fragmentation (see Figure 4). In Placozoa, the *cox1* gene is fragmented into five to nine exons, respectively, with the smaller number of exons representing the more ancestral state (21,22). In agreement with previous results (5,8), our data support the scenario that the *cox1* exon structure is identical in different lineages of the same clade. One case of a shared *cox1* exon structure even between members of different clades has previously been reported from the *Trichoplax*-group (i.e. between clade I and clade II) (6). However, our new data show for the first time that the *cox1* exon structure also can be identical between members of different clades in the *Hoilungia*-group (as seen between clade V and clade VII, Figure 4).

Following previous studies (7,21) the placozoan *cox1* gene can be subdivided into three different blocks A, B and C, each showing differential fragmentation patterns (Figure 4). Most strikingly, block B is conserved among all extant placozoans, indicating the split-off of this block already in their last common ancestor.

On the opposite, the *Polyplacotoma*-, the *Trichoplax*- and the *Hoilungia*-groups show unique fragmentation patterns in block A, respectively. The overall evolutionary tendency in this block is an increasing fragmentation into up to four exons, as seen in the *Trichoplax*-group (clade I and II).

A more complex fragmentation pattern can be observed in block C. While block C comprises only two exons in H0, this block is even fragmented into up to five exons (including the shared micro exon) in other placozoans. The exon pattern of block C in clades I, II and III, respectively, seems to match the exon pattern of the last common ancestor of the *Trichoplax*-/*Hoilungia*-groups. While clade III therefore still possesses this ancestral pattern within the *Hoilungia*-group, an increasing fragmentation in block C can be observed in clades V and VII. However, clade IV (i.e. haplotype H19) represents an exception in this

scenario, as its block C does not possess an otherwise conserved exon-exon boundary known from clades I, II, III, V and VII. Considering the 16S tree topology within the *Hoilungia*-group (9), the most parsimonious explanation for this pattern is the loss of the respective intron in clade IV, which resulted in the fusion of the previously separated exons. This is the first report of a putative intron loss in the placozoan *cox1* gene. In sum, although there seems to be a general evolutionary tendency for increasing gene fragmentation in placozoan mitogenomes, sporadic exceptions, which at least partially reverse this trend, can be found in some lineages. Future comparative approaches implementing *cox1* intron data from Porifera (e.g. 23) and Cnidaria (e.g. 24) will help to further reconstruct the evolution of *cox1* fragmentation at the base of Metazoa.

16S rDNA evolution

As already seen in the *cox1* gene, the 16S rDNA gene is likewise fragmented into multiple exons in Placozoa (4-8). The total lengths of 16S genes substantially differ between placozoan lineages due to multiple variable regions within exons. However, based on sequence alignments and the positions of exons within respective mitogenomes, the placozoan 16S gene can be separated into two distinct blocks A and B (6,7) (Figure 5). The 16S block A is shared among all placozoan lineages, including H0. This indicates that the separation of block B from block A has already occurred in the last common ancestor of all extant placozoans. The exon structure of block B, however, is more complex. The exon structure of block B is identical in H0 as well as in clade I and clade II (the latter both from the *Trichoplax*-group). In all these lineages, the 16S block B is split into two exons. This specific exon break point in block B also can be found in clade III (*Hoilungia*-group). However, in clade III, the 5`-exon of block B is further split, resulting in a block B that consists of in sum three exons. Surprisingly, the 16S block B in clades IV, V and VII consists of only one single exon. Given the 16S-based phylogeny (9), we deduce that the block B intron, which is shared between H0 and clades I-III, has been lost in the last common ancestor (LCA) of clades IV, V and VII (i.e. the LCA of subgroup A2, (10)).

Nad5 evolution

The *nad5* gene is the third placozoan mitochondrial gene, which can harbor an intron (see 3 for overview). With the exception of *P. mediterranea* H0 and haplotype H3, all placozoan lineages share an *nad5* intron at a conserved position (Figure 6). The absence of the *nad5* intron in H0 could be interpreted as primary absence, as phylogenetic analyses support H0 as the sister to all other extant placozoans (4). However, a secondary loss scenario of the *nad5* intron can not be completely excluded for *P. mediterranea* H0. In contrast to H0, haplotype H3 is branching-off deeply within placozoan lineages (9), which all posses an *nad5* intron. Therefore, the *nad5* intron in H3 has likely been secondarily lost, while all other *Trichoplax*-/*Hoilungia*-group mt genomes retained this *nad5* intron, which has then been gained along the stem leading to both groups. Another (less likely) scenario suggests a primary absence of an *nad5* intron in all *Trichoplax*-/*Hoilungia*-group mt genomes and therefore would imply an independent gain of an *nad5* intron in clade I as well as in the *Hoilungia*-group, respectively.

In sum, while all intron-containing genes (i.e. *nad5*, *cox1* and 16S) show the general evolutionary tendency for increasing fragmentation, sporadic intron losses have been found in all three genes in distantly related placozoan taxa. We would like to emphasize that the sporadic intron loss (and therefore the secondary re-compaction of genes) does not necessarily predate the general scenario of secondary size amplification of placozoan mitogenomes. The sporadically observed intron losses might rather emphasize the high structural dynamics within placozoan mitogenomes, although further analyses have to confirm this hypothesis.

Conclusions

The comparative analyses of mitochondrial genomes from the *Hoilungia*-group provide new insights into the genetic diversity and complex evolution of placozoan mitogenomes. Future studies on nuclear genomes as well as functional studies on mitochondrial DNA replication will help to better understand the mechanisms underlying the genetic radiation processes in placozoan mitochondria. Pending comprehensive phylogenetic analyses based on different

mitochondrial data sets will help to further clarify the taxonomic status within the *Hoilungia*-group.

Acknowledgements

HJO acknowledges a doctoral fellowship of the Studienstiftung des Deutschen Volkes. We acknowledge support from the German Science Foundation (DFG Schi-277/26, Schi-277/27, Schi-277/29). We thank Ulrike and Günter Oberjatzas (Barsinghausen, Lower Saxony, Germany) for kindly providing live animals of haplotype H23. We also thank Sacha Hanig for kindly providing rock samples containing haplotype H25.

Digital appendix

Further information related to this chapter can be found in the Digital appendix of this thesis.

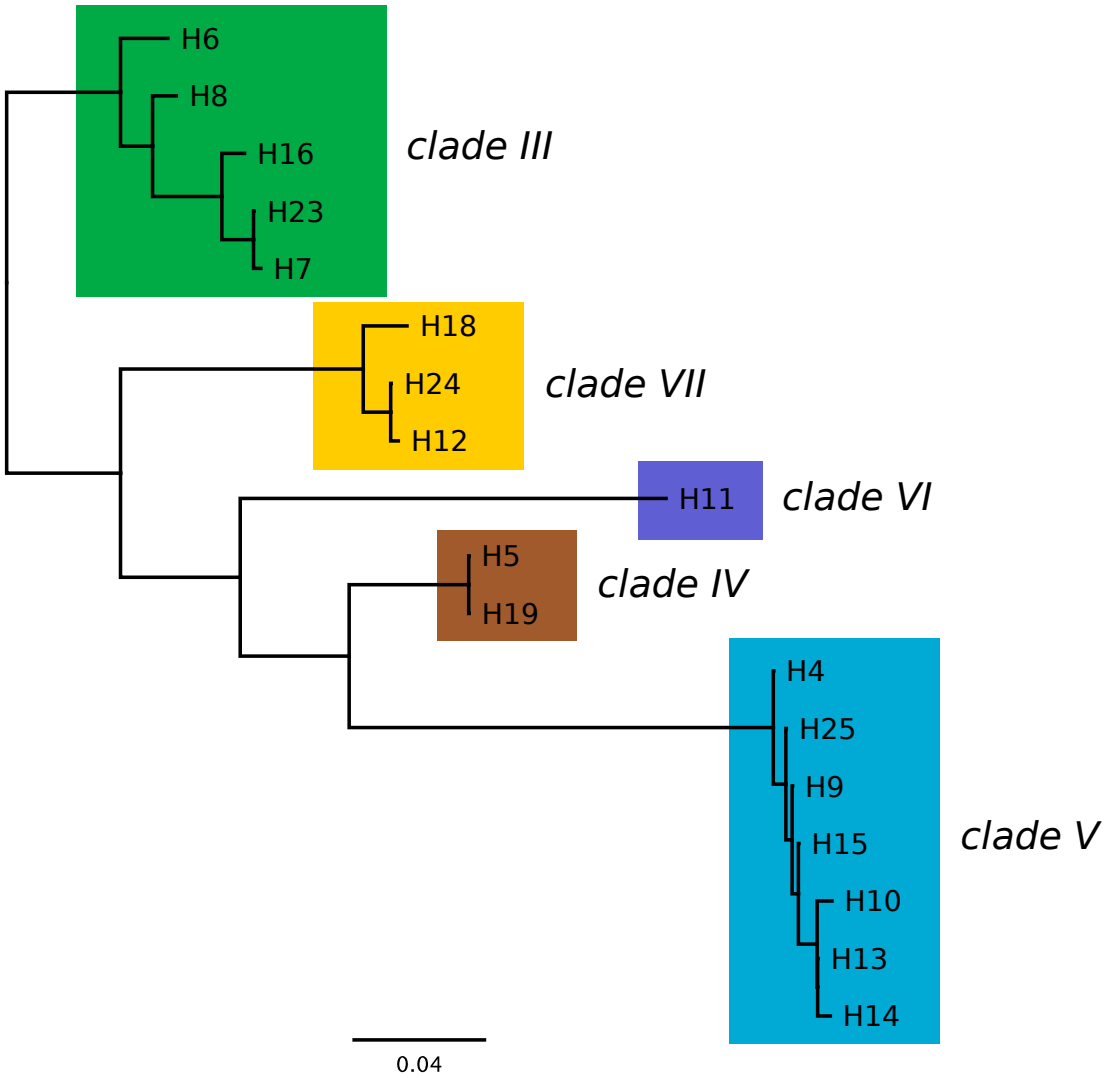

Figure 1

Phylogenetic relationships of placozoan haplotypes within the *Hoilungia*-group. Shown is an approximately Maximum Likelihood tree based on FastTree-analyses of the diagnostic 16S rDNA fragment. The haplotypes H18, H19, H23, H24 and H25 have been characterized in the course of this thesis. The colors of the 16S clades III-VII correspond to colors already used in previous studies (9,10).

clade	haplotype	reverse transcriptase/ maturase	LAGLIDADG homing endonuclease (Placozoa)	LAGLIDADG homing endonuclease (non-Placozoa)	DNA polymerase B	additional ORFs
	H0		+			0
I	H1	+	+			11
	H2	+	+			16
	H17	+	+			13
II	H3	+	+			3
III	H8		+			2
	H23		+	+		3
IV	H19	+	+			2
V	H4	+	+		+	3
	H13	+	+		+	3
	H15 Shi	+	+		+	5
	H15 M2	+	+		+	2
	H25	+	+	+		2
VII	H24	+	+	+		1

Figure 2

Distribution of mitochondrial open reading frames in Placozoa.

The figure shows the distribution of mitochondrial open reading frames in distantly related placozoan haplotypes. Some of the listed predicted ORFs of unknown function partially overlap with other ORFs, which possess the same transcriptional direction. Listed ORFs, which are completely nested within other ORFs, must possess an opposite transcriptional orientation. Please note that the absence of specific ORFs in some lineages might imply that rudimental fragments of these ORFs are still detectable in respective mitogenomes.

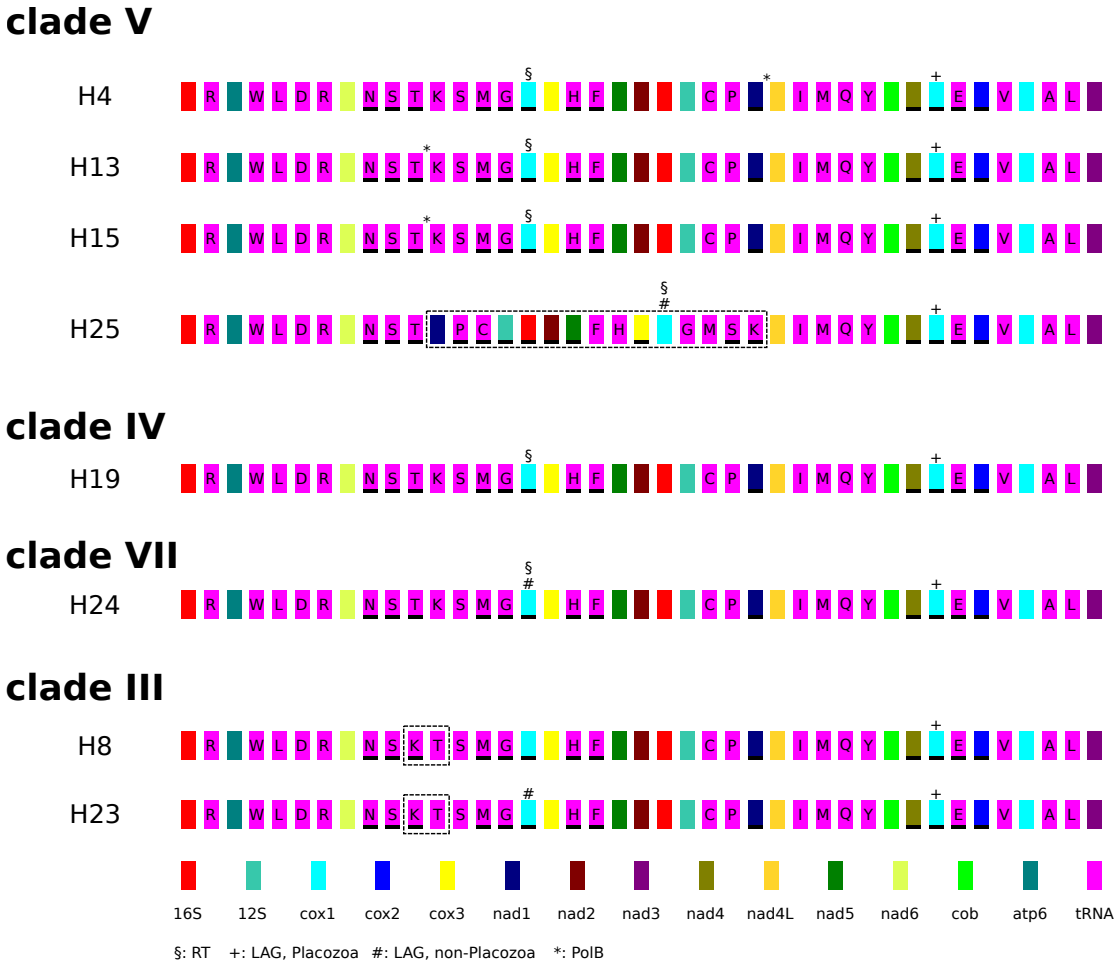

Figure 3

Positional alignment of linearized mitochondrial genomes from *Hoilungia*-group placozoans.

Typical mitochondrial coding genes are illustrated as color-coded boxes (not to scale). Genes with opposite orientation (i.e. 3'-5' instead of 5'-3') are labeled with a black bar at the bottom of the respective gene icon. According to the standard code, single letters name the corresponding amino acid of tRNA genes. Please note that some neighboring exons of *cox1*, *nad5* and 16S, respectively, have been merged. Multiple *cox1* and 16S icons at different loci, however, still indicate the fragmentation of these genes. Positions of potentially functional open reading frames are labeled by respective symbols, while ORFs of unknown function are not shown for clarity. Gene orders which deviate from the reference mitogenome (i.e. from *Hoilungia hongkongensis* H13) are highlighted with dotted line boxes. (RT: reverse transcriptase/maturase, LAG: LAGLIDADG- homing endonuclease, PolB: DNA polymerase B).

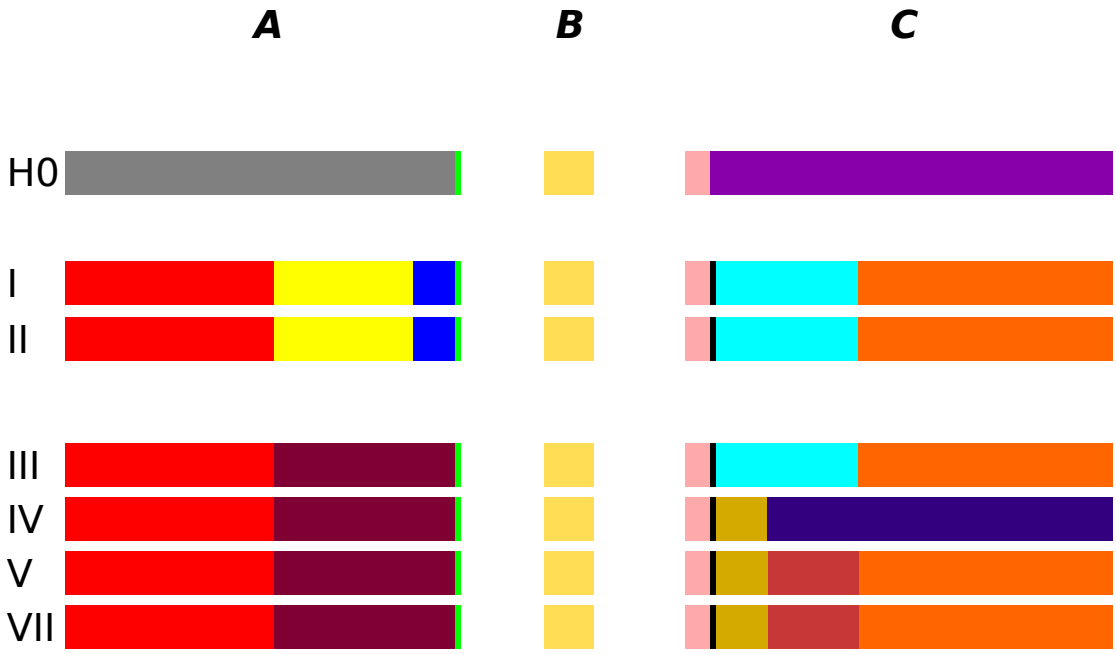

Figure 4

Exon structures of the fragmented *cox1* gene in the placozoan 16S haplotype H0 and the 16S clades I-VII. Exons with identical boundaries have the same color. Neighboring exons have been strung together. The *cox1* gene is separated into three different blocks A, B and C. The size of respective exons is approximate to scale, except for the *cox1* micro exon, which is indicated by a black line in block C in clade I-VII, but which is absent in H0.

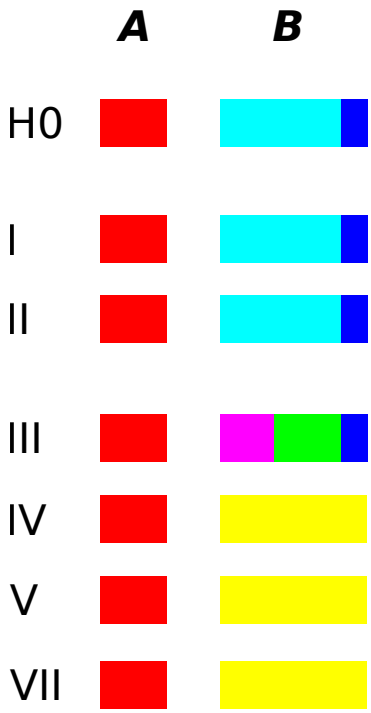

Figure 5

Exon structures of the fragmented 16S gene in the placozoan 16S haplotype H0 and the 16S clades I-VII. Exons with identical boundaries have the same color. Neighboring exons have been strung together. The 16S gene is separated into two different blocks A and B. Exons are only shown as relative approximations in unified size, and homologous exon boundaries have been aligned.

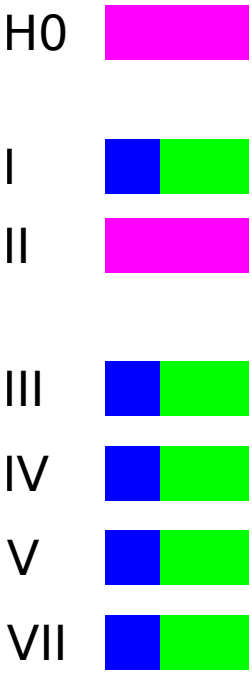

Figure 6

Exon structures of the fragmented *nad5* gene in the placozoan 16S haplotype H0 and the 16S clades I-VII. Exons with identical boundaries have the same color. Neighboring exons have been strung together. Exons are approximate to scale.

References

1. Lavrov, D.V. and Pett, W. (2016) Animal Mitochondrial DNA as We Do Not Know It: mt-Genome Organization and Evolution in Nonbilaterian Lineages. *Genome Biol Evol*, **8**, 2896-2913.
2. DeSalle, R., Schierwater, B. and Hadrys, H. (2017) MtDNA: The small workhorse of evolutionary studies. *Front Biosci-Landmrk*, **22**, 873-887.
3. Osigus, H.J., Eitel, M., Bernt, M., Donath, A. and Schierwater, B. (2013) Mitogenomics at the base of Metazoa. *Mol Phylogenet Evol*, **69**, 339-351.
4. Osigus, H.J., Rolfes, S., Herzog, R., Kamm, K. and Schierwater, B. (2019) Polyplacotoma mediterranea is a new ramified placozoan species. *Curr Biol*, **29**, R148-R149.
5. Eitel, M., Francis, W.R., Varoqueaux, F., Daraspe, J., Osigus, H.J., Krebs, S., Vargas, S., Blum, H., Williams, G.A., Schierwater, B. *et al.* (2018) Comparative genomics and the nature of placozoan species. *PLoS Biol*, **16**, e2005359.
6. Signorovitch, A.Y., Buss, L.W. and Dellaporta, S.L. (2007) Comparative genomics of large mitochondria in placozoans. *PLoS Genetics*, **3**, e13.
7. Dellaporta, S.L., Xu, A., Sagasser, S., Jakob, W., Moreno, M.A., Buss, L.W. and Schierwater, B. (2006) Mitochondrial genome of Trichoplax adhaerens

supports placozoa as the basal lower metazoan phylum. *Proc Natl Acad Sci U S A*, **103**, 8751-8756.
8. Miyazawa, H., Yoshida, M.A., Tsuneki, K. and Furuya, H. (2012) Mitochondrial genome of a Japanese placozoan. *Zoolog Sci*, **29**, 223-228.
9. Eitel, M., Osigus, H.J., DeSalle, R. and Schierwater, B. (2013) Global diversity of the Placozoa. *PLoS One*, **8**, e57131.
10. Eitel, M. and Schierwater, B. (2010) The phylogeography of the Placozoa suggests a taxon-rich phylum in tropical and subtropical waters. *Mol Ecol*, **19**, 2315-2327.
11. Ender, A. and Schierwater, B. (2003) Placozoa are not derived cnidarians: evidence from molecular morphology. *Molecular Biology and Evolution*, **20**, 130-134.
12. Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C. *et al.* (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, **28**, 1647-1649.
13. Herzog, R., Osigus, H.J., Feindt, W., Schierwater, B. and Hadrys, H. (2016) The complete mitochondrial genome of the emperor dragonfly Anax imperator LEACH, 1815 (Odonata : Aeshnidae) via NGS sequencing. *Mitochondrial DNA Part B*, **1**, 783-786.
14. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol*, **215**, 403-410.
15. Lowe, T.M. and Chan, P.P. (2016) tRNAscan-SE On-line: integrating search and context for analysis of transfer RNA genes. *Nucleic Acids Res*, **44**, W54-57.
16. Voigt, O., Collins, A.G., Pearse, V.B., Pearse, J.S., Ender, A., Hadrys, H. and Schierwater, B. (2004) Placozoa -- no longer a phylum of one. *Curr Biol*, **14**, R944-945.
17. Katoh, K. and Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol*, **30**, 772-780.
18. Price, M.N., Dehal, P.S. and Arkin, A.P. (2010) FastTree 2--approximately maximum-likelihood trees for large alignments. *PLoS One*, **5**, e9490.
19. INKSCAPE. (2017). 0.92.2 ed.
20. Lavrov, D.V. (2010) Rapid proliferation of repetitive palindromic elements in mtDNA of the endemic Baikalian sponge Lubomirskia baicalensis. *Mol Biol Evol*, **27**, 757-760.
21. Burger, G., Yan, Y., Javadi, P. and Lang, B.F. (2009) Group I-intron trans-splicing and mRNA editing in the mitochondria of placozoan animals. *Trends Genet*, **25**, 381-386.
22. Osigus, H.J., Eitel, M. and Schierwater, B. (2017) Deep RNA sequencing reveals the smallest known mitochondrial micro exon in animals: The placozoan cox1 single base pair exon. *PLoS One*, **12**, e0177959.
23. Schuster, A., Lopez, J.V., Becking, L.E., Kelly, M., Pomponi, S.A., Worheide, G., Erpenbeck, D. and Cardenas, P. (2017) Evolution of group I introns in Porifera: new evidence for intron mobility and implications for DNA barcoding. *BMC Evol Biol*, **17**, 82.

24. Chuang, Y.Y., Kitahara, M., Fukami, H., Tracey, D., Miller, D.J. and Chen, C.A. (2017) Loss and Gain of Group I Introns in the Mitochondrial Cox1 Gene of the Scleractinia (Cnidaria; Anthozoa). *Zool Stud*, **56**.

3. General discussion

Placozoan mitogenomic data are a valuable resource for targeting multiple fields of research at the base of Metazoa. Major open questions relate to the phylogenetic position of placozoans within Metazoa, the placozoan biodiversity and inner systematic as well as to the molecular evolution of placozoan mitochondrial genomes. These questions have been addressed in this thesis.

Mitochondrial metazoan phylogenetics

Previous phylogenetic analyses using concatenated mitochondrial respiratory chain genes have indicated that mitochondrial protein coding sequences are a valuable data set to reconstruct relationships at the base of Metazoa (e.g. 1,2). However, previous studies suffer from incomplete taxon sampling and/or the usage of inadequate evolutionary substitution models and have led to contradictory results (e.g. 3,4). To overcome these shortcomings five comprehensive metazoan data sets with proper non-metazoan outgroups have been generated, and subsequent phylogenetic analyses have been conducted using appropriate substitution models (Chapter II). The outcome of the analyses generally support a sister group relationship of bilaterian and non-bilaterian animals. This principal topology has been observed in previous mitochondrial (1,2,5) as well as total-evidence based analyses (6). However, this topology has not been recovered for instance in a nuclear gene based phylogenetic analysis (Chapter VI), which instead supports a sister group relationship of sponges to all other animals, with Placozoa as a sister to a clade formed by Cnidaria+Bilateria. To investigate these discrepancies, a closer look at the mitochondrial-based analyses is justified. It turns out that mitochondrial sequence evolution rates vary substantially between and within non-bilaterian phyla (7). Primary targets for long-branch attraction (LBA) artifacts (8) are the phylum Ctenophora (9-11) and the two poriferan classes Hexactinellida (12) and Calcarea (13). Another striking example for a problematic taxon is the cnidarian class Anthozoa (Hexacorallia+Octocorallia), which comes out paraphyletic in our mitochondrial data analyses (Chapter II) as well as in previous studies (e.g. 14). Despite these problematic taxa, several well-supported nodes like monophyletic Placozoa or Demospongiae (phylum Porifera) highlight the resolution of mitogenome data

analyses to resolve topologies at higher taxonomic levels. Metazoan phylogenetic analyses reflect a trade-off between broad taxon sampling and the depth of subsequent analyses (15,16). On the one hand, broad taxon sampling is generally considered to improve the outcome of phylogenetic analyses (see e.g. 17), while on the other hand, adding more data increases the amount of compositional heterogeneity across sites and/or lineages (see e.g. 18). The handling of large heterogenic data sets requires complex evolutionary substitution models, which are computationally highly demanding (e.g. 19,20,21). It seems that currently available models and computational resources are severely limited to deal with these issues, especially for mitochondrial data.

RNA sequencing data uncover the placozoan *cox1* micro exon

Deep RNA sequencing (RNAseq) is a modern technique to understand mitochondrial transcript processing in early branching metazoans (e.g. 22). As a complement to placozoan mtDNA data, the placozoan mitochondrial RNAseq data generated in this thesis reveal new insights into the unique mRNA processing and gene structure evolution of the placozoan *cox1* gene (Chapter IV). In detail, a previously postulated *cox1* mRNA editing mechanism, which has been deduced from expressed sequence tag (EST) data in *Trichoplax adhaerens* H1 (4), is not supported by *Trichoplax* sp. H2 RNAseq data. In contrast, RNAseq as well as mtDNA intron predictions from *Trichoplax sp.* H2 reveal the existence of a previously overseen single base pair *cox1* micro exon in placozoans. This is the first report of an ultra-short single base pair mitochondrial exon in animals. The unusual *cox1* micro exon originates from an intron insertion event, which separated the single nucleotide from the remaining exon. This intron insertion must have occurred after the split-off of *Polyplacotoma*, as the micro exon in *Polyplacotoma mediterranea* H0 is still merged with the original exon (Chapter VII). The existence of a single base pair micro exon is a remarkable example of extreme mitochondrial gene fragmentation in a non-bilaterian animal and highlights the crucial role of Placozoa as a genetic model system. This case study also highlights the importance of high coverage RNAseq data to identify rare transcripts and intermediate splicing stages.

Biodiversity and inner systematic of Placozoa based on mitochondrial and nuclear genome data

Comparative studies on the early evolution of metazoan traits require a representative choice of placozoan taxa to minimize potential analyses artifacts (Chapter I). In order to address this task, worldwide field sampling (23) has been conducted to maximize the probability of collecting highly diverse placozoan lineages (Chapter III, VII, VIII and IX). Six new placozoan lineages identified in this thesis have substantially widened our knowledge on placozoan biodiversity but at the same time call for new approaches to formally describe new placozoan species.

The traditional biological species concept could be an appropriate species concept for placozoans, although sexual reproduction experiments on placozoans under laboratory conditions are still problematic (24). The indirect proof of reproductive isolation by the grade of allele sharing between two placozoan lineages (see also 25), however, is a practical solution to overcome this issue (Chapter VI). Although future economic approaches should focus on mtDNA, the haploid nature of mitogenomes requires the initial implementation of nuclear genome data to test for reproductive isolation. Such approaches have been applied to the placozoan lineages H2 (Chapter V) and H13 (Chapter VI), respectively, which are closely (H2) and distantly (H13) related to the only so far named species *Trichoplax adhaerens* H1 (23). While the H2 nuclear genome reveals a high degree of allele sharing with H1, the allele content of the H13 nuclear genome suggests a long time of independent evolution due to reproductive isolation from H1. These general similarity patterns have also been found in whole mitogenome comparisons as well as in 16S based phylogenetic analyses (Chapter III, VI, VIII and IX). Consequently, molecular diagnostic characters extracted from the 16S marker have been used in a taxogenomic approach combined with the whole nuclear genome data to formally describe *Hoilungia hongkongensis* H13 gen. nov., spec. nov. (Chapter VI).

The formal description of the genus *Hoilungia* has been of substantial importance for subsequent taxonomic approaches to placozoans. The reference mitochondrial genomes of *Trichoplax adhaerens* H1 (1) and *Hoilungia hongkongensis* H13 can serve as landmarks and allow for the first time

taxonomic calibrations of comparative mitochondrial genome analyses. In detail, the H1 and H13 mitogenomes represent a maximum of mitogenomic separation between respective genera. The shared characteristics have been used to define mitochondrial genome synapomorphies for the entire *Trichoplax*/*Hoilungia* clade. The new placozoan lineage H0, which possesses a unique ramified morphological habitus, separates itself from both genera by substantial deviations from these mitogenome synapomorphies (Chapter VII). Phylogenetic analyses of both, mitochondrial and nuclear markers, support a sister group relationship of H0 to a clade formed by all other placozoans. Thus H0 was described as a new species, *Polyplacotoma mediterranea*, in a new genus (Chapter VII).

The three genera *Trichoplax*, *Hoilungia* and *Polyplacotoma* represent a new (and first) systematic framework for future taxonomic approaches. A large number of genetically still incompletely characterized placozoan lineages are awaiting a systematic classification and adumbrate the existence of further genera or even higher taxonomic ranks within the phylum Placozoa (Chapter III). Future comparative studies on the early evolution of Metazoa will clearly benefit from the implementation of taxa from at least these three placozoan genera.

Comparative placozoan mitogenomics

The evolution of mitochondrial genomes in the phylum Placozoa has previously been inferred from a limited number of two *Trichoplax*-group and three *Hoilungia*-group mtDNAs (1,2,26). The description of the first mitogenome from the new placozoan genus *Polyplacotoma* (Chapter VII) as well as the characterization of in sum 8 new placozoan mitogenomes from the *Trichoplax*-/*Hoilungia*-group in this thesis (Chapter VI, VIII and IX) more than doubles the number of completely sequenced placozoan mitogenomes. This substantially enlarged data set improved the resolution of comparative mitogenome analyses and revealed several remarkable independent evolutionary pathways.

The highly compact mitochondrial genome of *Polyplacotoma mediterranea* H0 challenges in many respects the textbook knowledge of 'typical' placozoan mitochondrial genome traits (Chapter VII). A putative mitochondrial control region, a deviating number of tRNA genes or the missing *cox1* micro

exon, are only some examples of the exceptional mtDNA characteristics of haplotype H0. Compared to *Trichoplax*- and *Hoilungia*-group mitogenomes, the mitochondrial genome of *P. mediterranea* seems to be less susceptible for hairpin proliferation, gain of ORFs or fragmentation of genes. It therefore retained several potentially more ancestral placozoan mtDNA features. Future studies on the nuclear encoded mitochondrial genes of H0 as well as on molecular data from other *Polyplacotoma* species will help to reconstruct the underlying factors, which led to the substantial mtDNA differences in this genus.

With a size of more than 43 kb, the mitochondrial genome of *Trichoplax adhaerens* H1 (clade I) represents an exceptionally large placozoan mtDNA (1). The characterization of two additional clade I mitogenomes in this thesis (Chapter VIII) supports the hypothesis, that an mtDNA size of more than 40 kb is a synapomorphy of this clade. A previously neglected feature, i.e. the accumulation of GC-rich hairpins, substantially contributes to the remarkable mt genome size. Although such hairpins are found in all *Trichoplax*- and *Hoilungia*-group mitogenomes, an enrichment of them apparently has only been occurred in clade I. The resulting differential distribution pattern within the *Trichoplax*-group (i.e. the enrichment of hairpins in clade I, but not in clade II) suggests a further subdivision of this group into two distinct taxa. In addition, molecular signatures indicate that hairpins might have played a role in previous placozoan mitogenome rearrangement events at least within the *Hoilungia*-group. The precise molecular mechanisms, however, which lead to the enrichment of hairpins or to mitogenome rearrangements, are target for future studies for instance on placozoan mtDNA replication.

The characterization of six new *Hoilungia*-group mtDNAs revealed a complex pattern of gene order evolution within this group (Chapter VI and IX). Despite possessing a variable number of additional ORFs, clades IV, VII and V (except for H25, see below) nevertheless share a conserved order of typical mitochondrial genes. In contrast, clade III seems to possess a clade-specific gene order within the *Hoilungia*-group. With special focus on clade V, the gene order of lineage H25 surprisingly differs from the other clade V members (including *Hoilungia hongkongensis* H13) due to the rearrangement of a large mitogenome fragment. Clade V therefore is the first clade within the phylum Placozoa, which

harbors two different mitochondrial gene orders. The observed exon patterns in the *cox1*, *nad5* and the 16S genes likewise reveal a complex history of gene fragmentation in Placozoa, which surprisingly even comprises sporadic intron loss events in distantly related taxa. The overall observed differences between *Hoilungia*-group mitogenomes clearly indicate the existence of additional species, which are awaiting their formal description.

Improved working hypotheses on the early evolution of metazoan mitochondrial genomes

The constantly increasing number of complete mt genomes reveals a high diversity of mitochondrial genomes in non-bilaterian phyla (7). This diversity raises the question about the characteristics of the mitochondrial genome in the urmetazoon as well as in the last common metazoan ancestor (LCMA) (27). In an earlier stage of this thesis, a scenario on the evolution of metazoan mitochondrial genomes has been discussed (Chapter I), which was subsequently revised (Chapter VIII). In both scenarios, however, the urmetazoan mitochondrial genome potentially was a large circular molecule harboring a large set of mitochondrial ribosomal proteins, while the mitogenome of the LCMA might have been a more compact circular molecule lacking any mitochondrial ribosomal protein (28). This relatively compact circular molecule could have been a parsimonious starting point for the independent evolution of mtDNA in Placozoa, Porifera, Cnidaria, Ctenophora and Bilateria, as all of these taxa still possess more or less compact mitogenomes at least in some subtaxa (7). However, the grade of compaction from the urmetazoon up to the last common metazoan ancestor remains controversial in both scenarios (29).

With focus on the more recent scenario (Chapter VIII), independent evolutionary pathways in the four non-bilaterian phyla become evident. In Porifera, the originally rather compact circular mitogenome as found e.g. in demosponges, evolved into multiple linear chromosomes for instance in Calcarea (13). The same independent tendency for mt genome linearization/fragmentation can be seen in the Cnidaria. In detail, while almost all Anthozoa still possess a single compact circular mtDNA (but see 30), linear/fragmented mitogenomes can be found in Medusozoa (e.g. 31). In

contrast, mitochondrial genomes in the phylum Ctenophora are an extreme case of secondary size reduction of mtDNA (9-11). Finally, placozoan mitochondrial genomes possess clear molecular signatures for secondary size amplification due to the gain of introns, ORFs and hairpin structures after the split-off of *Polyplacotoma mediterranea* (which still possesses a compact mtDNA) (Chapter VII). However, it should be highlighted that improved placozoan or choanoflagellate taxon sampling might lead to alternative evolutionary hypotheses, like the scenario discussed in Chapter I. Furthermore, all of these scenarios also depend to some extend on the phylogenetic trees, on which respective characteristics are mapped.

Conclusions

The generation of a comprehensive placozoan mitochondrial molecular data set together with the in-depth analyses in the course of this thesis revealed new important insights into the mtDNA evolution in the non-bilaterian phylum Placozoa. The results stress the power of comparative mitogenomics to address questions on phylogenetic relationships, molecular mitochondrial mechanisms, mitochondrial genome evolution and radiation processes at the base of Metazoa. Yet, knowledge on the overall mitochondrial genetic diversity in non-bilaterians as well as in non-metazoan outgroups is still patchy and future approaches must intensify taxon sampling to fill the gaps in current data sets.

References

1. Dellaporta, S.L., Xu, A., Sagasser, S., Jakob, W., Moreno, M.A., Buss, L.W. and Schierwater, B. (2006) Mitochondrial genome of Trichoplax adhaerens supports placozoa as the basal lower metazoan phylum. *Proc Natl Acad Sci U S A*, **103**, 8751-8756.
2. Signorovitch, A.Y., Buss, L.W. and Dellaporta, S.L. (2007) Comparative genomics of large mitochondria in placozoans. *PLoS Genetics*, **3**, e13.
3. Lavrov, D.V., Wang, X. and Kelly, M. (2008) Reconstructing ordinal relationships in the Demospongiae using mitochondrial genomic data. *Mol Phylogenet Evol*, **49**, 111-124.
4. Burger, G., Yan, Y., Javadi, P. and Lang, B.F. (2009) Group I-intron trans-splicing and mRNA editing in the mitochondria of placozoan animals. *Trends Genet*, **25**, 381-386.

5. Wang, X. and Lavrov, D.V. (2007) Mitochondrial genome of the homoscleromorph Oscarella carmela (Porifera, Demospongiae) reveals unexpected complexity in the common ancestor of sponges and other animals. *Molecular Biology and Evolution*, **24**, 363-373.
6. Schierwater, B., Eitel, M., Jakob, W., Osigus, H.J., Hadrys, H., Dellaporta, S.L., Kolokotronis, S.O. and Desalle, R. (2009) Concatenated analysis sheds light on early metazoan evolution and fuels a modern "urmetazoon" hypothesis. *PLoS Biol*, **7**, e20.
7. Lavrov, D.V. and Pett, W. (2016) Animal Mitochondrial DNA as We Do Not Know It: mt-Genome Organization and Evolution in Nonbilaterian Lineages. *Genome Biol Evol*, **8**, 2896-2913.
8. Felsenstein, J. (1978) Cases in Which Parsimony or Compatibility Methods Will Be Positively Misleading. *Syst Zool*, **27**, 401-410.
9. Pett, W., Ryan, J.F., Pang, K., Mullikin, J.C., Martindale, M.Q., Baxevanis, A.D. and Lavrov, D.V. (2011) Extreme mitochondrial evolution in the ctenophore Mnemiopsis leidyi: Insight from mtDNA and the nuclear genome. *Mitochondrial DNA*, **22**, 130-142.
10. Kohn, A.B., Citarella, M.R., Kocot, K.M., Bobkova, Y.V., Halanych, K.M. and Moroz, L.L. (2012) Rapid evolution of the compact and unusual mitochondrial genome in the ctenophore, Pleurobrachia bachei. *Molecular Phylogenetics and Evolution*, **63**, 203-207.
11. Arafat, H., Alamaru, A., Gissi, C. and Huchon, D. (2018) Extensive mitochondrial gene rearrangements in Ctenophora: insights from benthic Platyctenida. *BMC Evol Biol*, **18**, 65.
12. Haen, K.M., Lang, B.F., Pomponi, S.A. and Lavrov, D.V. (2007) Glass sponges and bilaterian animals share derived mitochondrial genomic features: a common ancestry or parallel evolution? *Mol Biol Evol*, **24**, 1518-1527.
13. Lavrov, D.V., Pett, W., Voigt, O., Worheide, G., Forget, L., Lang, B.F. and Kayal, E. (2013) Mitochondrial DNA of Clathrina clathrus (Calcarea, Calcinea): six linear chromosomes, fragmented rRNAs, tRNA editing, and a novel genetic code. *Mol Biol Evol*, **30**, 865-880.
14. Kayal, E., Roure, B., Philippe, H., Collins, A.G. and Lavrov, D.V. (2013) Cnidarian phylogenetic relationships as revealed by mitogenomics. *BMC Evol Biol*, **13**, 5.
15. Philippe, H., Brinkmann, H., Lavrov, D.V., Littlewood, D.T., Manuel, M., Worheide, G. and Baurain, D. (2011) Resolving difficult phylogenetic questions: why more sequences are not enough. *PLoS Biol*, **9**, e1000602.
16. Philippe, H. and Roure, B. (2011) Difficult phylogenetic questions: more data, maybe; better methods, certainly. *Bmc Biol*, **9**.
17. Dunn, C.W., Hejnol, A., Matus, D.Q., Pang, K., Browne, W.E., Smith, S.A., Seaver, E., Rouse, G.W., Obst, M., Edgecombe, G.D. *et al.* (2008) Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature*, **452**, 745-749.
18. Yang, H., Li, T., Dang, K. and Bu, W. (2018) Compositional and mutational rate heterogeneity in mitochondrial genomes and its effect on the phylogenetic inferences of Cimicomorpha (Hemiptera: Heteroptera). *BMC Genomics*, **19**, 264.

19. Pisani, D., Pett, W., Dohrmann, M., Feuda, R., Rota-Stabelli, O., Philippe, H., Lartillot, N. and Worheide, G. (2015) Genomic data do not support comb jellies as the sister group to all other animals. *P Natl Acad Sci USA*, **112**, 15402-15407.
20. Simion, P., Philippe, H., Baurain, D., Jager, M., Richter, D.J., Di Franco, A., Roure, B., Satoh, N., Queinnec, E., Ereskovsky, A. *et al.* (2017) A Large and Consistent Phylogenomic Dataset Supports Sponges as the Sister Group to All Other Animals. *Current Biology*, **27**, 958-967.
21. Feuda, R., Dohrmann, M., Pett, W., Philippe, H., Rota-Stabelli, O., Lartillot, N., Worheide, G. and Pisani, D. (2017) Improved Modeling of Compositional Heterogeneity Supports Sponges as Sister to All Other Animals. *Current Biology*, **27**, 3864-+.
22. Shimpi, G.G., Vargas, S., Poliseno, A. and Worheide, G. (2017) Mitochondrial RNA processing in absence of tRNA punctuations in octocorals. *Bmc Molecular Biology*, **18**.
23. Eitel, M. and Schierwater, B. (2010) The phylogeography of the Placozoa suggests a taxon-rich phylum in tropical and subtropical waters. *Mol Ecol*, **19**, 2315-2327.
24. Eitel, M., Guidi, L., Hadrys, H., Balsamo, M. and Schierwater, B. (2011) New insights into placozoan sexual reproduction and development. *PLoS One*, **6**, e19639.
25. Signorovitch, A.Y., Dellaporta, S.L. and Buss, L.W. (2005) Molecular signatures for sex in the Placozoa. *Proceedings of the National Academy of Sciences USA*, **102**, 15518-15522.
26. Miyazawa, H., Yoshida, M.A., Tsuneki, K. and Furuya, H. (2012) Mitochondrial genome of a Japanese placozoan. *Zoolog Sci*, **29**, 223-228.
27. Burger, G., Forget, L., Zhu, Y., Gray, M.W. and Lang, B.F. (2003) Unique mitochondrial genome architecture in unicellular relatives of animals. *Proc Natl Acad Sci U S A*, **100**, 892-897.
28. Gray, M.W., Burger, G. and Lang, B.F. (1999) Mitochondrial evolution. *Science*, **283**, 1476-1481.
29. Lavrov, D.V. (2010) In DeSalle, R. and Schierwater, B. (eds.), *Key transitions in animal evolution*. CRC Press, pp. 34-53.
30. Dubin, A., Chi, S.I., Emblem, A., Moum, T. and Johansen, S.D. (2019) Deep-water sea anemone with a two-chromosome mitochondrial genome. *Gene*, **692**, 195-200.
31. Kayal, E., Bentlage, B., Collins, A.G., Kayal, M., Pirro, S. and Lavrov, D.V. (2012) Evolution of linear mitochondrial genomes in medusozoan cnidarians. *Genome Biol Evol*, **4**, 1-12.

4. Statement

Erklärung

Hiermit erkläre ich, dass ich die Dissertation “Comparative mitochondrial genomics in basal metazoans: new phylogenetic and functional approaches” selbstständig verfasst habe. Die jeweiligen Beiträge der Koautoren zu den Chaptern I-IX wurden im Abschnitt „1. General introduction“ ausführlich dargestellt. Darüber hinaus wurden jegliche anderweitigen Hilfen Dritter in den jeweiligen Danksagungen der einzelnen Chapter kenntlich gemacht. Ferner sind in die bereits publizierten Chapter I-VII kritische Kommentare von Editoren bzw. unabhängigen Gutachtern eingeflossen.

Ich habe keine entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder anderer Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar entgeltliche Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Ich habe die Dissertation an der folgenden Institution angefertigt: Institut für Tierökologie, Stiftung Tierärztliche Hochschule Hannover, Hannover, Deutschland.

Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht. Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

_____

Ort, Datum eigenhändige Unterschrift (Hans-Jürgen Osigus)

5. Acknowledgements

First of all, I would like to thank my supervisor and "academic father" Prof. Dr. Bernd Schierwater for giving me the opportunity to work on this exciting project. He provided me a fantastic scientific environment for my work and his guidance was always a great help for me.

I also would like to thank Prof. Dr. Dieter Steinhagen who kindly agreed to review this thesis.

A very special thanks goes to Dr. Michael Eitel for his friendship and for being my "academic wingman" for so many years.

Another special thanks goes to Dr. Wolfgang Jakob for his friendship since my very first days at the ITZ in August 2006.

I also would like to thank PD Dr. Heike Hadrys for her stimulating intellectual input and for introducing me into the fascinating world of Odonata research.

A special thanks also goes to our present and former lab technicians and secretaries.

I also would like to thank all other present and former members of the ITZ for their help, encouragement and the friendly atmosphere in the institute. I really had a great time with all of you!

I would like to thank Prof. Dr. Stephen Dellaporta for inviting me to his lab at Yale University, as well as Dr. Maria Moreno and Dr. Christopher Heffelfinger for their help during my visit at Yale. I also would like to thank Prof. Dr. Rob DeSalle (AMNH, New York) for fruitful discussions.

I greatly acknowledge the financial support by the Studienstiftung des Deutschen Volkes, the Böhringer Ingelheim Fonds and the Stiftung Tierärztliche Hochschule Hannover. I also would like to thank the German Science Foundation (DFG) for the grants given to Prof. Dr. Bernd Schierwater.

Finally, I would like to thank my parents, Sigrid and Hans-Jürgen Osigus, for always supporting me the best way I can imagine! I love you!