A Comprehensive Molecular Phylogeny of Dalytyphloplanida (Platyhelminthes: Rhabdocoela) Reveals Multiple Escapes from the Marine Environment and Origins of Symbiotic Relationships

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Abstract

In this study we elaborate the phylogeny of Dalytyphloplanida based on complete 18S rDNA (156 sequences) and partial 28S rDNA (125 sequences), using a Maximum Likelihood and a Bayesian Inference approach, in order to investigate the origin of a limnic or limnoterrestrial and of a symbiotic lifestyle in this large group of rhabditothoran flatworms. The results of our phylogenetic analyses and ancestral state reconstructions indicate that dalytyphloplanids have their origin in the marine environment and that there was one highly successful invasion of the freshwater environment, leading to a large radiation of limnic and limnoterrestrial dalytyphloplanids. This monophyletic freshwater clade, Limnotyphloplanida, comprises the taxa Dalyelliidae, Temnocephalida, and most Typhloplanidae. Temnocephalida can be considered ectosymbiotic Dalyelliidae as they are embedded within this group. Secondary returns to brackish water and marine environments occurred relatively frequently in several dalyeliid and typhloplanid taxa. Our phylogenies also show that, apart from the Limnotyphloplanida, there have been only few independent invasions of the limnic environment, and apparently these were not followed by spectacular speciation events. The distinct phylogenetic positions of the symbiotic taxa also suggest multiple origins of commensal and parasitic life strategies within Dalytyphloplanida. The previously established higher-level dalytyphloplanid clades are confirmed in our topologies, but many of the traditional families are not monophyletic. Alternative hypothesis testing constraining the monophyly of these families in the topologies and using the approximately unbiased test, also statistically rejects their monophyly.

Introduction

With about 1530 described species, Rhabdocoela is one of the most species-rich taxa of non-neodermatan flatworms. Classical taxonomy, mainly based on the presence of a proboscis and the pharynx morphology, recognises four large groups within Rhabdocoela: Kalyptorhynchia (with a frontal proboscis and a pharynx rosulatus, i.e. a mostly globular pharynx with a muscular septum and a more or less vertical axis), Thalassotyphloplanida (with pharynx rosulatus, without proboscis), Dalyellioida (with pharynx doliiformis, i.e. a barrel-shaped, anteriorly-situated pharynx with a muscular septum and a horizontal axis) and Temnocephalida (with a pharynx doliiformis and ectosymbiotic on freshwater invertebrates and cheloniids). Willems et al. [1] were the first to thoroughly and intentionally explore rhabdocoel phylogenetic relationships using 18S rDNA sequences. In contrast to the traditional morphology-based phylogenies, this study divided Rhabdocoela into two large clades: Kalyptorhynchia (±530 species) and Dalytyphloplanida (±1000 species). Dalytyphloplanids are found worldwide in marine, brackish water, limnic and limnoterrestrial environments. It comprises the Typhloplanoida, Dalyellioida and Temnocephalida. Table 1 summarizes the traditional classification of the families and subfamilies in the Dalytyphloplanida. Yet, Willems et al. [1] showed that both the “Typhloplanoida” and “Dalyellioida” are polyphyletic and their representatives are scattered all over the dalytyphloplanid tree (Fig. 1). Consequently, the clades and names established by Willems et al. [1] will be followed here. As such, Dalytyphloplanida is divided into Neodalyellida and Neotyphloplanida, the former roughly consisting of all marine “Dalyellioida”, while the latter being further divided into the well-supported Thalassotyphloplanida and a poorly-supported, trichotomous freshwater clade comprising Typhloplanidae, Dalyelliidae and Temnocephalida [1] (Fig. 1). The monophyly of this freshwater clade was already
suggested by Jondeius and Thollesson [2], though needed further support because it was based on methodologically ill-founded conclusions [3]. Later on, Willems et al. [1] re-addressed the monophyly of this clade, but could not definitively resolve the issue because of too limited taxon sampling. Hence, the existence of the freshwater clade in the Neotyphloplanida needs to be explored further using more representatives than Willems et al. [1] did. Addressing the monophyly of the neotyphloplanid freshwater clade is interesting because it would show whether or not the Dalytyphloplanida follow a pattern of repeated independent invasions of freshwater habitats by marine ancestors, as observed in other major clades of Platyhelmintes (e.g. Catenulida, Macrostomida, Prohychnida, Contenticola, Kalyptrhyynchia; see [4]).

Hundreds of species of non-neodermatan flatworms in 35 families live in symbiosis with other organisms, ranging from commensalism to obligate parasitism. With $\approx 250$ species, the majority of these symbionts are dalytyphloplanids: Temnocephalida, Umagillidae, Pterastericolidae and Graffillidae. A few species of rhabdocoel symbionts were included in the analysis of Willems et al. [1], showing that they are not closely related to each other. This raised questions on the origins of commensalism and parasitism in dalytyphloplanids. Moreover, it would be interesting to further explore the relationships of the symbiotic rhabdocoels.

Against this background, the present work aims at extending and deepening the work of Willems et al. [1] in order to: (a) establish a sound molecular phylogeny of the Dalytyphloplanida using more taxa and gene fragments, (b) explore the transition from the marine to the freshwater environment in the Dalytyphloplanida, and (c) corroborate the phylogenetic positions of symbiotic taxa and their implications for the evolution of commensalism and parasitism in free-living flatworms.

Materials and Methods

1. Collection and selection of taxa
   From 2007 to 2011, 157 dalytyphloplanid taxa were collected worldwide for DNA sequence analysis of 18S and 28S rDNA. They represent 10 of the 13 traditional families, 17 of the 24 traditional subfamilies and 57 of the 168 genera of free-living taxa. In addition, sequences of 11 symbiotic taxa were also included (4 Temnocephalidae, 2 Graffillidae, 2 Pterastericolidae and 3 Umagillidae). This taxon sampling involved 81 marine and 76 freshwater taxa, yet many marine and some limnic species are also found in brackish water, while five “limnic” species are actually

![Figure 1. Dalytyphloplanid phylogeny redrawn after Willems et al. [1]. Dalytyphloplanida consists of Neotyphloplanida and Neodalyellida. Numbers of species per terminal taxon are given in parentheses if >1. doi:10.1371/journal.pone.0059917.g001](image-url)
limnoterrestrial. All necessary permits were obtained for the described field studies. Permission for sampling in protected areas was granted by the governing authorities (Don˜ana Biological Station, Don˜ana National Park, Spain; Ezemvelo KZN Wildlife, iSimangaliso, South Africa; Museum and Art Gallery of the Northern Territory, NT, Australia). No specific permits were required for the described field studies in other locations, which were not privately owned or protected. The field studies did not involve the collection of endangered or protected species.

Marine and some brackish water animals were collected from sediment and algae using the MgCl2 decantation method, while the oxygen depletion method was deployed to obtain limnic and brackish water specimens from aquatic vegetation, mud and organic material [5]. Limnoterrestrial animals were extracted from mosses and forest soils with the Baermann pan method [6]. Specimens were identified by studying live animals, whole mounts and stained serial sections. Additional specimens were fixed in 95% ethanol and stored at 2°C until DNA extraction.

Specimen collection and sequence data and GenBank accession numbers are provided in Table S1. Dalytyphloplanid sequences from previous studies [1,7–9] were taken from GenBank and are also listed in Table S1 with their GenBank accession numbers.

Some specimens could not be identified to genus or species level because either there was not enough material available or they were new to science and not yet formally described. Nevertheless we include these taxa in our analyses in order to recover as much phylogenetic information as possible. The results of Willems et al. [1] showed that Kalyptorhynchia is the sister group of the Dalytyphloplanida. Therefore Acrorhynchides robustus (Polycystidiidae) and Placorhynchus octaculeatus (Placorhynchidae), the only two kalyptorhynch species for which both 18S rDNA and 28S rDNA sequences are available, were chosen as outgroup.

2. DNA extraction and amplification

Genomic DNA was extracted from entire specimens using the QIAamp® DNA Micro Kit with QIAamp MinElute® columns (QIAGEN) and following the manufacturer’s protocol. Extracts were stored in duplicate (40 and 20 μl) for each specimen.

For most specimens complete 18S rDNA (±1780–1800 bp) and partial 28S rDNA (±1600–1700 bp) gene fragments were amplified using the primers and PCR protocols listed in Tables 2

### Table 1. Traditional classification of the taxa comprising Dalytyphloplanida.

| Typhloplanoida Bresslau, 1933 | Dalyellioida Bresslau, 1933 |
|-------------------------------|-------------------------------|
| Promesostomidae Den Hartog, 1964 | Dalyelliidae Graff, 1908 |
| Adenorhynchinae Ax and Heller, 1970 | Proptistidae Beklemischew, 1927 |
| Brinkmanniellinae Luther, 1948 | Neokirgellinae Oswald et al., 2010 |
| Promesostominae Luther, 1948 | Proptorhynchidae Luther, 1962 |
| Gazella De Clerck and Schockaert, 1995 | Haplovedovskyninae Luther, 1962 |
| Paraproboscifer De Clerck, 1994 | Eldenia reducta Ax, 2008 |
| Vauclusia Willems et al., 2004 | Hypoblepharinidae Böhmig, 1914 |
| Trigonostomidae Graff, 1905 | Luridae Sterrer and Rieger, 1990 |
| Mariplanellinae Ax and Heller, 1970 | Graffilliidae Graff, 1908 |
| Paramesostominae Luther, 1948 | Bresslauliinae Bresslau, 1933 |
| Trigonostominae Luther, 1948 | Pseudograffilliinae Meixner, 1938 |
| Typhloplanidae Graff, 1905 | Graffilliidae Graff, 1905 |
| Ascohorinae Findeneck, 1924 | Pterasterichelidae Meixner, 1926 |
| Cephalopharynginae Hochberg, 2004 | Umgallidinae Wahl, 1910 |
| Mesopoophorinae Norena et al., 2006 | Bicladinae Stunkard and Corliss, 1950 |
| Mesostominae Bresslau, 1933 | Collastominae Wahl, 1910 |
| Olisthanellinae Bresslau, 1933 | Umgallidinae Wahl, 1910 |
| Opistostominae Luther, 1963 | Temnocephalida Bresslau and Reisinger, 1933 |
| Phaeocorinae Wahl, 1910 | Temnocephalidae Monticelli, 1899 |
| Protoplanellinae Reisinger, 1924 | Diceratocephalidae Joffe et al., 1998 |
| Rhyhchosostominae Bresslau, 1933 | Bysorphilebidae Graff, 1905 |
| Typhloplanidae Bresslau, 1933 | Didymorchidae Bresslau and Reisinger, 1933 |
| Bytorhynchidae Rieger, 1974 | Actinodactylelidae Benham, 1901 |
| Solenopharyngidae Graff, 1882 | Scutariellidae Annandale, 1912 |
| Anthopharynginae Ehlers, 1972 | |
| Lenopharynginae Ehlers, 1972 | |
| Solenopharynginae Ehlers, 1972 | |
| Carcharodonpharyngidae Bresslau, 1933 | |
| Cilopharyngiellidae Ax, 1952 | |

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and S1. Most rDNA fragments were easily amplified using the standard primer pairs TimA/TimB for the 18S rDNA gene [9] and LSU/LSUd6.3 for the partial 28S rDNA gene [10] and their respective standardised PCR protocols. Nevertheless, for a relatively large number of taxa PCR conditions had to be optimised. This could involve nothing more than increasing the number of PCR cycles to developing new primers (with often a reduction of the number of amplifiable bp as a consequence). In particular, specimens of Promesostoma, Katalagia, some Trigonostomidae, Solenopharyngidae and many marine Dalayellioida could only be amplified with taxon-specific primer pairs (Tables 2 and S1). Amplicons yielded ±1700 bp for Prom18SFb/Prom18SRb, ±1640 bp for Neodal18SF/Neodal18SR, ±1300 bp for Neodal28SFa/Neodal28SRb and ±1600 bp for SolenoFI/SolenoR.

Most PCR reactions were carried out using the 0.2 ml PuReTaq Ready-To-Go PCR beads (GE Healthcare). For each reaction 2.5 μl of each primer (5 μM), 3 μl of DNA and 17 μl of purified water were assembled in the RTG-PCR tubes to make up a volume of 25 μl. Some reactions were performed in 0.2 ml tubes using the HotStar Taq® Plus DNA polymerase (QIAGEN) kit. Each of these reactions contained 2.5 μl dNTPs (2 mM), 2.5 μl PCR buffer (10 x) with 15 mM MgCl₂, 2.5 μl of each primer (5 μM), 0.25 μl Taq DNA polymerase, 3 μl of DNA and 11.75 μl purified water. All PCR reactions were carried out in an Eppendorf Mastercycler gradient or a Techne TC-5000 thermocycler. PCR products (5 μl) were verified on a 1.4% agarose gel and stained with ethidium bromide or GelRed®. Some products were excised and purified using the QIAquick® PCR Purification Kit (QIAGEN) following the manufacturer’s instructions, but for most PCR products, purification was performed by Macrogen (Korea). In the rare cases when multiple bands occurred while checking the PCR product, the bands corresponding with the target fragment were excised and purified using the QIAquick® Gel Extraction Kit (QIAGEN) according to the manufacturer’s protocol. Sequencing was done by Macrogen (Korea) using BigDye™ terminator cycling conditions. Reaction products were purified with ethanol precipitation and run with a 3730XL Automatic DNA Sequencer 3730XL.

3. Alignment and dataset

Before alignment, consensus sequences were assembled from contigs in Staden v1.6.0 [11] and subjected to a BLAST search [12] on the NCBI website (http://www.ncbi.nlm.nih.gov) to check for possible contaminations. Subsequently, sequences were aligned with the structural Q-INS-i algorithm in MAFFT [13–14]. Manual editing of the alignments was avoided by selecting and filtering ambiguous positions with the alignment masking program Aliscore using the settings described by Misof and Misof [15]. The 5’ and 3’ alignment ends of the 18S and 28S rDNA datasets were trimmed and subsequently both alignments were concatenated in Geneious Pro 5.3.4 (Biomatters Ltd). Processed (aligned and masked) sequences that were identical, were removed from the alignments before phylogenetic analysis (Table S2). Final alignments were tested for substitution saturation with DAMBE v5.2.57 according to Xia’s method for more than 32 OTUs [16]. Proportions of invariant sites were obtained in jModeltest v0.1.1 [17] when testing the best fitting substitution model for our dataset. Saturation tests were performed on all sites with gaps treated as unknown data (Table S3).

4. Phylogenetic analyses

Maximum likelihood (ML) analyses were conducted separately for the three final datasets in RAxML v7.2.8 performing 100 independent runs of thorough searches and 1000 non-parametric bootstrap replicates under the GTR+CAT model [18–19]. This model of evolution is a more workable approximation for GTR+CAT and recommended by the program instead of GTR+Γ+I. The latter was the most appropriate model of evolution for the concatenated and 28S dataset as selected in jModeltest 0.1.1 [17] by both the Akaike (AIC) and Bayesian (BIC) information criteria. Although the lnL value was better for the GTR+Γ+I model, AIC and BIC for the 18S alignment were slightly more in favour of the TIM2+Γ+I model. Gaps were treated as missing data. Bootstrap replicates were used to construct majority rule consensus trees and bootstrap support values (BS) were plotted on best-scoring trees in SumTrees v2.0.2 of the DendroPy package [20].

Bayesian inferences (BI) were done in MrBayes v3.1.2 [21] under the GTR+Γ+I model (nst = 6; rates = invgamma), using default prior settings. Metropolis coupled Markov chain Monte Carlo sampling with one cold and three heated chains (default; temp = 0.2) in two independent simultaneous runs for 10 million generations. Gaps were treated as missing data. Convergence was determined based on the logl, values and the average standard deviation of split frequencies. Trees were sampled every 100th generation after a burnin of 2,500,000 generations (burnin = 25,000). Majority rule consensus trees were constructed from the remaining 75,000 trees. All Bayesian analyses were performed on the Biportal at Oslo University [22].

Figtree v1.3.1 [23] was used for tree drawing and displaying bootstrap support values (BS) and posterior probabilities (PP). Identical sequences removed pre-analysis were manually reinserted into the final phylogenetic trees. We consider BS and PP significant above a 0.70 and 0.95 threshold respectively, as was proposed by Alfaro et al. [24].

5. Hypothesis testing

To test alternative hypotheses on the monophyly of some dalythphloplanid families, the ML searches in RAxML as described above (100 independent runs under the GTR+CAT model) were repeated with different constrained topologies. These constrained topologies were subsequently tested against the unconstrained ML topologies with the approximately unbiased (AU) test [25]. Site-wise log-likelihoods were calculated in TreePuzzle v5.2 [26] and processed in Consel v0.1i [27] to calculate p-values for the AU test.

6. Ancestral state reconstructions

To explore habitat shifts between marine/brackish water environments and limnic environments, we followed the procedure of Pagel et al. [28]. Using Acerophychnides robustus as enforced outgroup MrBayes v3.1.2. was rerun on the concatenated dataset with settings and indications of convergence as in the phylogenetic analysis proper. MrBayes parameter files (pp) were then analysed using Tracer v1.5 [29] to obtain the lnL integrated autocorrelation time (IACT = 202) and effective sampling size (ESS = 371) [30]. After burnin (25,000), the posterior of 75,000 trees (.t) was consequently “thinned” with Burntrees v0.1.9 [31] by subsampling every 202nd tree to approximate the ESS. A majority rule consensus tree was constructed from the resulting 372 trees in MrBayes v2.0.2 and compared to the original consensus tree to ensure that topology, branch lengths and posterior probabilities remained stable. Acerophychnides robustus was pruned from all trees in the thinned tree file with Simmap v1.0 [32] because branch lengths between ingroup and outgroup have been shown to affect ancestral state reconstructions [33–34].

Ancestral states were reconstructed for 11 well-supported nodes (PP>0.95) in the 18S+28S phylogeny in BayesTraits v1.0 [28]. The binary traits, “exclusively or predominantly marine/brackish
Table 2. Primers and usage.

| Primers & Regime | Direction | Primer sequence (5’-3’) | Usage | Reference |
|------------------|-----------|-------------------------|-------|-----------|
| **18S/SSU Primers** |           |                         |       |           |
| TimA             | Forward   | AMCTGGTTGATCCTGCCAG     | PCR/Sequencing | [82]       |
| TimB             | Reverse   | AGGTGAACTGCAATGAGTA     | PCR/Sequencing | [82]       |
| S30              | Forward   | GCTTGTCTCAAAGATTAAGCC   | Nested PCR | [82]       |
| SFK              | Reverse   | TTCCTGCAAAATGTCTTCG     | Nested PCR/Sequencing | [82]       |
| 4FB              | Forward   | CGACGAGCGCTGATACGCC     | Nested PCR   | [82]       |
| 1806R            | Reverse   | CTTGTTAGCTTTTTATCTTC    | Nested PCR   | [82]       |
| PCR regime TimA/TimB |        |                         |       |           |
| DK18S53cycI:     |           | 95°C at 5 min 10 s, 30 × (94°C at 30 s, 55°C at 30 s, 72°C at 1 min 30 s), 72°C at 5 min |       |           |
| Prom185Fb        | Forward   | AGGTGAGACCCGAATGGGC     | PCR/Sequencing | This study |
| Prom185Rb        | Reverse   | AACAAGTTCCTGGAACATCT    | PCR/Sequencing | This study |
| PCR regime Prom185Fb/Prom185Rb | | DK18S5: 95°C at 5 min 10 s, 30 × (94°C at 30 s, 65°C at 30 s, 72°C at 1 min 30 s), 72°C at 5 min | |           |
| Neodal18SF       | Forward   | TGTTGATCTCGACGATATCT    | PCR/Sequencing | This study |
| Neodal18SR       | Reverse   | CCCCCGTGCTACTACGATT     | PCR/Sequencing | This study |
| PCR regime Neodal18SF/Neodal18SR | | DK18S5: 95°C at 5 min 10 s, 30 × (94°C at 30 s, 65°C at 30 s, 72°C at 1 min 30 s), 72°C at 5 min | |           |
| 600F             | Forward   | GTGCCAGCACGGCGGTT       | Sequencing | [1]       |
| 600R             | Reverse   | ACCGGCGCTGCTGGCAC       | Sequencing | [1]       |
| 1100F            | Forward   | CAGGGTCGGACGGATC        | Sequencing  | [82]       |
| 1100R            | Reverse   | GATCTGGCTAACCCTG        | Sequencing  | [82]       |
| 1857F            | Forward   | GCAATAAAGCTGTGATGC      | Sequencing  | [82]       |
| 1857FK           | Reverse   | GACATCACAGACCTGTATGC    | Sequencing  | [82]       |
| 5F               | Forward   | GCGAAGCATTTGCCAAGAAG    | Sequencing  | [82]       |
| 7F               | Forward   | GCAATAACAGCTGTGTAGTG    | Sequencing  | [82]       |
| 7FK              | Reverse   | GCATCACAGACGTGTTATGC    | Sequencing  | [82]       |
| **28S/LSU Primers** |          |                         |       |           |
| LSU5             | Forward   | TAGTGAGACCCCGCTAAYTTA   | PCR/Sequencing | [10]     |
| LSUD6.3          | Reverse   | GACTGAAGTGGAGAAGGGTTCC  | PCR/Sequencing | [10]     |
| PCR regime LSU5/LSU6.3 |     | DK18S5: 95°C at 5 min 10 s, 30 × (94°C at 1 min, 50°C at 1 min 30 s), 72°C at 1 min 30 s, 72°C at 5 min | |           |
| DK28S33cycI:     |           | 95°C at 5 min 10 s, 35 × (94°C at 1 min, 50°C at 1 min 30 s, 72°C at 1 min 30 s), 72°C at 5 min | |           |
| LSUD6.3B         | Reverse   | GAAAGGGTGTCATGGAACACG   | PCR/Sequencing | This study |
| PCR regime LSU5/LSU6.3B | | DK18S5: 95°C at 5 min 10 s, 30 × (94°C at 1 min, 50°C at 1 min 30 s), 72°C at 1 min 30 s, 72°C at 5 min | |           |
| Neodal28SFa      | Forward   | AGCGCAAGTGACAGGAAGAAGC  | PCR/Sequencing | This study |
| Neodal28SRb      | Reverse   | AGACAGCACGGTGCTCCAAG    | PCR/Sequencing | This study |
| PCR regime Neodal28SFa/Neodal28SRb | | DK18S5: 95°C at 5 min 10 s, 30 × (94°C at 1 min, 65°C at 1 min 30 s, 72°C at 1 min 30 s), 72°C at 5 min | |           |
| SolenoF1         | Forward   | CGCCGAGTGAACAGGAATGAC   | PCR/Sequencing | This study |
| SolenoR          | Reverse   | AGGCCGATGTTGGAGAAGGGT   | PCR/Sequencing | This study |
| PCR regime SolenoF1/SolenoR | | DK28S68: 95°C at 5 min 10 s, 30 × (94°C at 1 min, 68°C at 1 min 30 s, 72°C at 1 min 30 s), 72°C at 5 min | |           |
| L300F            | Forward   | CAAGTACGCTGGAGAAGTGG    | Sequencing  | [10]       |
| L300R            | Reverse   | CAATCTCCCTGACGTGTCCT    | Sequencing  | [10]       |
| L1200F           | Forward   | CCGAAGATGTCGTAACCTG     | Sequencing  | [10]       |
| L1200R           | Reverse   | GCATAGTCACGTCTTCTC      | Sequencing  | [10]       |
| L1600F           | Forward   | GCAGGAGCTGTGGCCATGAA    | Sequencing  | [10]       |
| L1600R           | Reverse   | CTCCATGGCCACCGTCTTC     | Sequencing  | [10]       |

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Water (M/B)" and "exclusively or predominantly limnic/limno-terrestrial (L/LT)" were treated as habitat states. Each of the analysed nodes represents the most recent common ancestor (MRCA) of a certain number of descendants from both marine/brackish water and freshwater habitats. MRCA-MCMC analyses were run for each node (AddMRCA) using a multistate model with two states. A reversible-jump hyperprior approach with an exponential prior seeded from a uniform distribution between 0 and 30 (rjhp 0 30) was applied to reduce uncertainty and arbitrariness of prior choice [28]. Trial runs allowed to estimate a
value for the rate deviation parameter (rd 8) and to obtain an acceptance rate between 20–40%. MCMC chains were run for 50.1 million iterations (50,100,000) and sampled every 1000 iterations (sa 1000) after a burnin of 100,000 iterations (bi 100,000). Nodes were subsequently fossilised (fossil) to test whether one of the two habitat states is significantly more supported. For some analyses with fossil priors, rate deviations parameters were set to 15 (rd 15) to secure acceptance rates between 20–40%. Each analysis was repeated three times because the harmonic means of log-likelihoods can be unstable. Bayes factors were used as test statistics and calculated from the average harmonic means for the fossilised states [28].

Results

The combined dataset initially consisted of 2890 bp for 159 sequences (incl. the outgroup); 18S and 28S datasets comprised 1614 bp for 156 sequences and 1276 bp for 125 sequences respectively. After removing identical sequences (Table S2) from the initial alignments, the numbers of sequences were 155, 145 and 121 for the combined, 18S and 28S alignments respectively. There were no indications of substitution saturation (Table S3). ML topologies were generally congruent with the BI phylogenies, which are shown in Figs. 2, S1 and S2. The concatenated data yielded better-resolved trees with overall higher support values than the single gene trees (see Figs. 2, S1 and S2). Dalythyphloplanida consists of two well-supported sister clades: Neodalyellida and Neothyphloplanida.

All trees provide strong support for three clades within Neodalyellida: (a) the Einaeella+Solenopharyngidae clade, (b) the Provorticinae+Umagillidae clade and (c) a “mixed” clade including Grafillidae, Pterastericolidae, different provorticid genera (Canetello, Baciaelledl, Poguana, Balgetia, Eldenia, Provorticidae sp.) and some undescribed taxa. Some of the traditional families, i.e. Solenopharyngidae, Pterastericolidae and Umagillidae, are monophyletic with high support, while Provorticinae and Grafillidae are polyphyletic.

Most ML and BI trees (Figs. 2 and S2) support the division of Neothyphloplanida in two large clades: (a) a marine clade, Thalassotyphloplanida s.l. consisting of Kytorhynchiidae, Trigonostomidae, Promesostomidae (except for Einaeella argyrophyla), Byrsophlebiidae and Gazzella sp. (Promesostomidae), and (b) a freshwater clade, for which we propose the new name Limnotyphloplanida, which is defined as the last common ancestor of the taxa Dalyyllidae, Tennocephalida, most Typhloplanidae, and all of the descendants of that common ancestor.

The 28S and 18S+28S (Figs. 2 and S2), but not the 18S trees (Fig. S1) support Kytorhynchiidae as sister taxon to all other Thalassotyphloplanida s.s. The position of Gazzella sp. is somewhat doubtful, yet both the 18S and 18S+28S data (no 28S data available) suggest it to be the sister taxon of the remaining Thalassotyphloplanida s.s. The latter clade consists of a polytomy of several well-supported clades: (a) a clade with some Promesostomidae (Laticine, Coronhelmis) and all Trigonostominae (Trigonostomidae), (b) a clade with Promesostominae (Promesostomidae) and Paramesostominae (Trigonostomidae), and (c) a clade with Byrsophlebiidae and some peculiar Typhloplanidae (Styloplana strongylodotomodes, Thalassoplanella collaris, Katalugia sp.). The position of Brunknanniella palmita and Kynacraea sp. (both Promesostomidae) in these clades is uncertain. Anyway, the traditional families Trigonostomidae, Promesostomidae and Byrsophlebiidae are clearly not monophyletic. Conversely, most trees and support values support the monophyly of the thalassotyphloplanida genera represented by two or more species in this study: Coronhelmis, Trigonostomum, Psychopera, Ctenopera, Beklemischevella, Proxenetes and Promesostoma.

The monophyly of the freshwater clade, Limnotyphloplanida, is generally very well supported for all datasets (BS 98–100; PP 1.00) except for the 28S ML tree (BS 64). Within this freshwater clade, all topologies and support values (BS 98–100; PP 1.00) confirm the monophyly of three major clades: (a) a clade comprising Tennocephalida and the marine Dalyyllidae (Jensonia angulata and Halamoworx sp.), (b) a clade comprising all freshwater Dalyyllidae, and (c) a clade comprising nearly all Typhloplanidae and Carbohydrophyriana (Carcharodophyrianae). ML and BI trees of the concatenated and 18S data point to a clade (BS 76–96; PP 0.99–1) grouping the Temnocephalida+Jensonia+Halamoworx clade and the other Dalyyllidae as the sister clade of the Typhloplanidae+Carcharodophyriana clade (Figs. 2 and S1). For 28S (Fig. S2), this clade is weakly supported (BS 60; PP 0.88). Within Dalyyllidae, all genera with two or more representatives in this study (Microdalyellia, Dalyyella, Castella and all species of Gieyszoria except for Gieyszoria cf. billabungensis) are monophyletic with high support values (BS 93–100; PP 0.99–1), but their more detailed relationships remain partly unresolved. Only the position of Gieyszoria cf. billabungensis, which forms a separate clade with an unidentified species from India, jeopardises the monophyly of Gieyszoria. Relations within the Typhloplanidae+Carcharodophyriana clade are conflicting and often unresolved. Conversely, the positions of Acrhochordonoposthia conica, Opistomon arsenii and Carcharodophyriana as hierarchically nested sister taxa relative to all other freshwater Typhloplanidae are well-supported in most topologies (except for the position of O. arsenii and Carcharodophyriana in the 28S trees). The remaining taxa in the freshwater Typhloplanidae are either unresolved, paraphyletic or receive various degrees of support.

The AU tests rejected the constrained trees forcing the monophyly of all “traditional” families that appear para- and polyphyletic in our trees (Table 3). Only the AU test of the 28S tree constrained by monophyly of Hyphrophlebiidae was not rejected, possibly because fewer taxa were used in this analysis. The results of the ancestral state reconstructions in the 18S+28S phylogeny are summarised in Table 4 and Fig. 2. Bayes factors clearly provide statistical support for the expected habitat state of the most recent common ancestor of the analysed clades, i.e. exclusively or predominantly marine/brackish water (M/B) for Dalythyphloplanida (node 1), Neodalyellida (node 2), Neothyphloplanida (node 4), Thalassotyphloplanida s.l. and s.s. (nodes 5 and 6), the Byrsophlebiidae+“marine” Typhloplanidae clade (node 7) and the “mixed” neodalyellid clade (node 3); exclusively or predominantly limnic/limnoterrestrial (L/LT) for Limnotyphloplanida (node 8), Dalyyllidae s.l. (node 9) and Gieyszoria (node 11). Only for the Temnocephalida+Jensonia+Halamoworx clade (node 10), the support for the best ancestral state model is average (BF 1.64).

Discussion

With “only” 41 Dalythyphloplanida 18S rDNA sequences, Willems et al. [1] established a phylogeny of Dalythyphloplanida that was very different from the traditional classification of rhodococci into “Thalassoplaneida”, “Dalyyliida”, Temnocephalida and Kalytorhynchiida. These findings invoked important questions on the origin of the freshwater taxa and the phylogenetic position of symbiotic dalythyphloplanids. Our phylogenies and ancestral state reconstructions suggest that Dalythyphloplanida, Neotyphloplanida and Thalassotyphloplanida have [eury]haline origins and that apart from some smaller colonisation events in both Neodalyellida

Phylogeny of Dalythyphloplanida

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Figure 2. Majority-rule consensus tree from the Bayesian analysis of the concatenated 18S+28S rDNA dataset. Dalytyphloplanida consists of Neotyphloplanida and Neodalyellida. Symbols above the branches indicate bootstrap values from the ML analysis. No symbols indicate support values below the thresholds in the legend. Branches have been collapsed when both the posterior probabilities and bootstrap support values are below the thresholds in the legend. Scale bars represent numbers of substitutions/site. Node numbers correspond with the most recent common ancestors of the ancestral state reconstructions.

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Table 3. p-values of the AU tests in Consel v0.11.

| Trees | 18S+28S | 18S | 28S |
|-------|---------|-----|-----|
| Constrained monophyly | 0.002 | 4e-04 | 0.738 |
| Bysophlebiidae | | | |
| Optimal ML tree | 0.998 | 1.000 | 0.262 |
| Constrained monophyly Dalyvellidae | 2e-06 | 1.000 | 2e-96 |
| Optimal ML tree | 1.000 | 1.000 | 1.000 |
| Constrained monophyly | 2e-10 | 5e-54 | 0.003 |
| Promesostomiidae | | | |
| Optimal ML tree | 1.000 | 1.000 | 0.997 |
| Constrained monophyly | 6e-07 | 5e-06 | 2e-28 |
| Provironicidae | | | |
| Optimal ML tree | 1.000 | 0.994 | 1.000 |
| Constrained monophyly | 6e-05 | 5e-06 | 0.003 |
| Trigonostomiidae | | | |
| Optimal ML tree | 1.000 | 1.000 | 0.997 |
| Constrained monophyly | 3e-67 | 3e-06 | 1e-05 |
| Typhloplanidae | | | |
| Optimal ML tree | 1.000 | 1.000 | 1.000 |
| Constrained monophyly | 3e-132 | 2e-05 | 0.065 |
| Brinkmannellidae | | | |
| Optimal ML tree | 1.000 | 1.000 | 0.935 |

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Table 4. Ancestral state reconstruction using BayesTraits v3.1.2.

| Node (mrca) | Best model | BF | Support for best model |
|-------------|------------|----|-----------------------|
| 1 | M/B | 6.88 | strong |
| 2 | M/B | 12.67 | very strong |
| 3 | M/B | 12.70 | very strong |
| 4 | M/B | 7.17 | strong |
| 5 | M/B | 9.83 | strong |
| 6 | M/B | 12.63 | very strong |
| 7 | M/B | 11.38 | very strong |
| 8 | L/LT | 2.16 | positive |
| 9 | L/LT | 2.37 | positive |
| 10 | M/B | 1.64 | average |
| 11 | L/LT | 28.71 | very strong |

Habitat states are categorised as marine/brackish water (M/B) or limnic/limnoterrestrial (L/LT). Analysed nodes representing most recent common ancestors (MRCA) are visualised in Fig. 2. Bayes Factors (BF) were calculated using the harmonic means (HM) of the fossilised states: BF = 2*(HMbest model – HMworse model). Support for the best model is described as “average” (BF>0), “positive” (BF>2), “strong” (BF>5) or “very strong” (BF>10).

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and Thalassotypyphloplanida, one very successful invasion of the freshwater environment by a common ancestor of Dalyellididae, Typhloplanidae and Temnocephalida took place. The analyses presented here are the first to specifically deal with these issues using DNA sequence data. In addition, they provide a wealth of new phylogenetic insights and taxonomic information.

1. Single escape from the marine environment?

1.1. Major radiation after a single colonisation event. Except for the 28S ML topology (BS 64), the monophyly of Limnotyphloplanida, the clade roughly comprising Dalyellididae, most Typhloplanidae and Temnocephalida, is well-established. This freshwater clade was already suggested by Jondelius and Thollesson [2] and Zamparo et al. [35] based on morphological data. Willems et al. [1] also retrieved these three freshwater clades within Neotyphloplanida, but could not corroborate limnotyphloplanid monophyly with 18S sequence data. The present study, however, strongly suggests a major ecological shift and a spectacular evolutionary radiation when the common ancestor of Temnocephalida, Dalyellididae and Typhloplanidae invaded the limnic environment. This is supported by the fact that more than half of the currently known species occur in this environment and the majority of them belong to one of the limnotyphloplanid genera present in this study.

Dalyellididae, Typhloplanidae and Temnocephalidae include some of the most species-rich rhabdococell genera (e.g. Gieysztoria, Castrada, Temnocephala) possibly suggesting evolutionary radiation took place once freshwater environments were colonised. Many of these freshwater genera have representatives on all continents and some species seem to be widespread or even cosmopolitan (e.g. Mesostoma lingua, Rhychnomesostoma rostratum, Gieysztoria cuspidata). No less than 53 dalytyphloplanid species, mostly Protoplanellinae (Typhloplanidae), are limnoterrestrial [36]. This is remarkable, since the limnoterrestrial taxa (e.g. Acrochordonoposthia, Carcharodopharynx, Byzopla, Protplanella, and Khambachia) form a polyphyletic assemblage suggesting multiple independent colonisations of limnoterrestrial habitats.

1.2. From marine and brackish water to freshwater and back? Surprisingly, our trees suggest several secondary returns to brackish water and marine habitats within Limnotyphloplanida. Species of Halammovortex and Jensenia are euryhaline. They appear as possible sister taxa of the limnic temnocephalids, but since many other dalyelliid genera that might be more closely-related to either Temnocephalida (e.g. Varsoviella and Alexkleria; see section 2.1) or Halammovortex and Jensenia have not been included in the analysis, the origins of symbiosis and a secondary return to brackish and marine environments remain highly speculative in this clade. In addition, four species of Gieysztoria exclusively occur in brackish water or marine environments (G. expeditoides, G. maritima, G. reggae and G. suboasica; see [37–39]). These species were not available for this study, but their morphology suggests that they undoubtedly belong to Gieysztoria. Other dalyelliid species are limnic, but occur facultatively in brackish water (e.g. M. arnigera, M. fusca, G. cuspidata, G. knapoci, G. troiqueta; [39]). Similar examples of limnic species that may also inhabit brackish water of up to 5‰, can be found within the limnic Typhloplanidae (e.g. Castrada hofmanni, C. intermedia, C. lanceola, Mesostoma lingua, Strongylostoma elongatum and Typhloplana viridata; [39,40]). This suggests that recolonisation of brackish habitats may be relatively
easy and may have occurred independently in different clades.

1.3. Freshwater invasions by thalassotyphloplanids and neodalyellids. In addition to the single-origin freshwater colonisation of the Limmotyphloplanida, independent shifts from marine to limnic environments also occurred within Thalassotrephoeploplanida and Neodalyellida. Many, predominantly marine genera in these clades have obligate or facultative brackish water representatives (Thalassotrephoeploplanida: Beklemishevia, Brinkmanvialla, Brysophlebs, Coronhelmis, Maehrenthalia, Promeostoma, Proxenetes, Ptychopera, Thalassotrepho, Troganostoma; Neodalyellida: Bicaelellia, Balgeta, Bresslauella, Canetella, Pogaina, Proxertes, Pseudograffilla, Vploksy; [39,40]), but only few have become limnic (see [41] for an overview). The sole holomimic thalassotyphloplanid is Styloplanella strangleaetamoides, a species apparently confined to alpine-boreal bogs. Although only 18S data were available for this taxon, Bayesian analyses support it as a member of the Brysophlebidae (+marine) Typhloplanidae clade (PP 1.0), Findenegg [42] already stressed its problematic position within Typhloplaninae and even expressed doubts on its position within Typhloplanidae. The presence of a stylette and the species’ overall morphology is similar to that of other marine Typhloplanidae, suggesting a phylogenetic reassessment of the other marine representatives of this taxon is needed.

Obligate limnic neodalyellids are very rare. We included an unidentified species of Provorticidae (Provorticidae sp.) from Hawaiian freshwater ponds, with a morphology that was somewhat reminiscent of the poorly-described Provortex sphagnorum. Some marine and euryhaline neodalyellid species have also been found in limnic habitats. For Bresslauella velicita many inland records from freshwater habitats are known [39,43], while the euryhaline Vploksyya ignava and Coronhelmis multispinosa locally invade the limnic zones of the Elbe estuary [39,44,45]. Several hypotheses on how these colonisations may have taken place, were tentatively suggested by Kolasa [41]. Euryhaline habitats such as estuaries, old land-locked seawater basins and salt marshes could have acted as stepping stones between marine and limnic habitats. Possibly Bresslauella velicita and the limnic provorticid from Hawaii, which are both closely related to many neokergilline taxa that are euryhaline (Bicaelellia brevitha, Balgeta semicirculifera, Canetella beauchampii) or exclusively known from salt marshes (Edenia reticulata), fit into this hypothesis.

2. Symbiosis

Symbiotic taxa are scattered throughout several clades in our trees, confirming previous findings that symbiotic life strategies have evolved multiple times independently in both marine and freshwater dalyellid taxa [1,2]. Ecological and evolutionary triggers for the origins of symbiotic relations and the transition from facultative commensalism to obligate parasitism are far from clear in non-neodermatan flatworms in general and dalyellid planids in particular (for an interesting hypothesis see [46]).

2.1. Temnocephalida. Temnocephalida, ecysymbionts of various freshwater invertebrates and chelonians, display nearly all intermediate steps of transition from commensalism to parasitism. As corroborated by our phylogenies, temnocephalids are in fact ecysymbiotic dalyellids.

The phylogenetic position of Temnocephalida has always been controversial. Various studies linking them to Neodermata have later been rejected (see [47] and references therein). A close relationship between Dalyellioida and Temnocephalida was suggested by Karling [48], who proposed the stylette morphology of Aleslumbrina as an ancestral “template” for the Temnocephalida in general and for Didymorchis in particular. Later, a sister group relation between free-living Dalyellid and Temnocephalid was tentatively confirmed by Ehlers [49], Jondelius and Thollesson [2] and Zamparo et al. [33]. Ehlers and Sopott-Ehlers [50] also supported this point of view based on stylette morphology and the ultrastructural synapomorphies of Jensenia and the temnocephalids (duo-gland adhesive system and lamellated rhabdites). Although very different life strategies have been adopted by the free-living Dalyellioida and by the ecysymbiotic Temnocephalida, the position of Temnocephalida within Dalyellidae is plausible considering the very similar internal morphology of both taxa. As such, the temnocephalid genus Didymorchis has even been linked to either Dalyellioida or Temnocephalida, but cladistical analyses of morphological data and ultrastructural studies on the epidermis confirms that it belongs to Temnocephalida [51,52]. This temnocephalid relationship is fully supported by our DNA data. Several morphological synapomorphies have been defined for Temnocephalida, including a multisyncytial epidermis, a posterior adhesive organ, genito-intestinal communication and a split shaft of sperm cells [51,53].

2.2. Pterastericolidae. Pterastericolidae are parasites of starfish, which have been linked to different dalyelliid groups and even to Neodermata (see [2] and references therein, [54–56]). The latter hypothesis was, however, rejected by DNA sequence data [57] and different ultrastructural characters (spemminogenesis, sperm, protonephridia; see [50] and references therein). One pterastericid species was included in the analyses of Willems et al. [1], which showed it to be firmly embedded within Neodalyellida as the sister taxon of the ecysymbiotic graffillid Graffilla buccincola. Our data confirm its position within Neodalyellida and also suggest that pterastericolids are closely related to some neokergilline provorticids (Canetella, Bicaelellia) and some graffillids (Graffilla, Pseudograffilla).

2.3. Graffillidae. This family is a polyphyletic amalgam of endosymbiotic and free-living species. Graffilla buccincola lives in the digestive glands of carnivorous gastropods. It predominantly feeds on its host’s partly-digested food and almost entirely depends on its host’s digestive enzymes [59]. Pseudograffilla arenicola and a yet unidentified marine dalyellid species (Dalyellia “houdini” sp.), both free-living, emerge as the sister taxa of G. buccincola in our topologies. Pseudograffilla is known to be microphagous rather than predatory, while for Dalyellia “houdini” sp. stylettes of other rhhabdocoels were among its gut contents (Willems, unpublished observations). The fact that Dalayellia “houdini” sp. is related to G. buccincola and P. arenicola opens interesting perspectives for future research on the evolution of nutritional strategies in this group [60].

2.4. Umagillidae. Umagillidae is the most species-rich taxon of symbiotic non-neodermatan flatworms [61,62]. Umagillids are common endosymbionts of echinoderms, with few species also living inside sipunculids. They demonstrate a wide variety of feeding behaviours, ranging from endozaic predation of symbiotic protozoans to full endoparasitic feeding on its host’s tissues. Little in-depth information (e.g. physiology, host specificity, nutritional preferences) is available about life history strategies of umagillids.

Most species, including species of Seritia and Wahita are intestinal, while species of Amphodiium are confined to the coelomic regions of its host. Cannon [61] suggests that species of Amphodiium must have been derived from intestinal inhabiting forms. With only three umagillid taxa represented in this study, none of these hypotheses could be corroborated. Yet, the phylogenetic position of Umagillidae confirms that symbiotic relations in marine rhhabdocoels originated multiple times independently involving different nutritional strategies that eventually led to full endoparasitism in some clades.
3. Taxonomic implications

Our phylogenetic inferences have important implications for the taxonomy of rhabdocoels in general and of Dalytyphloplanida in particular. The addition of new taxa and a second gene fragment (28S rDNA) in this study compared to that of Willems et al. [1], largely confirms the new clades by Willems et al. [1], i.e. Neodalyellida, Neodalyellida and Thalassotyphloplanida. The monophyly of taxa that have long been regarded as such based on morphology (Dalyeliidae, Umagillidae, Perastericolidae, Temnopharyngidae, Ptychopariidae, Solenopharyngidae), is firmly supported by our DNA sequence data, while many of the uncertain taxa (Graffillidae, Provorticidae, Promesostomidae, Trigonostomi-
dae, Typhloplanidae, Byrsophlebididae; see [2] for a morphological overview) are clearly not monophyletic in our trees. The division of Dalytyphloplanida in Neodalyellida and Neodalyellida is well-supported.

3.1. Neodalyellida.

The Einarellidae+Trisacophyrrinae, Anoplo-
dium+Provortex and Graffillidae+Perastericolidae clades suggested by Will-
em's et al. [1], are confirmed and extended in the present analysis. The position of the promesostomid Einarella argillophyla as the sister group of Solenopharyngidae is one of the several indications of the polyphyly of Promesostomidae, which is also supported by our DNA sequence data (see below). Solenopharyngidae has been well diagnosed by Ehlers [63] and is likely monophyletic. However, our analysis included species of only one of the three recognised subfamilies (Solenopharyngidae), except for Solenopharyngidae sp., which could not be placed in any subgroup because of a lack of data.

Provorticidae and Graffillidae are clearly not monophyletic, since species belonging to these families are scattered over the two other major neodalyellid clades. As some authors pointed out, the monophyly of Provorticidae is not supported by morphological synapomorphies [64,65]. Moreover, the taxonomic mixing of Provorticidae and Graffillidae is not surprising, since both taxa were originally regarded as subfamilies of a single family that was first named Graffillidae, and later on Provorticidae [66–68]. The present taxonomic separation into Graffillidae and Provorticidae is solely based on the position of the gonopore [68,69], a character considered of poor phylogenetic value by Karling [48,70]. Two peculiar sister taxa of the “mixed” clade (see results), Neodalyella sp. 1 and Neodalyella sp. 2 could not be placed in any of the existing families. Their overall morphology suggests that they belong to one of the marine dalyelliod clades, but the presence of an anterior male copulatory organ with a stylet is a unique feature within Dalytyphloplanida. Only graffillids also have a male copulatory organ in the anterior body half, though without a stylet. The provorticid Eldenia reducta is closely related to the free-living graffillid Brescanella rectica. However, Eldenia reducta has a caudal stylet accompanied by an accessory stylet, a feature that is unique within Provorticidae. The other well-defined taxa within the “mixed” clade, Bucicella, Balgita, Cantellia and Poganna, are phylogenetically closely related. Traditionally they are grouped within Neokirgellinae, which is clearly not monophyletic because of the position of the Perastericolidae.

Provorticinae (Provortex, Vejdovskya) is well-separated from the Neokirgellinae in our trees. This separation is also supported by morphology based on the relative position of prostate and seminal vesicle [70,71]. The close relation between Provortex and Vejdovskya, the only difference being separate ovaries and ovicellaria in Provortex as opposed to ovovitellaria in Vejdovskya, was already suggested by Luther [72] and Marcus [68] and is confirmed in our 18S and combined trees (no 28S sequences of Provortex available), though in a polytomy with the endosymbiotic Umagillidae in the concatenated analysis. Only one umagillid species (Anoplogium stichopi) was included in the study of Willems et al. [1], where it appeared as the sister taxon of Provortex. The close relationship of umagillids with Provorticinae was already discussed in Karling [49] and is now firmly supported by our molecular phylogenies.

3.2. Thalassotyphloplanida.

The 20S and combined data suggest that Kytorhynchidae is the sister taxon of all other thalassotyphloplanids. Kytorhynchidae have a glandular “false” proboscis derived from a permanent anterior terminal invagination of the body wall. The differentiation of the anterior tip into a proboscis-like structure is a feature they share with some other “typhloplanoids” (e.g. Trigonostomum, Astrotrochynchus, Microvahine, Rhychnomesostoma, Adenorhynchus, Hoplophrychna, Microcalypthychnus, Prothoracella, Parathychna [73–75]). The presence of a proper muscular proboscis is typical of Kalyptorhynchia. Based on “proboscis” structure and other morphological data, Rieger [75] placed Kytorhynchidae within the non-kalyptorhynch rhabdocoels. The present phylogenetic study confirms that kytorhynchids are indeed not kalyptorhynchs, but dalytyphloplanids.

The internal relationships of Thalassotyphloplanida show that many of the traditional marine typhloplanoid genera (Trigonosto-
umum, Promesostoma, Proxnetes, Ceratophylla) and subfamilies (Promesostominae, Paramesostominae, Trigonostominae) are monophy-
etic, but that the two largest traditional families are not. Indeed, both Trigonostomidae and Promesostomidae appear to be polyphyletic. In the cladogram, Trigonostominae is represented by two clades: Trigonostominae, sistergroup to a clade containing the promesostomid taxa Lituccis (Adenorhynchinae) and Colina andyonei (Brinkmanniellinae), and Paramesosto-
minae, which forms a well-supported sistergroup relationship with Promesostoma (Promesostominae, Promesostomidae). This Parame-
stoaminae+Promesostoma clade is part of a larger unresolved clade containing among others Brinkmanniella palma (Brinkmanniellinae, Promesostomidae), clearly showing the polyphyletic nature of the taxon Promesostomidae, a member of which is even found within Neodalyellida (Einarella argillophyla, see above). Another clade within the polytomy is formed by species of Byrsophlebidae, a number of marine Typhloplanidae and one freshwater typhlopla-
 nid, Styloplanella strongigastosonoides. Both Typhloplanidae and By-
rsophlebidaceae share the presence of one single ovary, but species of Byrsophlebidae have separate male and female genital pores, whereas species of Typhloplanidae have a common genital pore. Species of Typhloplanidae are nearly all limnic, but some, including Thalassoplanella and Kytalagia, occur in brackish water and marine environments. Although Luther [76] classified Thalassoplanella into Typhloplanidae, he firmly resisted to do so because of certain morphological features (e.g. the presence of a reduced second ovary). A similar situation applies to Kytalagia, which Willems et al. [77] considered to be very closely related to Thalassoplanella. In addition, many authors [75,78–81] state that the taxonomic position of other marine typhloplanid genera is doubtful (e.g. Haloplanella, Gallinamella, Notonomphonophum, Thalassoplanella, Tauridella, Lionella, Runians, Stygo-
planellina, Pratopiana, Magnetia, and Hoplodidymus). With the presence of a stylet in the male genital system (unusual for Typhloplanidae) most of these brackish water and marine taxa appear similar to Byrsophlebidae, except that Byrsophlebidae have separate male and female genital pores.

This clearly shows that the higher-level phylogeny (i.e. above genus) of the taxa in question is highly in need of revision, which was already discussed by Karling et al. [73] and many of the above-mentioned authors (e.g. [73]).
Gieszytoria. This species forms a separate clade with Dalyelliidae n. gen. n. sp., a yet unidentified species from India. Irrespective of whether or not this Indian taxon is the same as the Australian one, it is clear that based on our molecular data, both taxa are not part of the Gieszytoria clade. In general, intergeneric dalyelliid relations are far from clear, illustrated by the conflicting or unresolved position of several genera (e.g. Castrella, Dalyella, Microdalyella, Pseudodalyella, Gieszytoria) in the 18S and 28S trees.

Conclusions

Our phylogenies clearly show that: (a) dalytyphloplanids have their origins in the marine environment, (b) colonisation of a wide range of limnic and limnoterrestrial habitats took place when the common ancestor of all species of Limnotyphloplanida (comprising all Dalyelliidae, Temnocephalida, and nearly all limnic Typhloplanidae) escaped its marine environment; (c) this colonisation was followed by a spectacular radiation, resulting in speciose genera of Limnotyphloplanida, (d) some thalassotyphloplanids and neodalyelids also invaded limnic environments, though very sporadically and not followed by speciation events as in Limno-
typhloplanida; (e) secondary returns to brackish water and marine environments occurred relatively frequently in several dalyelliid and typhloplanid taxa; (f) some well-diagnosed rhobdocel families or subfamilies are monophyletic (freshwater Dalyelliidae, Umagilliidae, Pterastericolidae, Temnocephalidae, Solenopharyngidae, Trigonostominae, Paramesostominae, Promesostominae), while others are clearly polyphyletic (Graffillidae, Prosvorticidae, Promesostomidae, Trigonostomidae, Typhloplanidae, Bysophlebiidae); (g) Kytor-
hylnchiidae, an enigmatic “typhloplanoid” taxon, is most likely the sister taxon to all other thalassotyphloplanids; (h) Carcharodopharynx can be assigned to Typhloplanidae.

Although many new dalytyphloplanid clades can be formally recognised based on this molecular phylogeny, we provisionally refrain from erecting a new formal (DNA-based) classification. We believe that this should ideally be backed up by thorough morphological studies in which clear (syn)apomorphies for each newly-established clade are delineated.

Supporting Information

Figure S1 Majority-rule consensus tree from the Bayesian analysis of the 18S rDNA dataset. Legend identical to Fig. 2. (TIF)

Figure S2 Majority-rule consensus tree from the Bayesian analysis of the 28S rDNA dataset. Legend identical to Fig. 2. (TIF)

Table S1 List of species, sampling locations and amplification primers as used in this study. Additional sequences that were taken from GenBank are also listed with their GenBank accession number and when known, their sampling location. M: marine; F: freshwater; B: brackish water; L: limnoterrestrial; S: symbiotic.

Table S2 Identical sequences removed from the analyses.

Table S3 Test of substitution saturation with DAMBE v5.2.57 according to Xia’s method for more than 32 OTUs. Analyses performed on all sites with gaps treated as unknown data. Subsets of 4, 8, 16 and 32 OTUs were randomly sampled 60 times and the test was performed for each subset. Iss: simple index of substitution saturation; Iss,c:Sym: critical Iss assuming a symmetrical topology; Iss,c:Asym: critical Iss assuming an asymmetrical topology. If Iss is significantly smaller than Iss,c, little substitution saturation is present. Although Iss[18S+28S] does not significantly differ from Iss,c:Asym[18S+28S], asymmetrical trees are highly unlikely for our datasets.

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Author Contributions

Conceived and designed the experiments: NVS TA. Performed the experiments: NVS BT WW UJ TA. Analyzed the data: NVS TA. Contributed reagents/materials/analysis tools: NVS BT WW UJ TA. Wrote the paper: NVS TB TA.

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