A Mitogenomic Re-Evaluation of the Bdelloid Phylogeny and Relationships among the Syndermata

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Abstract
Molecular and morphological data regarding the relationships among the three classes of Rotiferida (Bdelloidea, Seisonidea, and Monogononta) and the phylum Acanthocephala are inconclusive. In particular, Bdelloidea lacks molecular-based phylogenetic appraisal. I obtained coding sequences from the mitochondrial genomes of twelve bdelloids and two monogononts to explore the molecular phylogeny of Bdelloidea and provide insight into the relationships among lineages of Syndermata (Rotiferida+Acanthocephala). With additional sequences taken from previously published mitochondrial genomes, the total dataset included nine species of bdelloids, three species of monogononts, and two species of acanthocephalans. A supermatrix of these 10–12 mitochondrial proteins consistently recovered a bdelloid phylogeny that questions the validity of a generally accepted classification scheme despite different methods of inference and various parameter adjustments. Specifically, results showed that neither the family Philodinidae nor the order Philodinida are monophyletic as currently defined. The application of a similar analytical strategy to assess syndermate relationships recovered either a tree with Bdelloidea and Monogononta as sister taxa (Eurotatoria) or Bdelloidea and Acanthocephala as sister taxa (Leonisca). Both outgroup choice and method of inference affected the topological outcome emphasizing the need for sequences from more closely related outgroups and more sophisticated methods of analysis that can account for the complexity of the data.

Introduction
Rotiferida and Acanthocephala (Syndermata) persist as phylogenetically problematic invertebrate taxa despite numerous attempts to decipher their evolutionary history. Here, I present the first mitogenomic exploration of relationships within the rotifer class Bdelloidea in an attempt to resolve one piece of the puzzle. Additionally, I evaluate how increased taxonomic-breadth of sampling, outgroup selection, and different methods of inference alter the phylogeny of Syndermata and compare these results to previous mitogenomic analyses. The phylum Rotiferida traditionally includes three classes: Monogononta, Bdelloidea, and Seisonidea, representing cyclical parthenogens, apparently strict parthenogens, and obligate bisexuals, respectively. Monogononta and Bdelloidea are free-living and found in aquatic or ephemerally aquatic environments [1]. While there are hundreds of monogonont and bdelloid species [2], Seisonidea consists of only three described species, each of which forms an ecto-symbiotic/commensal bdelloid species [3,4]. Under the traditional classification system, Philodinidae and Habrotrochidae belong to the same order, Philodinida, sharing similar organization of the corona (ciliated wheel organ) and rostrum (an adhesive structure that aids in creeping movement with the foot) [6,7]. Similarly, a most parsimonious tree derived from 60 morphological, embryological, and biochemical characters revealed a monophyletic Philodinida with Adinetidae as sister to this group [8]. However, molecular analyses of the bdelloid phylogeny, which could contribute to proper identification and classification of these small invertebrates, are severely lacking [9,10].

I sought clarification of the bdelloid phylogeny and specifically tested the evidence for monophyletic relationships among bdelloid families as well as sister relationships among genera by analyzing a supermatrix of 10–12 mitochondrial coding sequences from 13 bdelloids (representing 8 species and three out of four bdelloid families), three monogononts, and two acanthocephalans. Animal mitochondrial genomes typically consist of 12–13 coding sequences - atp6, cox1, cox2, cox3, cytb, nd1, nd2, nd3, nd4, nd4l, nd5, nd6, and sometimes nd8, a large and small ribosomal subunit (16s and 18s), and 22 tRNAs – usually found as a single circular molecule [11]. Mitochondrial genes are maternally inherited as a single unit and as such, usually reflect the same evolutionary history [12]. Because bdelloids are degenerate tetraploids [13,14], the potential exists for
incorporating paralogous nuclear sequences into a dataset and generating trees that reflect a chimeric history. An analysis of mitochondrial genomes circumvents this issue.

In addition to avoiding the problems of paralogy, I performed several different phylogenetic analyses on my mitochondrial dataset in an attempt to overcome any inherent biases. Several factors could contribute to model violations and result in long-branch attraction (LBA; the artificial attraction of two rapidly evolving or compositionally similar taxa that are not each other’s closest relatives [13], including taxonomic sampling, outgroup choice, rate variation across sites, and compositional heterogeneity [16,17,18]. I attempted to address these issues by increasing the taxonomic sampling of Bdelloidea, selecting closely related outgroups (Monogononta and Acanthocephala), choosing appropriate models of amino acid substitution, and accounting for rate and compositional heterogeneity. My results provide little justification for maintaining the order Philodinida or the family Philodiniidae and emphasize the need for increased taxonomic sampling, more phylogenetically informative data, and better models of protein evolution to resolve the bdelloid phylogeny.

Finally, I rooted Syndermata with Platyhelminthes and Chaetognatha to recover relationships among Acanthocephala, Monogononta, and Bdelloidea. Because it is difficult to assess how these relationships might change with the introduction of sequences from Scisnoida, I compared my results with two recent mitogenomic analyses and a study that included >1000 cox1 sequences from the same syndermate lineages [9,19,20]. Both previous mitogenomic analyses support a sister relationship between Acanthocephala and Bdelloidea (Lemnisacea) [19,20]. The results from the large-scale cox1 study are inconclusive: topology tests support Lemnisacea but the best maximum likelihood and Bayesian trees support a sister relationship between Bdelloidea+Monogononta (Eurotatoria) [9]. I incorporated the two strategies employed by these three studies – more sequences and greater taxonomic representation – in attempt to improve resolution. My analyses reveal that Acanthocephala consistently falls sister to Eurotatoria when site-homogeneous, empirical amino acid substitution models are applied to a syndermate dataset rooted by Platyhelminthes. With a more complex model and/or a more slowly evolving outgroup (Chaetognatha), a sister relationship between Acanthocephala and Bdelloidea emerges. Relationships among subtaxa of Syndermata appear rather unstable and highly influenced by outgroup selection. Thus, care should be taken to reduce the effects of systematic biases.

Materials and Methods

Rotifer Culture and Molecular Techniques

Thirteen bdelloids, three monogononts, and two acanthocephalans represented Syndermata in the present analyses (Table 1). Members of Bdelloidea include: Adineta vaga and Adineta riecie representing the family Arinidae, Macrotrachela quadriornifera, Philodina roseola, Philodina acuticornis, and Rotaria rotatoria representing the family Philodinidae, and Habrotrocha rosa and Habrotrocha constricta representing the family Habrotrochidae (Table 1). Brachionus plicatilis, Brachionus manjavacas, and Brachionus calyciflorus represented Monogononta (Table 1). Cultures of Adineta vaga, Habrotrocha constricta, and six distinct but closely related clonal cultures of Macrotrachela quadriornifera (“MM”, “MA”, “CR”, “LH”, “HR”), and “MQ” obtained from M. Meselson) grew in filtered or autoclaved Poland Spring water and subsisted on E. coli strain M28. I extracted DNA using a bead-beater and 0.1 mm silica beads to break open the rotifers, followed by lysis in an SDS/proteinase K (20 mg/ml) solution and a standard phenol chloroform extraction with ethanol/sodium acetate precipitation. Degenerate primers from Min and Park (2009) initially amplified small fragments of the cox1, cox2, and 16s mitochondrial genes from M. quadriornifera, A. vaga, and H. constricta [20]. The sequences generated from these amplicons then directed the design of primers specific to these taxa. The NEB LongAmp PCR kit (NEB, Ipswich, MA) generated 5–12 Kb amplicons. Either the hydro-luminescent (GeneMachines, San Carlos, CA, USA) or the Covaris S2 (Covaris Inc, Woburn, MA, USA) sheared PCR products into 500–1500 Kb pieces. T4 DNA polymerase (Invitrogen, Carlsbad, CA) end-repair, dephosphorylation of 5’ ends with Antarctic phosphatase (NEB, Ipswich, MA, USA), and 5’ A tail addition using GoTaq polymerase (Promega, Madison, WI, USA) proceeded DNA shearing. Qiagen PCR Qiagen quick purification columns removed enzymes after each reaction (Qiagen, Valencia, CA, USA). Amplicons were then ligated in to the Topo TA 2.1 PCR or Topo TA 4.0 sequencing vector and transformed into TOP10 electrocompetent cells (Invitrogen, Carlsbad, CA, USA). Colonies were grown in 96-well plates and the Biomek FX liquid handling robot (Beckman Coulter, Fullerton, CA, USA) purified positive plasmids using a standard alkaline lysis protocol. The Applied Biosystem 3730 XL capillary sequencer (Applied Biosystems, Foster City, CA, USA) performed all sequencing, which included both forward and reverse reads generated with M13 primers and ABI BigDye 3.1 chemistry.

Total RNA was isolated from clonal cultures of A. riecie, P. acuticornis, P. roseola, H. rosa, and B. manjavacas using RNaseAqueous Micro Kit (Ambion). Synthesis of cDNA with first strand primer 5’-CTA GAG GCC GAG GCG GAT TTT TTT TTT TTT TTT–3’ was used as the template switching property of Superscript II (Invitrogen) to incorporate barcoded, biotintylated 5’ adapters that matched the “A” sequence primer used 5’ GCC TTC CTC GCG CCA TCA Gxx xxx GG, where xxxxx is CACTG for B. manjavacas). To prepare libraries for pyrosequencing, 3–5 µg of cDNA was sheared using an Aeromist Nebulizer (Allied Healthcare Products) for 3–4 min at 50 psi N2, and the biotintylated 5’ EST ends of the fragmented library were captured with Dynabeads M-280 Streptavidin (Invitrogen). The captured DNA was end repaired using a Quick Blunt Kit (New England BioLabs) and ligated to a modified 454 FLX “A” primer adapters ([AAC CCT TGC CAG CCC GCT CAG T] following A-tailing with Taq polymerase. Libraries were quantified and sequenced on a Roche GS FLX using the manufacturers protocols. Resting eggs of Brachionus calyciflorus Florida strain (Florida Aqua Farms Inc., USA) were hatched in MBL medium. Neontes were collected, washed with sterile DI water, and pelleted by centrifugation. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions, and purified using the Microcon Centrifugal Filter device YM-100 (Millipore, Billerica, MA). A genomic DNA library was prepared using the Nextera DNA Sample Prep Kit (Illumina, San Diego, CA, USA) and sequenced using the Roche 454 GS-FLX with Titanium chemistry.

Sequences were trimmed of the 5’ barcode and 3’ cDNA primer (if present) using in-house Perl scripts, and assembled using Newbler v2.6 [21]. TBLASTX similarity searches [22] of these transcriptomic and genomic libraries against an MA mitochondrial reference identified putative mitochondrial coding sequences.

Sequence Editing and Genome Assembly

For M. quadriornifera, A. vaga, and H. constricta, Phred v.1.08 called bases and assigned quality values, crossmatch v.1.08 removed all vector sequence, and an in-house Perl script assembled forward and reverse reads together when present
Mitochondrial Sequence Annotation and Alignment

Additional mtDNA Sequences

The acanthocephalan mt genomes from *Leptothyroides thecatus* (GenBank # NC_016754; [19]) the monogonont mt genome of *Brachionus plicatilis* (GenBank #s NC_010472 and NC_010484; [28]), and a previously sequenced bdelloid genome from *Rotaria rotatoria* (GenBank # NC_013568; [20]) were downloaded from GenBank and included in phylogenetic analyses (Table 1). Outgroup sequences derived from the Platyzoonines *Benedenia hishinaui* and *Schistosoma mansoni* (GenBank #s NC_014591 and NC_002545) and the Chaetognatha *Spadella cephaloptera* and *Paraspadella gatoi* (GenBank #s AV545549 and AY619710; Table 1). Mitochondrial sequences generated or mined in this study were submitted to GenBank and given the accession numbers JX183989–JX184083 and JW861079–JW861112.

Mitochondrial Sequence Annotation and Alignment Properties

GLIMMER v.3.0 [29] identified open reading frames while BLAST similarity searches using TBLASTX [22] against *R. rotatoria* coding sequences distinguished legitimate and non-legitimate ORF calls. Musc v.3.8.31 [30] generated amino acid alignments and MEGA v5.0 [31] generated in-frame nucleotide alignments for individual mitochondrial genes. The CLC DNA workbench (http://www.clcbio.com) or an in-house Python script concatenated gene alignments into a supermatrix. MEGA also provided summary statistics such as GC and amino acid compositions for coding sequences and the relative rate test for estimating different rates of evolution among taxa [32]. TREE-PUZZLE v.5.2 [33] provided estimates of compositional homogeneity among sequences under JTT, MtREV, and WAG models of amino acid substitution with site rate variation modeled by a gamma distribution.

Phylogenetic Analyses

I employed Bayesian and maximum likelihood (ML) analyses to reconstruct the relationships among lineages of Bdelloidea and Syndermata and a variety of strategies to reduce compositional biases, decrease the effects of saturation, and assess the reliability of my results (summarized in Table 2). These strategies included applying different models of amino acid evolution with and without data partitioning, choosing different outgroups, and recoding amino acid alignments into Dayhoff categories [34]. Dayhoff recoding helps mitigate the effects of compositional heterogeneity by binning amino acids into 6 groups (AGPST, C, DENQ, FWY, HKR, ILMV) based on biochemical properties, such as being a positively charged amino acid [18,34]. These bins reflect amino acids that tend to replace each other [18,34].

RAxML v.7.0.4 or v.7.2.8 [35,36] reconstructed maximum likelihood trees from bdelloid and syndermate amino acid alignments (Table 2). All analyses ran under a gamma distribution of among site rate-heterogeneity with four rate categories (the PROTGAMMA option) with 100–500 bootstrap replicates, a random seed, and amino acid substitution models chosen by ProtTest v.2.4 [37,38,39] (Table S1). ML analyses reconstructed bdelloid and syndermate phylogenies from partitioned and non-partitioned data (Table 2). The model(s) assigned to the majority of the mitochondrial proteins (JTT, see Table S1) or chosen by the relative rate test for estimating different rates of evolution among taxa [32]. TREE-PUZZLE v.5.2 [33] provided estimates of compositional homogeneity among sequences under JTT, MtREV, and WAG models of amino acid substitution with site rate variation modeled by a gamma distribution.

Table 1. Species, taxonomic classification, and sequencing coverage of mitochondrial genes.

| Species                     | Phylum/Class/Family          | Missing genes | AA sequence length |
|-----------------------------|------------------------------|---------------|-------------------|
| Macrotrachea quadricornifera | Rotifera/Bdelloidea/Philodinidae* | None          | 3306              |
| Habrotrocha constricta      | Rotifera/Bdelloidea/Habrotrichidae* | nd2           | 3341              |
| Habrotrocha rosa            | Rotifera/Bdelloidea/Habrotrichidae* | None          | 3257              |
| Philodina roseola           | Rotifera/Bdelloidea/Philodinidae* | None          | 3062              |
| Philodina acuticornis       | Rotifera/Bdelloidea/Philodinidae* | Truncated cox1 & nd5 | 2952              |
| Rotaria rotatoria          | Rotifera/Bdelloidea/Philodinidae | None          | 3340              |
| Adineta ricciae            | Rotifera/Bdelloidea/Adinetidae* | None          | 3305              |
| Adineta vaga                | Rotifera/Bdelloidea/Adinetidae* | None          | 3348              |
| Brachionus plicatilis       | Rotifera/Monogononta/Brachionidae | None          | 3413              |
| Brachionus manjavacas       | Rotifera/Monogononta/Brachionidae* | nd6           | 3160              |
| Brachionus calyciflorus     | Rotifera/Monogononta/Brachionidae* | nd2, nd3      | 2698              |
| Leptothyroides thecatus     | Acanthocephala/Paracanthocephala/Rhadinorhyncha | None | 3410              |
| Oncicola luehei             | Acanthocephala/Archiacanthocephala/Oligacanthorhynchaideae | None | 3457              |
| Benedenia hoshinaui        | Platyhelminthes/Monogenea/Capsalidae | None | 3289              |
| Schistosoma mansoni         | Platyhelminthes/Trematoda/Schistosomatidae | None | 3306              |
| Paraspadella gatoi         | Chaetognatha/Phragmarphora/Spadellidae | atp6 | 3201              |
| Spadella cephaloptera       | Chaetognatha/Phragmarphora/Spadellidae | atp6 | 3206              |

*“None” indicates the presence of a complete mitochondrial coding sequence repertoire (atp6, cox1-cox3, cytb, nd1-nd6, and nd4). Mitochondrial sequences generated from this study; AA sequence length, total length of concatenated amino acid sequences from mitochondrial genes. The mitochondrial genomes of all six clonal cultures of *Macrotrachea quadricornifera* were sequenced completely. The gene, atp6 is not present in Chaetognatha. doi:10.1371/journal.pone.0043554.t001
over-fitting the data, which might occur if each protein was assigned to its own partition [40]. Additionally, RAxML and the MEGA implementation of parsimony analysis generated phylogenies based on Dayhoff-recoded alignments (Table 2).

MrBayes v.3.1.2 [41] and Phylobayes v.3.2e [42] performed all Bayesian inferences (Table 2). MrBayes analyses comprised of two runs of 1 or 2 million generations sampled every 100th generation with the first 200,000–250,000 generations discarded as burn-in. Bayesian analyses included an alpha-shaped gamma distribution across site-rate heterogeneity and a proportion of invariant sites (I+G). In lieu of certain models specified by ProtTest but not available in MrBayes (such as JTT), I assigned the WAG model to the bdelloid alignment and the mixed model of evolution to bdelloid and syndermate alignments (Table 2). The mixed model of evolution allows the MCMC sampler to choose the best amino acid substitution model (MrBayes manual) – in these cases MrRev for bdelloid and syndermate phylogenies. Tracer v.1.5.0, from the BEAST package, graphically displayed the trace files from MrBayes analyses and allowed evaluation of chain mixing and run convergence and confirmed the adequacy of burn-in times [43].

Phylobayes offers an advantage over MrBayes and RAxML by applying the CAT model of amino acid evolution, which more accurately infers amino acid substitutions [42,44]. Essentially, CAT is a site heterogeneous model that estimates site-specific equilibrium frequencies and provides a greater robustness to LBA artifacts than the site-homogeneous models offered in MrBayes and RAxML [42,44]. Phylobayes ran with the following default parameters for the site-homogeneous models offered in MrBayes and RAxML (such as JTT), I assigned the WAG model to the bdelloid alignment and the mixed model of evolution to bdelloid and syndermate phylogenies. Tracer v.1.5.0, from the BEAST package, graphically displayed the trace files from MrBayes analyses and allowed evaluation of chain mixing and run convergence and confirmed the adequacy of burn-in times [43].

I rooted Bdelloidea with sequences from the most closely related taxa available - Monogononta and Acanthocephala - to provide topological balance and decrease the chances of homoplasy [16] although Monogononta and Acanthocephala still fall on relatively long branches with respect to Bdelloidea. I chose Platymbelmintes to serve as an outgroup to Syndermata because Platymbelmintes shows a close association with Syndermata [19,45,46,47,48,49] and mitochondrial genomes from representatives of Gnathifera (a clade comprised of Gnathostomulida and perhaps Cyclophora, Micrognathoza, and Gastrotricha) - a probable sister group to Rotifera [5,8,50,51,52,53] - have not been sequenced yet. Despite being the most closely related group to Syndermata available for outgroup sequences, most members of Platymbelmintes are rapidly evolving, which potentially promotes LBA to rapidly evolving members of the ingroup [46,54]. Two members of Chaeognatha - another phylogenetically elusive invertebrate phylum - served as an alternative root to assess whether outgroup choice affected the topology of Syndermata.

### Topology Testing

The approximately unbiased (AU) test and the Shimodaira-Hasegawa (SH) test, as implemented in CONSEL v.0.1i [55] evaluated the possibilities of monophyletic Adinetidae+Philodinidae, Adinetidae+Habrotrochiidae, and Philodinidae+Habrotrochiidae clades as well as whether all possible sister relationships among bdelloid genera were equally plausible. RAxML reconstructed the most likely trees with these groups constrained as monophyletic and calculated the site likelihood values for these trees. I also tested all possible relationships of Acanthocephala to Monogononta and Bdelloidea to determine whether any relationships could be definitively ruled out.

### Results

#### Bdelloid Mitochondrial Genomes

I sequenced the complete mitochondrial genomes of six *M. quadricornifera* clonal cultures (MA, MM, LH, HR, MQ, and HR), which form a monophyletic clade as well as the complete
mitochondrial genome of *A. vaga* and 11/12 genes from *H. constricta* (Table 1). Average assembly coverage was 14X with an average read length of 733 bp using both forward and reverse reads. The mitochondrial genomes of *M. quadricornifera* are ~1 Kb shorter than *R. rotatoria* and are very similar in size to each other, ranging from 14,201 bp – 14,204 bp in length. The genome of *A. vaga* is the shortest at 14,032 bp. I obtained all or most mitochondrial coding sequences for *A. ricciae*, *H. rosa*, *P. roseola*, and *P. acuticornis* (Table 1) from transcriptome libraries. Because all complete bdelloid mitochondrial genomes sequenced thus far contained the same 12 coding sequences (*atp6*, *cox1–cox3*, *cytb*, *nd1–nd6*, and *nd4l*) and 2 ribosomal subunits (*ssu* and *lsu*) and shared complete synteny, I chose to focus on phylogenetic analysis of mitochondrial proteins instead of gene order or gene presence/absence. Additionally, I could not assess mitochondrial genome sizes or gene order information from transcriptomic datasets except in the instances of polycistronic sequences that contained gene order information for 2–4 genes.

### Brachionus Mitochondrial Sequences

I obtained mitochondrial sequences from *B. manjavacas* and *B. calyciflorus* transcriptomic and genomic datasets, respectively and compared these sequences to the previously published mitochondrial genome of *B. plicatilis*. I found all mitochondrial coding sequences except *nd6* for *B. manjavacas* and *nd2* and *nd3* for *B. calyciflorus* (Table 1). The contigs assembled from reads sequenced by 454 technology could not provide confirmation of synteny among monogeneont mitochondrial genomes (with the exception of polycistronic contigs) nor could they establish whether *B. manjavacas* and *B. calyciflorus* divided their mitochondrial genomes between two circular chromosomes as in *B. plicatilis* [28].

### Nucleotide and Amino Acid Composition

Syndermata and outgroup sequences displayed variable intra and inter-group GC compositions (Monogononta 29–36% GC, Acanthocephala 28–41% GC, Bdelloidea 20–26% GC, Platyhelminthes 26–31% GC, and Chaetognatha 27–36% GC). The disparate GC composition in the datasets and the long evolutionary periods of history being considered recommended analysis at the protein level, where extreme compositional biases are potentially mitigated and the potential for substitution-rate saturation decreases. However, chi-square tests for compositional homogeneity indicated that the amino acid frequencies of monogonont, acanthocephalan, and many bdelloid mitochondrial sequences potentially undermines the validity of the trees generated with these matrices depending on the severity of the violation. While Dayhoff-recoding enabled all bdelloids to pass the chi-square test for homogeneity, monogonont and acanthocephalan sequences still failed (Table 3). Both outliers to Syndermata (Platyhelminthes and Chaetognatha) failed the test of compositional homogeneity whether recoded or not (Table 3).

| Taxon                | Original data P-values (%) | Recoded data P-values (%) |
|----------------------|-----------------------------|---------------------------|
| *A. vaga*            | 28                          | 97                        |
| *A. ricciae*         | 0.96                        | 94                        |
| *H. rosa*            | 0.02                        | 83                        |
| *H. constricta*      | 0.07                        | 64                        |
| *M. quadricornifera* (CR) | 6.35                   | 89                        |
| *M. quadricornifera* (HR) | 0.02                   | 90                        |
| *M. quadricornifera* (LH) | 7.85                   | 92                        |
| *M. quadricornifera* (MA) | **4.58**               | 90                        |
| *M. quadricornifera* (MM) | **4.94**               | 91                        |
| *M. quadricornifera* (MQ) | 9.86                   | 89                        |
| *P. roseola*         | **0.31**                    | 84                        |
| *P. acuticornis*     | 0.5                         | 65                        |
| *R. rotatoria*       | 99                          | 99                        |
| *B. calyciflorus*    | 0                           | 0                         |
| *B. manjavacas*      | 0                           | 0                         |
| *B. plicatilis*      | 0                           | 0.01                      |
| *O. luehei*          | 0                           | 0                         |
| *L. thecatus*        | 0                           | 0                         |
| *P. gotoi*           | 0                           | 0                         |
| *S. cephaloptera*    | 0                           | 0                         |
| *B. hashinai*        | 0                           | 0                         |
| *S. mansoni*         | 0                           | 0                         |

Bold type indicates significantly deviating amino acid compositions. doi:10.1371/journal.pone.0043554.t003
Phylogenetic Relationships of Bdelloidea

No strategy employed recovered a monophyletic order Philodinida or monophyletic family Philodinidae due specifically to the fact that *Macrotrachela* and *Philodina* never formed a monophyletic association (Fig. 1). Additionally, *Habrotrocha* frequently broke up the possible monophyletic relationship among the remaining Philodinidae by falling sister to *Rotaria* or *Macrotrachela* instead of *Rotaria* and *Macrotrachela* branching as each other's closest relatives.

For all ML analyses except one, *Philodina* emerged as the earliest diverging bdelloid lineage (Fig. 1, Table 2). When *Philodina* emerged at the root of the bdelloid tree, *Adineta* fell sister to a clade comprised of Habrotrochidae (*Habrotrocha*) and the remaining Philodinidae (*Rotaria* and *Macrotrachela*) but the relationships within this RHM clade varied (Fig. 1, Table 2). The application of the JTT model recovered *Macrotrachela* at the root of the bdelloid tree instead (Fig. 1; Table 2). Bootstrap support values remained low for inter-genus, familial, and ordinal relationships regardless of the model of evolution or the partitioning scheme applied to the data (Fig. 1; Table 2), suggesting a lack of phylogenetic information at these levels. Only splits between species of the same genus were strongly and consistently supported (Fig. 1). ML analysis of a Dayhoff-recoded amino acid alignment improved the bootstrap support for internal nodes, particularly for the placement of the root between *Philodina* and the rest of the bdelloids (Fig. 1; Table 2). Parsimony analysis of Dayhoff-recoded sequences recovered the same topology (Fig. 1; Table 2). All 15 most parsimonious trees indicated that *Philodina* was the earliest diverging bdelloid lineage with RHM forming a monophyletic group. In fact, the only variation among these 15 trees was the position of the six *Macrotrachela* sequences with respect to each other.

Depending upon the amino acid substitution model, MrBayes analyses recovered *Philodina* or *Macrotrachela* at the root of the bdelloid tree (Fig. 1; Table 2). The WAG model supported an RHM clade (as seen in most ML analyses) and *Philodina* as the earliest diverging lineage (Fig. 1, Table 2). In contrast, the MtRev model in MrBayes (selected by the MCMC sampler as the best-fitting model under the mixed model parameter) produced a tree with *Macrotrachela* at the root; the same model under ML analysis recovered *Philodina* in this position (Fig. 1, Table 2). Both topologies produced by MrBayes showed strong but conflicting support for internal nodes (Fig. 1, Table 2).

Under a CAT+Poisson model of evolution, Phylobayes recovered the bdelloid root between *Macrotrachela* and the rest of the bdelloids (Fig. 1; Table 2). A combination of GTR (general time reversible) and CAT, which should account for more complexity, recovered *Philodina* at the base of the bdelloid tree, albeit both topologies lack strong support (Fig. 1; Table 2). Essentially, the

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**Figure 1. Bdelloid phylogenies.** Maximum Likelihood and Bayesian phylogenies reconstructed with concatenated mitochondrial proteins and rooted with Monogononta and Acanthocephala. Left and right trees represent two predominant topologies, where the root lies between *Macrotrachela* and the rest of the bdelloids (left) or *Philodina* and the rest of the bdelloids (right). The left tree represents the topology recovered from three analyses: 1) ML+JTT, 2) MrBayes+MtRev, and 3) Phylobayes with CAT+Poisson model. Bootstrap support values and posterior probabilities for the position of the root are listed in the order given above for each analysis. The right tree represents the topology recovered from six analyses: 1) ML+MtRev, 2) ML+Partitioned data (models were assigned to each partition and partitions were generated by grouping together genes that shared the same model), 3) ML+JTT on Dayhoff-recoded data, 4) Maximum Parsimony, 5) MrBayes+WAG, and 6) Phylobayes with CAT+GTR model. Bootstrap support values and posterior probabilities for the position of the root and the RHM clade (*Rotaria, Habrotrocha, and Macrotrachela*) are listed in the order given above for each analysis. Thicker branches indicate clades strongly supported by most analyses (bootstrap support or posterior probability >90). Red taxon labels indicate members of the family Philodinidae, green taxon labels indicate the family Habrotrochidae, and black indicates the family Adinetidae. Neither Philodinidae nor the order Philodinida is monophyletic. Line represents 0.25 amino acid substitutions per site. Summaries of strategies and support value provided in Table 2. doi:10.1371/journal.pone.0043554.g001
advantage of employing the CAT model allows equilibrium frequencies to vary among sites. All other analyses in this study employ stationary frequencies—an unrealistic assumption given that some amino acids will tend to replace others more frequently depending on the position. Although the results of Phylobayes are as equally conflicting as ML and MrBayes analyses, the universal themes are that Macrotrachelidae never forms a monophyletic association with Philodina. Additionally, in all analyses, the placement of Habrotrocha and Adineta disrupt the remaining associations among members of Philodiniidae and Philodinidae (Fig. 1; Table 2).

To determine whether any relationships could be rejected as significantly worse than the traditional schema, I obtained the best ML trees constrained to reflect all possible sister relationships between bdelloid families (Adinetidae+Philodiniidae, Adinetidae+Habrotrochidae, and Habrotrochidae+Philodiniidae) and evaluated their topologies using the AU and SH tests in CONSEL. No sister relationship could be rejected as implausible using the AU and SH tests, but a sister relationship between Habrotrochidae and Philodiniidae was the least likely and on borderline of being rejected (at a significance level of 0.05) according to the AU test (Table 4). The conflicting signal between the likely legitimate relationship of Habrotrochidae and some members of Philodiniidae (Macrotrachelida and Rotaria) and the unlikely monophyletic relationship of Macrotrachelida and or Habrotrocha with Philodina probably contributes to this result. To test this hypothesis, I also evaluated the plausibility of every possible sister relationship among genera. While the best tree with Philodina and Macrotrachelida as sister taxa falls within the confidence set of trees, it is not as likely as other scenarios (Table 4) and is never observed in my analyses. The AU test rejects a topology with Philodina and Habrotrocha as sister at a significance level of 0.05 (the SH test fails to reject this relationship but is more conservative Table 4; [56]).

Table 4. Bdelloid topology tests.

| Relationship tested | lnL | AU | SH |
|---------------------|-----|----|----|
| Habrotrocha+Adinetidae | -49632 | 0.77 | 0.82 |
| Adinetidae+Philodiniidae | -49634 | 0.32 | 0.36 |
| Habrotrocha+Philodiniidae | -49636 | 0.09 | 0.21 |
| Habrotrocha+Macrotrachelida | -36270 | 0.74 | 0.95 |
| Adineta+Philodina | -36270 | 0.58 | 0.93 |
| Rotaria+Habrotrocha | -36275 | 0.5 | 0.75 |
| Philodina+Rotaria | -36275 | 0.43 | 0.72 |
| Rotaria+Macrotrachelida | -36278 | 0.29 | 0.63 |
| Philodina+Macrotrachelida | -36279 | 0.36 | 0.56 |
| Adineta+Rotaria | -36282 | 0.08 | 0.48 |
| Adineta+Macrotrachelida | -36288 | 0.07 | 0.25 |
| Adineta+Habrotrocha | -36293 | 0.02 | 0.13 |
| Philodina+Habrotrocha | -36294 | 0.03 | 0.13 |

First three rows represent family relationships tested for Bdelloidea. The remaining rows represent all possible sister relationships among genera. lnL, log likelihood; AU, P-value for approximately unbiased test; SH, P-value for Shimodaira-Hasegawa test. Boldface indicates statistical significance at the alpha level of <0.05.

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Phylogenetic Analyses of Syndermata

Although lacking a complete representation of Syndermata due to the absence of Seisonidea, it is still informative to compare the relationships of Acanthocephala, Bdelloidea, and Monogononta recovered from my mitochondrial dataset to that of previous mitochondrial analyses. Min and Park consistently recovered a monophyletic Lemniscea in metazoan trees that included bdelloid families (Adinetidae+Philodiniidae, Adinetidae+Habrotrochidae, and Habrotrochidae+Philodiniidae) and recovered their topologies using the AU and SH tests in CONSEL. No sister relationship could be rejected as implausible using the AU and SH tests, but a sister relationship between Habrotrochidae and Philodiniidae was the least likely and on borderline of being rejected (at a significance level of 0.05) according to the AU test (Table 4). The conflicting signal between the likely legitimate relationship of Habrotrochidae and some members of Philodiniidae (Macrotrachelida and Rotaria) and the unlikely monophyletic relationship of Macrotrachelida and or Habrotrocha with Philodina probably contributes to this result. To test this hypothesis, I also evaluated the plausibility of every possible sister relationship among genera. While the best tree with Philodina and Macrotrachelida as sister taxa falls within the confidence set of trees, it is not as likely as other scenarios (Table 4) and is never observed in my analyses. The AU test rejects a topology with Philodina and Habrotrocha as sister at a significance level of 0.05 (the SH test fails to reject this relationship but is more conservative Table 4; [56]). This is consistent with the results of ML and Bayesian analyses as Habrotrocha never forms a sister relationship with Philodina (Fig. 1).

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In contrast, rooting Syndermata with Chaetognatha (as represented by P. gotoi and C. cephaloptera) – a phylum that does not show rapid mitochondrial evolution with respect to Rotifera–consistently recovered Lemniscea with strong bootstrap support, regardless of amino acid model, partitioning, or Dayhoff-recoding for ML analyses (Fig. 2; Table 2). All Bayesian analyses also strongly supported Lemniscea, providing total congruency among all models and methods of inference (Fig. 2; Table 2). The AU and SH tests soundly rejected Eurotatoria and a sister relationship between Acanthocephala and Monogononta when rooting the three possible syndermate topologies with Chaetognatha (Table 5).

Discussion

My results indicate that neither Philodiniidae nor Philodinidae represent phylogenetically informative entities. The placement of Philodina or Macrotrachelida at the base of the bdelloid tree single-handedly disrupts both classifications. The sister relationship frequently recovered between Habrotrocha and Macrotrachelida also scrambles the Philodiniidae family. However, the inconsistent results produced despite attempting to account for compositional biases and substitution rate saturation indicates that these mitochondrial sequences do not contain enough phylogenetic information for greater insight into bdelloid relationships. Better
methods of analysis to address the problems created by short internal branch lengths, compositional biases, and potential artifacts generated by the long branch between outgroup and ingroup are also necessary to increase resolving power. Recoding amino acid alignments into Dayhoff categories might be a cost-effective alternative to amassing more sequences for phylogenetic resolution but the loss of information incurred from recoding is an undesirable solution when dealing with a possible rapid radiation of bdelloid lineages. Site-heterogeneous models such as CAT, in combination with methods that assign different substitution models to different lineages (non-stationary models) appear to be the best solutions for uncovering relationships among problematic taxa but at the expense of computational time. Increased taxonomic sampling, particularly from members of the missing family, Philodinavidae might enhance the resolution of the bdelloid tree. Although my dataset improves the taxonomic resolution of bdelloids and the sample size of monogononts, all three monogononts represent the same order. Sampling other monogonont orders might provide a representative of this class that does not fail the test of compositional homogeneity. A less rapidly evolving member of Acanthocephala might also provide more topological stability and break up the long branch from outgroup (Monogononta+Acanthocephala) to ingroup (Bdelloidea). In the end, the ambiguous nature of the reconstructed bdelloid phylogeny suggests that close associations among members of different families and orders might be as equally probable as traditional ideas regarding bdelloid evolution. Members of Bdelloidea display unusual characteristics such as the ability to survive desiccation at any life stage [57] and extreme resistance to ionizing radiation [58], which have made them candidates for aging research (Meselson, Personal communication). Yet, little attention has been given to disentangling the bdelloid phylogeny, particularly at the molecular level [9,10]. Any study promoting bdelloids as model systems would greatly benefit from putting these traits into an evolutionary framework. In general, the classification of these small invertebrates that are often difficult to examine morphologically would greatly benefit from an accurate molecular-based phylogeny.

Current morphological and molecular data are inconclusive about the relationships among lineages of Syndermata. From a morphological perspective, Rotifera, sensu stricto (Monogononta, Bdelloidea, and Scisionidea), share more obvious synapomorphies with each other, such as coronas and trophi (jaw structures), than the highly modified Acanthocephala. However, relationships between Rotifera and Acanthocephala based on strict morphology are difficult to establish due to the lack of characters available for comparison, particularly since acanthocephalans are highly modified for a parasitic lifestyle [59]. While the presence of a syncytial epidermis appears to unite Rotifera and Acanthocephala [45], other morphological characteristics propose conflicting scenarios for the placement of Acanthocephala with respect to the three classes of Rotifera and for relationships among Rotifera. The lemnisci (sac-like structures involved in metabolism) and proboscis (protrudable feature that pierces the host) of Acanthocephala and the sac-like organs and rostrum (an adhesive structure that aids in creeping movement with the foot) in Bdelloidea either

Figure 2. Phylogenies of Syndermata. Syndermata phylogenies reconstructed with ML and Bayesian analyses of concatenated mitochondrial protein alignments and rooted with Platyhelminthes or Chaetognatha. A, Acanthocephala; B, Bdelloidea; M, Monogononta; E, Eurotatoria (Bdelloidea+Monogononta); L, Lemniscea (Bdelloidea+Acanthocephala). Summaries of the two predominant topologies recovered are presented. Only support values for Eurotatoria and Lemniscea are shown as the monophilies of Bdelloidea, Monogononta, and Acanthocephala were always fully supported. All analyses performed with Platyhelminthes, except for one, produced the top phylogeny. Bootstrap support values and posterior probabilities from these analyses are listed in the following order: 1) ML + MtRev, 2) ML + JTT, 3) ML + Partitioned data (models were assigned to each partition and partitions were generated by grouping together genes that shared the same model), 4) ML + JTT on Dayhoff-recoded data, and 5) MrBayes + MtRev. All analyses performed with Chaetognatha and one Platyhelminthes analysis recovered the bottom phylogeny. The first value represents the posterior probability from a Phylobayes analysis of Syndermata+Platyhelminthes under a CAT + Poisson model. The remaining values represent results from Syndermata+Chaetognatha analyses: 1) ML + MtRev, 2) ML + JTT, 3) ML + Partitioned, 4) ML + JTT on Dayhoff-recoded data, 5) MrBayes + MtRev, and 6) Phylobayes with CAT + Poisson model. Lines represent number of amino acid substitutions per site. Summaries of strategies and support values provided in Table 2.

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support a sister relationship between these two groups [60] or contribute to placing Acanthocephala as sister to Rotifera [8,61] depending on the interpretation of these structures as homologous (Fig. 3). Within Rotifera, analyses based primarily on trophi and sperm morphology support a sister relationship between Eurotatoria [52,62] (Fig. 3). Other characters considered synapomorphic for Eurotatoria are: the absence of pores in the terminal organ of the protonephridia, unpaired retrocerebral glands, salivary glands integrated into the mastax, and the presence of a vitellarium (Fig. 3). Seisonidea falls sister to: 1) Bdelloidea based on the condition of paired female gonads [63] or 2) Monogononta based on the presence of males, a cellular stomach with microvilli, shared features in the syncytial integument, and the absence of a bladder [3] or 3) Acanthocephala based on the ultrastructural analysis of spermatozoa [45] (Fig. 3). In other words, morphological features recover every possible sister relationship among the four major clades of Syndermata except for a monogonont/acanthocephalan group.

Molecular analyses thus far support a monophyletic Syndermata but fail to recover consistent relationships among syndermate lineages [5,50,59,66,67,68]. The gene(s) comprising each dataset and the different analytical methods and/or taxonomic sampling strategies employed all contribute to different results. For example, the small ribosomal subunit (ssu rDNA) recovers a sister relationship between Lemniscea in the absence of Seisonidea but recovers a well-supported relationship between Seisonidea and Acanthocephala when Seisonidea is present [50,66,68]. A phylogenomic analysis of 79 ribosomal proteins recovers Lemniscea but does not include a representative of Seisonidea [69]. The nuclear-encoded heat shock protein, hsp82, supports a sister

Table 5. Syndermata topology tests.

| Relationship tested | lnL     | AU     | SH     |
|---------------------|---------|--------|--------|
| Lemniscea           | -63255  | -61772 | 0.24, 0.98 | 0.4, 0.99 |
| Eurotatoria         | -63252  | -61744 | 0.49, 0.02 | 0.54, 0.02 |
| Monogononta+Acantocephala | -63259, -61745 | 0.62, 0.01 | 0.65, 0.01 |

The two values for each entry represent the topology tests conducted with Platyhelminthes (first value) and Chaetognatha (second value) as outgroups. Relationships tested included Lemniscea (Bdelloidea+Acanthocephala), Eurotatoria (Bdelloidea+Monogononta), and Monogononta+Acanthocephala. lnL, log likelihood; AU, P-value for approximately unbiased test; SH, P-value for Shimodaira-Hasegawa test. Boldface indicates statistical significance at the alpha level of <0.05. doi:10.1371/journal.pone.0043554.t005
Similarly, all empirical models employed by MrBayes and bdelloids still fail the test for compositional homogeneity. Recover Lemniscea instead. The inability of Dayhoff-recoding to ing the CAT model - a more sophisticated method of inference – Eurotatoria in the Syndermata + data [73]. Dayhoff-recoding improves bootstrap support for probabilities of my Bayesian trees for the Syndermata bootstrap support values of my ML trees and the posterior not equivalent measures, such large differences between the two topologies with a monophyletic Lemniscea were most likely [9]. From these three studies, it is unclear whether increasing characters or increasing taxonomic sampling provided any benefit in resolving syndermate relationships. The inclusion of an outgroup contributes the strongest topological influence in my analyses. Lemniscea appears to be a legitimate clade pulled apart by the artificial attraction of Acanthocephala to Platyhelminthes as both lineages are rapidly evolving. Although bootstrap values and posterior probabilities are not equivalent measures, such large differences between the bootstrap support values of my ML trees and the posterior probabilities of my Bayesian trees for the Syndermata+Platyhelminthes dataset are reminiscent of the effects of LBA on simulated data [73]. Dayhoff-recoding improves bootstrap support for Eurotatoria in the Syndermata+Platyhelminthes tree but employing the CAT model - a more sophisticated method of inference – recovers Lemniscea instead. The inability of Dayhoff-recoding to recover Lemniscea is not surprising given that all taxa except the bdelloids still fail the test for compositional homogeneity. Similarly, all empirical models employed by MrBayes and RAxML suffer from the same problem – the assumption of a site homogeneous substitution process. In other words, all RAxML and MrBayes analyses are subject to same systematic bias and result in the same syndermate topology. Further support for Lemniscus stems from replacing Platylehminthes with Chaeto- natha - a less rapidly evolving outgroup. Regardless of data manipulation or model complexity, all strategies arrive at the same – a well-supported Lemniscus. Thus, my results highlight the importance of not only applying different methods of inference but also modifying the taxonomic representation of one’s dataset. Without reconstructing the syndermate phylogeny with Phyo- bayes or a Chaetognatha outgroup, majority rule would favor a most likely incorrect sister relationship between Bdelloida and Monogononta. Ultimately, key factors to confirming the monophyly of Lemniscus will be to obtain mitochondrial sequences from the missing rotifer class Sceenidae and to obtain sequences from a more closely related outgroup (such as Gnathostomluida) that reduces the potential for LBA. Resolving the phylogeny of Syndermata not only represents a unique opportunity to study the evolution of asexuality, parasitism, and commensalism within a single lineage and to examine the genomic modifications that occur in association with each lifestyle and mode of reproduction but resolving the phylogeny of Syndermata also contributes to our greater understanding of metazoan evolution in general.

Supporting Information

Table S1 ProtTest Results. (DOCX)

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Author Contributions

Conceived and designed the experiments: ELN. Performed the experiments: ELN. Analyzed the data: ELN. Contributed reagents/materials/analysis tools: ELN. Wrote the paper: ELN.

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