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The phylogeography of the Placozoa suggests a taxon-rich phylum in tropical and subtropical waters

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Abstract

Placozoa has been a key phylum for understanding early metazoan evolution. Yet this phylum is officially monotypic and with respect to its general biology and ecology has remained widely unknown. Worldwide sampling and sequencing of the mitochondrial large ribosomal subunit (16S) reveals a cosmopolitan distribution in tropical and subtropical waters of genetically different clades. We sampled a total of 39 tropical and subtropical locations worldwide and found 23 positive sites for placozoans. The number of genetically characterized sites was thereby increased from 15 to 37. The new sampling identified the first genotypes from two new oceanographic regions, the Eastern Atlantic and the Indian Ocean. We found seven out of 11 previously known haplotypes as well as five new haplotypes. One haplotype resembles a new genetic clade, increasing the number of clades from six to seven. Some of these clades seem to be *cosmopolitan* whereas others appear to be endemic. The phylogeography also shows that different clades occupy different ecological niches and identifies several euryoecious haplotypes with a cosmopolitic distribution as well as some stenoecious haplotypes with an endemic distribution. Haplotypes of different clades differ substantially in their phylogeographic distribution according to latitude. The genetic data also suggest deep phylogenetic branching patterns between clades.

Keywords: cryptic species, haplotypes, phylogeography, Placozoa, placozoan biodiversity, *Trichoplax*, worldwide distribution

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Introduction

Placozoans have been attracting increasing attention from almost all fields of biology. While their role as the simplest organized metazoan model system is hardly questionable (Schierwater 2005; Schierwater *et al.* 2009a), their phylogenetic position near or even at the very base of the metazoan tree of life has been subject of hot disputes (Hadrys *et al.* 2005; Schierwater & DeSalle 2007; DeSalle & Schierwater 2008; Miller & Ball 2008; Schierwater *et al.* 2008, 2009b,c; Srivastava *et al.* 2008; Blackstone 2009; Hejnol *et al.* 2009; de Jong *et al.* 2009; Philippe *et al.* 2009; Sidall 2009). Quite remarkably, the biology of placozoans is poorly known and their ecology very poorly known. The only described species within the phylum Placozoa is *Trichoplax adhaerens* (Schulze 1883). *Trichoplax* is a small disc-shaped animal with a diameter of up to 2 mm, which continuously changes its body shape. With a total of 98 Mb it has the smallest known metazoan genome (Srivastava *et al.* 2008) and represents the simplest metazoan bauplan with only five somatic cell types (Schierwater *et al.* 2009a). An extracellular matrix is absent, so are a basal membrane, muscle or nerve cells, and a primary and secondary body axis. The upper epithelium (or 'protection layer') of the bottom crawling animal is directed to the water. It is made up of a squamous epithelium with mono ciliated cells that sometimes harbour so called shiny spheres (Grell & Benwitz 1971, 1981; Grell & Ruthmann 1991), which are believed to function in antipredator defence (Jackson & Buss 2009). The lower epithelium (or 'nutrition layer') faces the bottom and is built up of mono ciliated cylindrical cells, that account

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for the 'slow' movement of the animal and gland cells, which secrete enzymes for extra cellular digestion of the underlying algae and biofilm (Ruthmann *et al.* 1986; Grell & Ruthmann 1991; Wenderoth 1994). Sandwiched between these two layers are the interconnected fibre cells, which represent some kind of contractive elements (Schulze 1883, 1892; Grell & Benwitz 1971, 1981; Behrendt & Ruthmann 1986; Grell & Ruthmann 1991). They are responsible for the coordinated body shape changes and the 'fast' movement (Schulze 1892; Grell & Ruthmann 1991). For further details and references on the morphology see Syed & Schierwater (2002a,b) and for images of placozoans see http://www.trichoplax.com.

The natural habitat of placozoans is mostly unknown because of the nearly invisible natural appearance of placozoans. We can draw a few conclusions on their ecology from a limited number of biogeographical and ecological studies (cf. Voigt *et al.* 2004; Pearse & Voigt 2007 and refs therein). Based on these studies placozoans are common in warm tropical and subtropical marine waters in a geographic latitudinal band approximately reaching from 30° North to 30° South. Placozoans are often found on mangrove tree roots, reefs, boat docks in the eulittoral and littoral zone, and at stony beaches but never on sandy surfaces or in areas with high wave activity or with abundant freshwater input. Very little is known about the population density of placozoans in their habitats and the habitats themselves (Fraschetti *et al.* 2008). Only a single study reports seasonality in the occurrence of placozoans in the Western Pacific Ocean (Okinawa) with high numbers in the summer months and very low numbers in the winter (Maruyama 2004). Growth rates and vegetative reproduction by budding and fission seem to be positively correlated to increasing temperatures. Vegetative reproduction by binary fission is the normal mode of reproduction in the laboratory and most likely also in the field. Sexual reproduction is rarely but regularly seen under laboratory conditions, but all efforts to complete the sexual life cycle in the laboratory have been unsuccessful yet (Grell 1984; Schierwater 2005). Like all other metazoans, which have invented vegetative reproduction as a complement to sexual reproduction, placozoans likely reproduce sexually in the field in preparation for less favourable conditions (cf. Schierwater & Hauenschild 1990; Blackstone & Jasker 2003; Signorovitch *et al.* 2005). The specific mode of sexual reproduction (mono- vs. bisexual, outcrossing vs. selfing), however, remains unknown.

Placozoans represent the only animal phylum that contains just a single described species. A second species, *Treptoplax reptans* (Monticelli 1893) was never found again since its original description and its existence must be doubted (Monticelli 1893; Syed & Schierwater 2002). Recent genetic studies have suggested, however, that there is an unknown, yet substantial biodiversity within the Placozoa (Voigt *et al.* 2004; Signorovitch *et al.* 2006, 2007; Pearse & Voigt 2007; Wolf *et al.* 2007). Using ribosomal DNA genes Voigt *et al.* (2004) were able to identify eight different genetic lineages (named haplotypes H1–H8 based on 16S sequence), which form five major clades. After this pilot study the number of haplotypes was subsequently increased to ten (Signorovitch *et al.* 2006) and finally to eleven (Pearse & Voigt 2007). No morphological differences are visible in light microscopy, suggesting the existence of so-called 'cryptic' species. For overview and references on the turbulent history of placozoan research see Schierwater (2005) and Schierwater *et al.* (2009a).

Phylogeography is the study of relationships among organisms in relation to their geographical distribution and local environmental traits. In this context molecular phylogeographic analyses have become a major tool for investigating historical aspects of biogeography and understanding genetic structuring among populations (e.g. Emerson & Hewitt 2005). It involves the analysis of gene genealogies in a spatial context for inferring historical processes that have shaped current population structures and the distribution of organisms. Phylogeography is also a key tool to define immediate conservation units and conservation areas in times where species extinction accelerates continuously (cf. Thomas *et al.* 2004).

For placozoans, the few existing phylogeographic data provide only a very patchy picture of their distribution. Only 15 sites worldwide have been genetically characterized to date, with most samples from the Caribbean and the bordering Pacific areas (Voigt *et al.* 2004; Signorovitch *et al.* 2006; Pearse & Voigt 2007). Very little data are available from the Mediterranean (Western Italy), the Pacific Ocean (Western Australia, Guam, Hawaii and the Pacific coast of the US and Panama) and the Western Atlantic Ocean (Bermudas) (Pearse 1989; Tomassetti *et al.* 2005; Signorovitch *et al.* 2006). No genetic data at all are available from the Indian Ocean and the Southern and Eastern Atlantic Ocean. The known clades do not show any obvious pattern of restricted geographic distribution and no hints for ecologically separated lineages. Several lineages seem to occur sympatrically. Although placozoan specimens have been reported from around the world (Sudzuki 1977; Ivanov *et al.* 1980; Grell & López-Ochoterena 1988; Grell & Ruthmann 1991; Tomassetti *et al.* 2005; Pearse & Voigt 2007), a genetic characterization is missing for most of the findings. The latter is crucial, however, for understanding the biodiversity, phylogeny and biogeography of one of the earliest (possibly the earliest) metazoan animals with presumably a few hundred million years

of dispersal and evolution. Unravelling placozoan phylogeography may also help to better understand phylogeographic distribution patterns of benthic tropical and subtropical organisms in general.

By means of a worldwide sampling effort and molecular characterization of the mitochondrial 16S gene we here report five new haplotypes and one new clade within 23 newly genotyped sampling sites. The data suggest an unexpected high biodiversity of possibly dozens to hundreds of placozoan haplotypes and species of Placozoa and support the former observation that the 16S gene as a single marker is sufficient to characterize the phylogenetic complexity of the Placozoa. The data unravel unique geographic distribution patterns of certain genetic lineages and suggest a genetic split of haplotypes by means of ecological niche separation and a differential latitudinal distribution of higher taxonomic units (clades).

Materials and methods

Placozoan sampling and culturing

Placozoan specimens were sampled worldwide in coastal tropical and subtropical waters in different depths up to 20 m. For choosing the collection sites we focused on poorly or nonstudied areas, including the Mediterranean Sea and the Indian and the Western Pacific Ocean (see Table 1 and Fig. 3). Specimens were collected using two different methods. In the first method stones and other hard substrates, such as coral parts and mussel shells were collected at a depth of up to 1 m and placed in plastic bottles with seawater from the sampling site. These samples are hereafter referred to as 'rock samples'. As a second method, standard microscopic glass slides (76 × 26 mm) were placed in plastic microscope slide boxes ('slide samples'), which were cut open at the top and the bottom to enable water circulation (Sudzuki 1977; Maruyama 2004; Voigt *et al.* 2004). Each rack contained five evenly spaced glass slides. Nylon ropes were used to attach single or groups of racks (2–5) to the bottom, boat docks or coral reefs at a water depth of 1–20 m. As reported before (Pearse & Voigt 2007), placozoans were found most abundantly on slides floating in the water column. Most of the racks at each sampling site were thus attached to float freely in the water. Racks were exposed to the marine environment from 3 days to 3 weeks. After recovery, single and combined slide samples from each site were placed separately into plastic bottles (0.5 –2 L volume) while still submerged. The samples were then transferred to the laboratory for culturing and genetic analyses. All slides from a single rack were transferred to a glass Petri dish (14 cm in diameter and 2 cm height) with one side placed on a new microscopic slide (to prevent the sample-slides from sitting on the bottom). All culture glass dishes were pre-filled with 200 mL of 50% seawater from the sampling site and 50% sterile artificial seawater (ASW) with a salinity of 35 ppt, supplemented with soil extract (see http://www.epsag-uni-goettingen.de), KNO_3 (0.2 g/L), K_2HPO_4 (20 mg/L) and Mg_2SO_4 (20 mg/L). To each dish 1–2 mL of diluted *Pyrenomonas helgolandii* (Chromalveolata, Cryptophyceae) algal culture was added. Algae thereafter kept dividing in the cultures. Both sides of each slide were screened for placozoans once a day for up to 4 weeks using a Zeiss Stemi SV 6 dissecting microscope. Every week 50% of the water was replaced by fresh ASW for slow acclimatization to the artificial seawater. Adult animals were found within this period with some slides positive for placozoans immediately and some only towards the end of this period. Identified placozoans from both, rock and slide samples, were either processed directly for DNA isolation or transferred to new culture dishes using artificial seawater only (see above). Clonal lineages were started with a single individual in a Petri dish in a climate regulated culture room at 23 °C at a long day light regime (LD 14:10) placed 40 cm below two 30 W neon lamps (Osram, Germany) (cf. Ender & Schierwater 2003; Jakob *et al.* 2004).

Molecular analyses

Genomic DNA was extracted from single animals using FTA Elute cards micro following the manufactures' recommendations (Whatman) or by using a chelex-isolation method described in Voigt *et al.* (2004). Isolation of genomic DNA from clonally cultured isolates was performed on 50–100 individuals using a HOM buffer isolation protocol (Ender & Schierwater 2003). A region of variable length of the mitochondrial 16S rDNA gene was amplified by PCR using the primers and PCR conditions described in Signorovitch *et al.* (2006). PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced directly in both directions using the dGTP BigDye (Applied Biosystems). Cycle sequencing reactions were read on an ABI PRISM 310 DNA sequencer. When the standard sequencing protocol failed because of a GC-rich hairpin secondary structure, PCR products were subcloned into pGEM-T (Promega) and sequenced using the sequencing service for difficult templates provided by Macrogen (Korea). Chromatograms and sequences were analysed using the LaserGene software package (DNASTAR). In order to obtain additional 5′ sequences with informative characters a different 16S fragment was amplified from several representatives of

Table 1 Newly genotyped placozoan isolates. Haplotypes (H1–H16) and clades (I–VII) are listed according to their oceanographic regions. Asterisks '*' mark samples derived from stone collections. A total of 78 specimens were genotyped. SL = Schierwater Lab: Stefanos Anastasiadis, Michael Eitel, Heike Hadrys, Wolfgang Jakob, Kai Kamm, Sara Khadjeh, Jessica Rach, Sven Sagasser, Bernd Schierwater, Tareq Syed, Janne Timm; Co = Collaborators: Dorothee Huchon, Jean-Pascal Quod, Paolo Tomassetti, Ng Wai Chuen, Gray Williams

Oceanographic Region	Clade	Haplotype	Sampling site	Habitat type	Genotyped isolates	No. in Fig. 2	Date of collection	Sampled by
Mediterranean Sea	I	H1	Cala Rajada (Majorca), Spain	Stone pool	1	12	10/2006	SL
		H2	Castiglioncello, W Italy*	Stony beach	4	13	05/2008	SL
		H2	San Felice Circeo, E Italy*	Muddy water pond	2	15	10/2007	Co
		H2	Kateríni, Greece	Boat dock/harbour	2	17	08/2008	SL
		H2	Ormos Panagias	Boat dock/harbour	1	17	05/2009	SL
		H2	Port of Hammamet, Tunisia	Boat dock/harbour	3	19	04/2006	SL
		H2	Zarzis, Tunisia	Stony beach	4	19	07/2008	SL
		H2	Caesarea, Israel	Stony beach	8	20	01/ 2007	Co
	V	H9	Turunç, Turkey	Stony beach	3	18	08/2007	SL
		H10	Otranto, E Italy*	Stony beach	4	16	08/ 2008	SL
Indian Ocean	I	H2	Réunion	Coral reef	4	23	12/2006	Co
	III	H16	Mombasa, Kenya	Coral reef	2	22	05/2007	SL
	V	H4	Laem Pakarang, Thailand	Stony beach	3	24	03/2008	SL
Indo-Pacific	I	H2	Bali, Indonesia (AS)	Unknown	3	26	?	SL
		H2	Indonesia (AS)	Coral reef	3	25	?	SL
	VII	H12	Indonesia (AS)	Coral reef	2	25	?	SL
W Pacific Ocean	I	H2	Chatan (Okinawa), Japan	Boat dock/harbour	2	30	03/2007	SL
	V	H4	Kota Kinabalu (Sabah), Malaysia	boat dock/harbour	3	28	09/2005	SL
		H4	Hong Kong, China	Mangroves	2	29	03/2007	Co & SL
		H13	Hong Kong, China	flow through seawater system	8	29	04/2006, 09/2007	Co & SL
		H14	Hong Kong, China	flow through seawater system	1	29	04/2006	Co & SL
		H15	Boracay, Philippines*	stony beach	4	31	09/2007	SL
C Pacific Ocean	III	H8	Oahu, Hawaii	Boat dock/harbour	1	1	05/2007	SL
Caribbean	II	H3	Bahamas	flow through seawater system	1	9	2001	SL
	III	H8	Bahamas	flow through seawater system	1	9	2001	SL
E Atlantic Ocean	I	H2	Puerto de la Cruz (Tenerife), Spain	stone pool	6	11	08/2007	SL

*Rock samples.

haplotypes H2, H9, H12, H13 and H14 using the primers and protocol from Voigt *et al.* (2004). This way we filled gaps in the alignment to other haplotypes from previous studies (Voigt *et al.* 2004). All DNA sequences were deposited into GenBank (accession numbers GQ901078–GQ901155; see Table S1, Supporting Information). Sequences were aligned by means of MAFFT (Katoh *et al.* 2005; Katoh& Toh 2008) using the 'E-INS-' option implemented online (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). This option improved the alignment for the 16S sequences with multiple conserved domains and stretches of weakly conserved regions. Indels commonly found among different placozoan clades in less conserved loop regions were removed manually from the alignment. As some haplotypes differ only in these regions of low conservation we maintained the alignment in all phylogentically informative regions.

To infer phylogenetic relationships among placozoan haplotypes we performed Bayesian likelihood, maximum likelihood (ML) and maximum parimony (MP) inference. For likelihood-based analyses a TrN + G model of nucleotide evolution (Akaike information criterion) was used as obtained from ModelTest 3.7 (Posada & Crandall 1998). Bayesian posterior probabilities were obtained from the parallel version of MrBayes 3.1.2

(Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) with two runs (Nchains = 8; Temp = 0.5). As the TrN + G model is not implemented in MrBayes, the model was set to GTR + G with changes according to ModelTest. We ran 10 000 000 Markov Chain Monte Carlo generations, sampling at every 100 generations. The first 25% of the obtained trees were discarded. The ML analysis was carried out with PhyML 3.0 (Guindon & Gascuel 2003; Guindon *et al.* 2009) including 500 bootraps replicates. The MP analysis was performed in PAUP* 4.0b10 (Swofford 2003) with default values and bootstrap support values obtained from 10 000 replicates (full heuristic search) with gaps scored as missing characters. A haplotype network analysis was performed in TCS 1.21 (Clement *et al.* 2000) with gaps scored as a fifth character state. In the absence of a suitable outgroup midpoint rooting was applied (cf. Voigt *et al.* 2004).

In order to compare 16S divergences between placozoan haplotypes to those between closely related Porifera and Cnidaria, additional 16S sequences were taken from GenBank (http://www.ncbi.nlm.nih.gov). Sequences were aligned using MAFFT (see above) with separate alignments for Porifera and Cnidaria, respectively. Mean uncorrected pairwise distances between families (within orders), genera (within families) and species (within genera) were calculated in MEGA version 4.0 (Tamura *et al.* 2007) and compared with distances within the Placozoa. We only compared orders of Porifera and Cnidaria that had at least two sequences from different families. Similarly, mean p distances within families (and genera) were calculated only for those families (or genera) with at least two representatives from different genera (or species).

In order to obtain first estimates of the completeness of haplotype sampling in the Placozoa we plotted the number of identified haplotypes against the total number of genotyped locations. A Coleman Rarefaction Curve (Coleman 1981; Coleman *et al.* 1982) was therefore calculated in EstimateS available online at http://viceroy.eeb.uconn.edu/EstimateS.

Results

Sampling and culturing

Using standard 'trap' sampling and rock sampling procedures a total of 78 isolates from 23 field-sampling sites were collected. In addition eight isolates from two aquarium samples were also genotyped (Table 1). Sampling efforts on the following sites yielded no placozoans: coasts of Costa Rica, Argentina, Uruguay, Chile, Peru, Colombia, Florida, Crete (Greece), Cyprus, Rovinji (Croatia), Cres (Croatia), Fano (W Italy), Saintes-Maries-de-la-Mer (France), Lanzarote (Spain), Perth (W Australia) and Townsville (E Australia). The overall sampling success of approximately 60% positive sites for placozoans indicates their worldwide distribution, whereas the negative sampling efforts are no valid indication of a lack of placozoans in the respective area. Sampling was mainly performed in the summer to increase the chances for finding placozoan specimens (see Table 1). From the Mediterranean Sea, however, we were also able to collect placozoans in January, indicating their occurrence throughout the year even in this temperate climate zone. In Hong Kong we performed repeated sampling at different time points to learn about the seasonality of placozoan occurrence. During spring the number of collected placozoans was low (n = 0–3 in March through May), whereas in September 15 individuals (eight of which were genotyped) were collected under comparable sampling conditions. Most sampling was performed in shallow waters with the exception of Kenya. Here the positive slide racks were attached to a reef at a depth of 20 m. Two specimens were isolated from this location indicating their abundance at least in the first 20 m. Another sampling effort in Kenya in a mangrove stream system at 3 m water depths yielded no placozoans.

Culturing of isolates in the laboratory was mainly successful for clade I samples. Most other haplotypes died after a short while (days or weeks) of culturing, although different culturing conditions were tried. The only sample from another clade for which year-round cultures was successfully established derived from the 'Kenya' clone (H16, clade III). For clade V only cultures of H4 and H13 were stable for a few weeks with increasing population density before declining and dying off.

Systematics

As known from three previous studies (Voigt *et al.* 2004; Signorovitch *et al.* 2006; Pearse & Voigt 2007) the 16S gene is well suited for identifying species lineages in placozoans. This marker has been successfully used in the Placozoa and has been known to provide good phylogenetic resolution. We could detect seven out of 11 previously known haplotypes: H1, H2, H3, H4, H8, H9, H10. In addition we found five new 16S haplotypes (Fig. 1). These new haplotypes were named in an increasing numerical order with higher numbers found later during the study (H12–H16). Haplotypes formerly named H4-2 and H4-3 are here referred to as H9 and H10, respectively, in accordance with the continuing numbering of new haplotypes proposed by Voigt *et al.* (2004). The haplotype numbering does not denote an affiliation of a certain haplotype to a specific clade. Partial sequences within one haplotype were always 100%

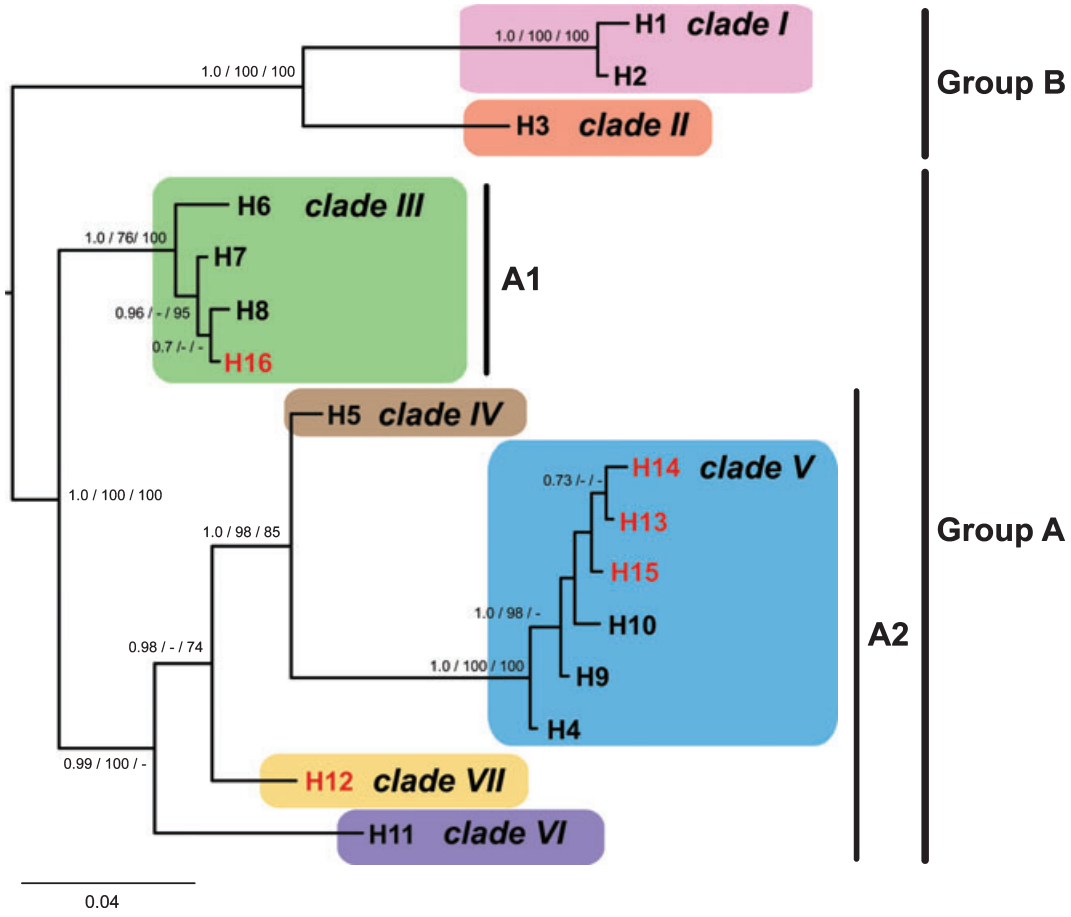

Fig. 1 16S haplotype cladogram of all known placozoan lineages. The cladogram shows a distinctive hierarchical arrangement independent of the tree-building algorithm applied. Haplotype numbers (H) refer to strains listed in Table 1. Numbers beside nodes are from left to right: Baysian posterior probabilities, Maximum likelihood and Maximum Parsimony bootstrap support. Values below 70% are marked with '–'. Two main groups ('A' and 'B') are found within the Placozoa probably representing higher taxonomic units. Within group 'A' two subgroups ('A1' and 'A2') are clearly distinguishable. Red labelling marks formerly undescribed haplotypes.

identical, independent of the isolates' origin. Thus the following 16 unique haplotype sequences were used in the alignments: *Trichoplax adhaerens*/H1 (NC_008151.1), H2 (GQ901079), H3 (NC_008834.1), H4 (NC_008833.1), H5 (AY652526), H6 (AY652527), H7 (AY652528), H8 (NC_008832.1), H9 (EF421454), H10 (GQ901128), H11 (EF421455), H12 (GQ901132), H13 (GQ901134), H14 GQ901136), H15 (GQ901137), H16 (GQ901141). The alignment contained 816 nucleotide positions including gaps. For subsequent analyses unalignable indel positions were removed, which resulted in a total of 536 nucleotide positions including gaps (see Fig. S1, Supporting Information).

Baysian inference, maximum likelihood (ML) and maximum parsimony (MP) analyses all resulted in the same overall tree topology with seven clearly separated clades, increasing the number of known clades from five to seven (I–VII; Fig. 1): five formerly described clades I–V and the new clades VI and VII. Clade VI was also recognized by Pearse & Voigt (2007) but not named. Differences between ML and MP analysis were only found within a single clade (clade V) where slightly different phylogenetic relationships were observed for haplotypes H9, H10, H13, H14 and H15 with low support (Fig. 1). In addition to the two new clades, we also found three new members of clade V (H13–H15) as well as one new member of clade III (H16). The overall phylogenetic analysis additionally reveals a separation of clades into two main groups (A and B), harbouring 13 (A) and three (B) haplotypes, respectively. Group A is furthermore subdivided into two subgroups, A1 and A2 (Fig. 1). This obvious separation of groups A and B is also immediately evident in the TCS haplotype network (Fig. 2). Haplotypes of group A1 and B are separated by at least 105 mutational steps (H2–H16). Between A2 and B the minimal number of mutational steps is 124 (H2–H11).

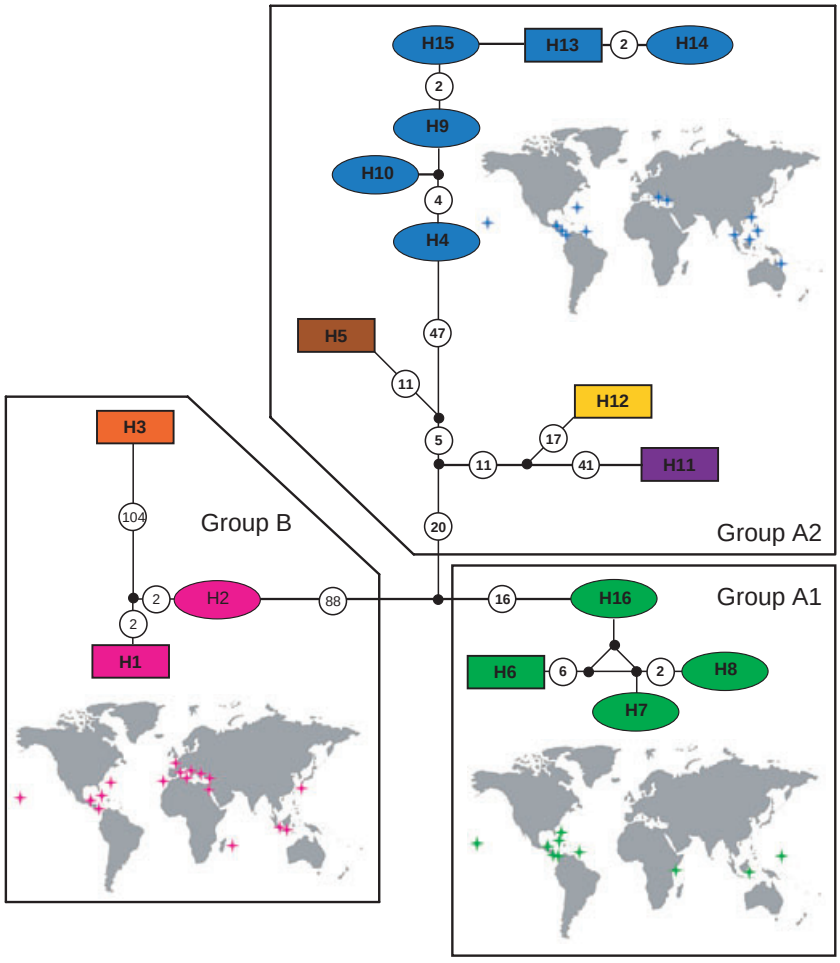

Fig. 2 TCS haplotype-network and phylogeographic distribution of clades. Based on 16S genetic distances (number of nucleotide exchanges given in circles between each haplotype) a clear grouping into groups A1, A2 and B is apparent. Colour code is the same as in Fig. 1. Putative ancestral haplotypes within each clade are marked by a rectangle. Within each group cosmopolitans are found represented by stars in the world maps. These cosmopolitan clades are clade III (group A1, green stars), clade V (group A2, blue stars), and clade I (group B, magenta stars). Stars in the world maps summarize all observed haplotypes within each clade to highlight its worldwide distribution.

For an overview of genetic differences between the seven placozoan clades and in order to provide a framework for subsequent systematic studies, we analysed mean uncorrected pairwise nucleotide distances within and between clades. The pairwise distances *within* a placozoan clade ranged from 1.6% in clade V to 2.1% in clade III (Table 2). In contrast to this intraclade variability mean distances *between* two clades ranged from 3.8% to 21.5% (Table 2 and Supporting Information Table S2).

For obtaining an ad hoc idea of the systematic importance of these values we compared them with established data from Porifera and Cnidaria. Distances between placozoan haplotypes were found to be at the same order of magnitude as seen between genera or families of Porifera and Cnidaria (Fig. 4). For instance, the highest observed value of placozoan sequence divergence of 27% is higher than any distance observed within genera, families or orders in the Porifera. Within the Cnidaria this value exceeds all comparable distances within genera and families and eight out of 10 distances among families within orders. The mean distance between placozoan clades of 13% reflects a number that separates higher taxonomic categories in other diploblastic animals (Fig. 4, Table 2 and Table S3, Supporting Information).

Table 2 The genetic distance between placozoan clades is substantially higher than within clades

Level of comparison	Distance
Highest pairwise distances within clade I	0.8
Highest pairwise distances within clade III	2.1
Highest pairwise distances within clade V	1.6
Lowest minimal pairwise distances between clades	3.8
Highest minimal pairwise distances between clades	21.5
Mean of all minimal pairwise distances between clades	13.0
Minimum of all pairwise distances between haplotypes	0.2
Maximum of all pairwise distances between haplotypes	26.7

Phylogeography

Placozoan isolates were found worldwide in tropical and subtropical waters including the Mediterranean Sea. First genetic information was obtained from the Indian Ocean (three samples) and Eastern Atlantic Ocean (one sample). In the Mediterranean Sea the sampling size increased from 1 to 12 and in the Western Pacific Ocean from two to six. The total number of genetically characterized worldwide sampling sites was thereby raised from 15 to 37. The biogeographic distribution of all known placozoan 16S haplotype lineages is summarized in Fig. 3. According to the phylogeographic distribution shown here, three groups of distributional range become obvious: (i) clades I, III, V show a worldwide distribution; (ii) clade II is restricted to the Caribbean; (iii) clades IV, VI and VII were found only on a single sampling site. The first genetic data from the Indian Ocean revealed a community of at least three different placozoan clades in this area. The aquarium samples from 'Indonesia' and 'Bali' (numbers 25 and 26 in Fig. 3 and Table 1) cannot be assigned to a specific location other than to the 'Indo-Pacific' region [compare the 'Indo' sample from Voigt *et al.* (2004)]. Thus the number of clades in this region was increased to three. Adding H12 to the Indian Ocean increases the number to four clades in this area, a number identical to the Caribbean, a known placozoan diversity hotspot (compare Fig. 3).

Our in-depth sampling of the Mediterranean revealed haplotypes from three different clades. Specimens from clade V were not previously found in this region and within this clade Haplotype H10 was only reported from the Bermudas. The phylogeographic distribution of clade III was also considerably increased by the new data. This clade was previously known from the Caribbean only, with the exception of an H8 sample from Guam and an H7 sample from the 'Indo-Pacific' (Voigt *et al.* 2004). The new data expand the distribution of clade III to the Indian Ocean (H16, Kenya), Bermuda and Hawaii (both H8). The new haplotypes H13–H15 were found in the tropical Western Pacific only, namely in Hong Kong (H13 and H14) and Boracay (Philippines; H15) increasing the number of haplotypes within the clade V to a total of six. In contrast to previous studies (Voigt *et al.* 2004; Signorovitch *et al.* 2006), we never found more than a single haplotype in a single sample from a single site. The only exception was an aquarium

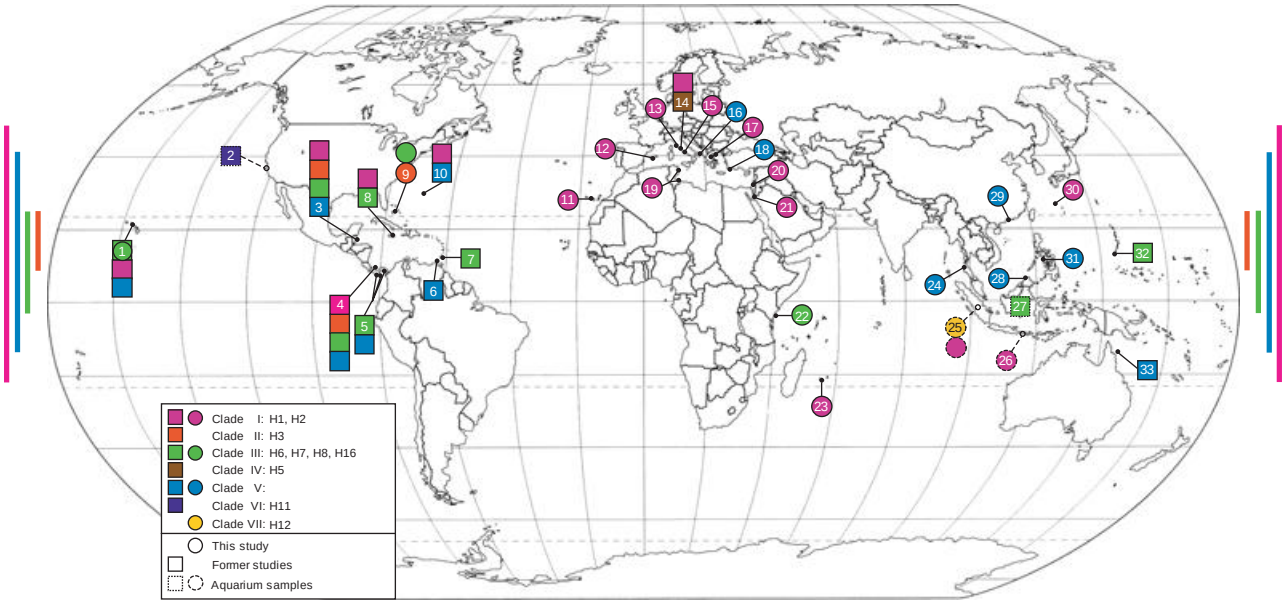

Fig. 3 Worldwide distribution of genetically characterized placozoan specimens. Circles denote the 23 newly and one additionally (Hawaii) genotyped sites from this study. Known genotypes from other studies are marked with squares. Aquarium samples (AS) with presumed origin are labelled with dashed lines. Note that several numbers combine multiple sampling sites (see text). (i) Oahu, Hawaii (US), (ii) Southern California (AS, US), (iii) Caribbean coast of Belize, (iv) Caribbean coast of Panama, (v) Pacific coast of Panama, (vi) Cubagua Island/Margarita Island (Venezuela), (vii) Grenada, (viii) Discovery Bay (Jamaica), (ix) Bahamas, (x) Bermuda (GB), (xi) Tenerife, Canary Islands (Spain), (xii) Majorca, Balearic Islands (Spain), (xiii) Castiglioncello (Italy), (xiv) Orbetello Lagoon (Italy), (xv) San Felice Circeo (Italy), (xvi) Otranto (Italy), (xvii) Kateríni and Ormos Panagias (Greece), (xviii) Bay of Turunç (Turkey), (xix) Gulf of Hammamet and near Zarzis (Tunisia), (xx) Caesarea (Israel), (xxi) Elat (Israel), (xxii) Mombasa (Kenya), (xxiii) Réunion (France), (xxiv) Laem Pakarang (Thailand), (xxv) 'Indonesia' (AS), (xxvi) Bali (AS), (xxvii) 'Indo-Pacific' (AS), (xxviii) Kota Kinabalu, Sabah (Malaysia), (xxix) Hong Kong (China), (xxx) Okinawa, Ryukyu Islands (Japan), (xxxi) Boracay (Philippines), (xxxii) Guam (US), (xxxiii) Lizard Island (NE Australia).

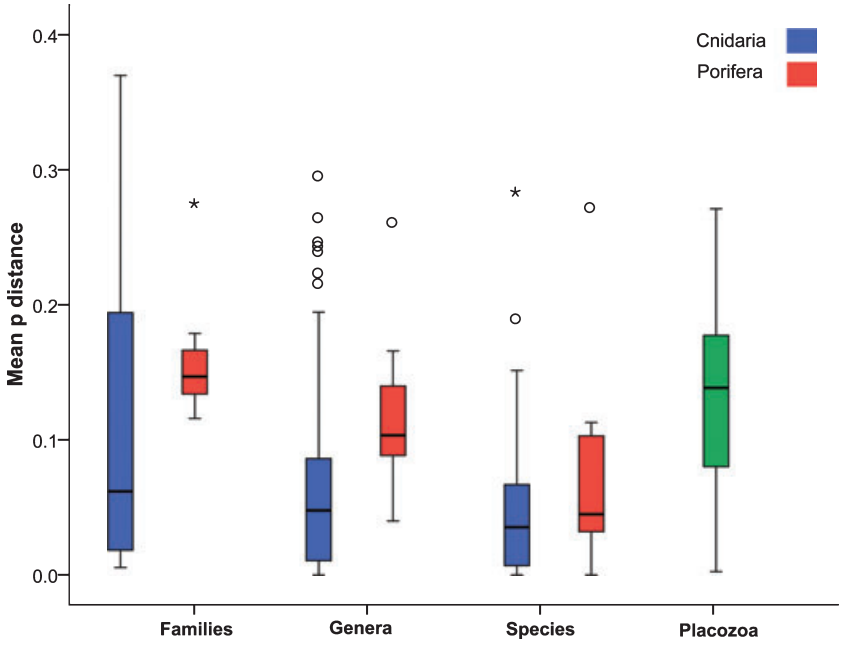

Fig. 4 Pairwise genetic distance between taxonomic ranks in Porifera, Cnidaria and Placozoa. Shown are mean uncorrected p distances in the 16S fragment between families (within orders), genera (within families) and species (within genera) of Cnidaria (blue) and Porifera (red). Mean distances between haplotypes of Placozoa (green) are at least as high as distances seen between families within orders in the other two diploblast phyla. Values lying just or clearly outside the upper quartile are marked with circles and asterisks, respectively.

sample, which revealed two different haplotypes (H2 and H12; number 25 in Fig. 3 and Table 1).

An analysis of the North–South distribution of the different clades revealed significant differences in their phylogeographic distribution. To test the hypothesis that clades differ in their temperature dependent latitudinal distribution and their specificity of niche occupation as shown in Fig. 3, we performed a Jonckheere–Terpstra test (Terpstra 1952; Jonckheere 1954) using the exact test module in PASW Statistics 18.0 (SPSS). Sea surface temperatures were downloaded for the year 2008 from the NEO homepage (http://neo.sci.gsfc.nasa.gov/Search.html) and the average, minimal and maximal temperatures were calculated for each location (see Fig. S2, Supporting Information). The Jonckheere–Terpstra test independently revealed highly significant monotonic trends ($P < 0.01$) for (i) the increasing latitudinal range and (ii) the temperature adaptation abilities (especially to the local minimal temperatures) for the clades in the following sequence: II < III < V < I; in other words clade I has the highest distributional range from North to South and the highest adaptive capacity to different water temperatures (temperature extremes); accordingly clade II has the smallest distributional range and the lowest adaptive capacity (cf. Fig. 3).

Discussion

Biodiversity and systematics

Our worldwide sampling effort led to the detection of several new haplotypes and one new placozoan clade. Comparative genetic analyses suggest the presence of a large number of placozoan species that must group into several distinct higher taxonomic units. Our data confirm the former observation that a single mitochondrial marker, the 16S gene, is both, highly suited and sufficient to identify placozoan lineages and to resolve placozoan relationships even among very closely related lineages. It must be noted that several other markers, including mitochondrial coding genes and nuclear ribosomal proteins, do not provide this level of resolution (Voigt *et al.* 2004; Signorovitch *et al.* 2007; Eitel & Schierwater, unpublished).

With this study the number of known 16S haplotypes has increased to 16, which form seven distinct clades. Given the numerous yet unsampled tropical and subtropical marine areas it is obvious that only a small fraction of placozoan species/haplotypes has been found yet. According to Fig. 5, which plots the number of total haplotypes against the number of screened locations the existence of at least several dozen haplotypes (and likely placozoan species) has to be assumed. The real number of unknown haplotypes, however, may be in the hundreds as repeated sequencing of already known haplotypes creates an artificial saturation effect. The important question what these haplotypes are in terms of systematic units (e.g. which of the haplotypes represent a separate species) cannot be addressed here and in our understanding requires additional studies that include characters from other disciplines, particularly morphology (cf. DeSalle *et al.* 2005; Suatoni *et al.* 2006; Beheregaray & Caccone 2007; Boero 2009). The relatively high genetic distance between haploytpes in comparison with Cnidaria and Porifera and the clear branching pattern suggests that the phylum Placozoa

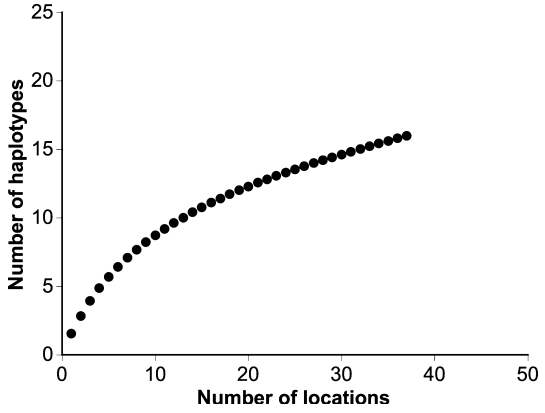

Fig. 5 Coleman rarefaction curve obtained from plotting the total number of different haplotypes against the number of genetically screened locations.

harbours at least several different taxonomic entities of yet undefined ranks. In our analyses two major groups are genetically distinguishable, group A and B, with group A being divided in two subgroups (A1 and A2). [Correction added after online publication 13 April 2010: in the preceding sentence the wording of the final clause was corrected to 'group A', 'A1' and 'A2'.] The same phylogenetic structure was also obtained from protein coding mitochondrial genes (Signorovitch *et al.* 2007). The term 'Placozoa sp.' for 16S haplotypes H2–H16 thus clearly is more reasonable than the misleading term '*Trichoplax* sp.' as this pretends a close phylogenetic relationship to the genus *Trichoplax*. Sequence variation within the 16S, ITS, 18S and 28S ribosomal RNA, (Voigt *et al.* 2004) and complete mitochondrial genome sequences (four species from Dellaporta *et al.* 2006; Signorovitch *et al.* 2007) further cement this view.

We are currently observing great confusion in placozoan taxonomy with each new sequence given a new '*Placozoan* sp./*Trichoplax* sp.' name. Currently GenBank lists 75 putative placozoan species—a number that is clearly far outside the real number of species supported by existing data. We thus propose to name placozoan specimens as 'Placozoa sp. Hx' with 'x' referring to the haplotype reference number (e.g. 2–16 for known haplotypes or $x > 16$ for new haplotypes) and *Trichoplax adhaerens* (H1), respectively. To ensure a subsequent correct assignment of an isolate to a species and to additionally provide geographic information, we suggest inclusion of the clone/isolate-ID in the taxonomic name. Accordingly the TUN-B clone from Tunisia is here named 'Placozoa sp. H2 (TUN-B clone)', for example. In order to avoid confusion when new haplotypes arise from parallel sampling we strongly suggest reporting any new haplotype to the editors of the World Placozoa Database at the World Register of Marine Species (WoRMS) (http://www.marinespecies.org/placozoa/) first.

For valid species assignment we suggest collection of morphological and ecological data for the different haplotypes and subsequent application of the taxonomic circle approach (DeSalle *et al.* 2005; Damm *et al.* 2010) before any new species is given a name. Only after the new species has been validly described by at least two different and *cum grano salis* independent datasets (e.g. 16S sequences and morphological data) we can address the question of the taxonomic ranks of the clades and groups. These morphological aspects are currently investigated, and will to be addressed in a different study. The ecological and phylogeographic aspects related to differential clade distribution, however, can be discussed here.

Phylogeography

In three former studies (Voigt *et al.* 2004; Signorovitch *et al.* 2006; Pearse & Voigt 2007) placozoans were genotyped from 15 sites of five major geographic regions: The Mediterranean Sea, the Caribbean, the Central and Western Pacific Ocean and the Western Atlantic Ocean. Our combination of slide and rock sampling led to the isolation of placozoan specimens from an additional 23 tropical and subtropical waters (including the Mediterranean) leading to the first genotyped placozoans from the Eastern Atlantic Ocean, the African coasts and the Indian Ocean. Placozoans have been known from tropical and subtropical waters but also from temperate sites with seasonally low water temperatures (11–14 °C in the Mediterranean Sea and Western Pacific; Sudzuki 1977; Tomassetti *et al.* 2005). We found samples in January in the Mediterranean Sea at 15 °C. The highest water temperature at which we found placozoans in our samples was 27 °C (Kenya, Indian Ocean).

One of the aims of this study was to find out whether the distribution of haplotypes/clades maps to geographic patterns, and whether different placozoan lineages may occupy different ecological niches. The observed genetic divergences suggest that different genetic strains are differentially adapted to certain environmental conditions. In our study we found an interesting distribution pattern of certain clades that support this view: clade I has the highest distributional range from North to South and thus can be termed a euryoecious clade with the most abundant and best adapted haplotype H2 belonging here. Not surprisingly H2 is by far the easiest to culture placozoan lineage. An example of the opposite, that is, a stenoecious lineage, is H13. This haplotype has been found at two different times and locations in Hong Kong but nowhere else. Possibly H13 is adapted to local environmental conditions. All

efforts to culture H13 in the laboratory for an extended period of time failed. Animals of haplotype H3 (clade II) have been exclusively found in the Caribbean and thus may be endemic to that region. The haplotypes H5 and H12-H16 have each been found in a single spot only and may also be endemics. Clade III representatives are restricted to a narrow latitudinal corridor ranging from 25°N (e.g. Bahamas) to 3.5°S (e.g. Kenya). While clade I likely harbours the most euryoecious and clade II possibly the most stenoecious species, clade V distributional patterns are difficult to interpret. Clade V shows a wide longitudinal distribution including tropical, subtropical and temperate regions. This cosmopolitan clade, however, has been very resistant to culturing under laboratory conditions. Besides water temperature other environmental factors such as salinity, fresh water and nutrient input from the land, water chemistry, light conditions, etc. likely affect lineage distribution and accessibility to culturing. Possibly clade V harbours a number of stenoecious species that have radiated to a broad spectrum of niches. Overall the first phylogeographic data suggest the presence of a large number of ecologically very different placozoan species lineages and at the same time highlight our poor knowledge of this group.

The above interpretations might present an underestimation of placozoan diversity and distribution for several reasons. Sample transportation and laboratory culturing prior to genetic characterization of placozoan specimen may lead to differential survival rates, as different haplotypes react differently to certain environmental conditions. Haplotypes with higher acclimatization abilities may have higher chances to survive and thus get genotyped. As we transported new samples in their natural water and reduced culturing times before analysis to a minimum, however, we do not expect that this to be significant in our study. Another factor that might affect the observed phylogeography is shipping traffic in a globalized economy, which has become a general problem for biogeography studies on marine invertebrates (Carlton & Geller 1993; Molnar *et al.* 2008; Miglietta & Lessios 2009). As ballast water of ships usually travels several days or weeks in the dark, however, placozoans are not likely to survive long routes in the absence of growing algae as food. Unfortunately we know little about other potential food sources for different placozoans.

A good, yet, underestimated source for collecting placozoans is aquaria. The new clade VI (H12), for example, derived from an aquarium sample, which was newly set up with stone/coral material from 'Indonesia'. The same is true for the 'Bali' samples. Despite the missing exact geographic assignment of these samples—and of aquaria samples in general—it is obvious, however, that they are a reasonable sources for placozoan specimens that are at least helpful for screening genetic diversity in Placozoa.

Based on the known data we can predict most placozoans are found between the equator and 20° North. Finally resolving placozoan phylogeography is a major task of unravelling species diversity and species distribution in this phylum. Given that our data suggest the presence of possibly several dozens or even hundreds of placozoan species the number of sampling locations needs to be substantially increased in future studies. Only a worldwide effort by several laboratories promises success in unravelling the biodiversity and ecological and phylogeographic distribution of the enigmatic Placozoa in detail. For this we endeavour to offer free genetic characterization of genotypes of new placozoan samples, haplotype assignment, and material and database storage (for details see http://www.marinespecies.org/placozoa/).

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M.E.'s research interest is on Placozoa, with special emphasis on the phylogenetic position, biodiversity, phylogeography and biology of the Placozoa. B.S.'s research covers (i) integrative approaches to the ecology and evolution of basal metazoans, (ii) evolutionary and applied genomics of Placozoa, and (iii) new approaches to conservation ecology.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Accession numbers of all genotyped isolates with associated clone identifier

Table S2 Pairwise genetic distances between placozoan 16S haplotypes (explanations see text). The minimal p-distance between clades (grey) is substantially higher than within clades (purple, green and blue for clades I, III and V, respectively). Note that values for H10 are misleadingly high compared to closely related haplotypes (H9, H13–H15) because of missing sequence information for H10 at the conserved 5′ end

Table S3 Poriferan and Cnidarian mean uncorrected pairwise distances (16S)

Fig. S1 16S alignment used in phylogenetic analyses in Fig. 1.

Fig. S2 Sea surface temperatures for the 37 genetically screened locations. The average temperature decreases with increasing distance from the equator. To show the differences in seasonal temperature fluctuations between tropical, subtropical and temperate habitats the minimal (min. temp.) and maximal (max. temp.) sea surface temperatures are given.

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