The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2014, **111**, 570–588. With 3 figures

Inclusive taxon sampling suggests a single, stepwise origin of ectolecithality in Platyhelminthes

CHRISTOPHER E. LAUMER* and GONZALO GIRIBET FLS

Museum of Comparative Zoology & Department of Organismic and Evolutionary Biology, Harvard University, 26 Oxford Street, Cambridge, MA 02138, USA

Received 16 September 2013; revised 7 November 2013; accepted for publication 11 November 2013

Ectolecithality is a form of oogenesis unique within Metazoa but common in Platyhelminthes, in which almost yolkless oocytes and tightly associated yolk cells are deposited together in egg capsules. Despite profound impacts on the embryogenesis and morphology of its beneficiaries, the origins of this developmental phenomenon remain obscure. Traditionally, all ectolecithal flatworms were grouped in a clade called Neoophora. However, there are also morphological arguments for multiple origins of ectolecithality and, to date, Neoophora has seen little support from molecular phylogenetic research, largely as a result of gaps in taxon sampling. Accordingly, we present a molecular phylogeny focused on resolving the deepest divergences among the free-living Platyhelminthes. Species were chosen to completely span the diversity of all major endo- and ectolecithal clades, including several aberrant species of uncertain systematic affinity and, additionally, a thorough sampling of the 'lecithoepitheliate' higher taxa Prorhynchida and Gnosonesimida, respectively, under- and unrepresented in phylogenies to date. Our analyses validate the monophyly of all classical higher platyhelminth taxa, and also resolve a clade possessing distinct yolk-cell and oocyte generating organs (which we name Euneoophora **new taxon**). Furthermore, implied-weights parsimony and Bayesian mixture model analyses suggest common ancestry of this clade with the lecithoepitheliates, implying that these taxa may retain a primitive form of ectolecithality. This topology thus corroborates the classical hypothesis of homology between yolk cells and oocytes in all Neoophora, and should serve to guide future evolutionary research on this unique developmental innovation in Platyhelminthes. *Biological Journal of the Linnean Society*, 2014, **111**, 570–588.

ADDITIONAL KEYWORDS: phylogenetics – Neoophora – Gnosonesimida – Prorhynchida – mixture model – heterotachy – implied weights parsimony.

INTRODUCTION

Flatworms (phylum Platyhelminthes) are among Earth's dominant invertebrate animals, with over 6500 described free-living (Tyler *et al*., 2012) and 20 000 parasitic species (Caira & Littlewood, 2013). Indeed, their total diversity may be over an order of magnitude larger as a result of their predominance as members of the marine meiofauna (Martens & Schockaert, 1986) and as parasites of vertebrate and invertebrate hosts (Poulin & Morand, 2000), two of the least-known reservoirs of global biodiversity. Although historically considered to retain characters predating the diversification of Bilateria (Rieger *et al*., 1991), a hypothesis that has seen no recent support in light of recent molecular phylogenies (Baguñà & Riutort, 2004), the most speciose flatworm groups (e.g. Tricladida, Rhabdocoela, and Neodermata) have diverged remarkably from the anatomical and developmental simplicity commonly evoked in discussions of this phylum. In particular, as hermaphrodites bestowed with diverse mechanisms for achieving internal fertilization, platyhelminths have developed some of the most elaborate reproductive systems among Metazoa (Rieger *et al*., 1991; Conn, 2000).

A key component of this reproductive complexity is the existence in most flatworm species and higher taxa of a condition known as ectolecithality, the spatial partitioning of the products of oogenesis into

*Corresponding author. E-mail: claumer@oeb.harvard.edu

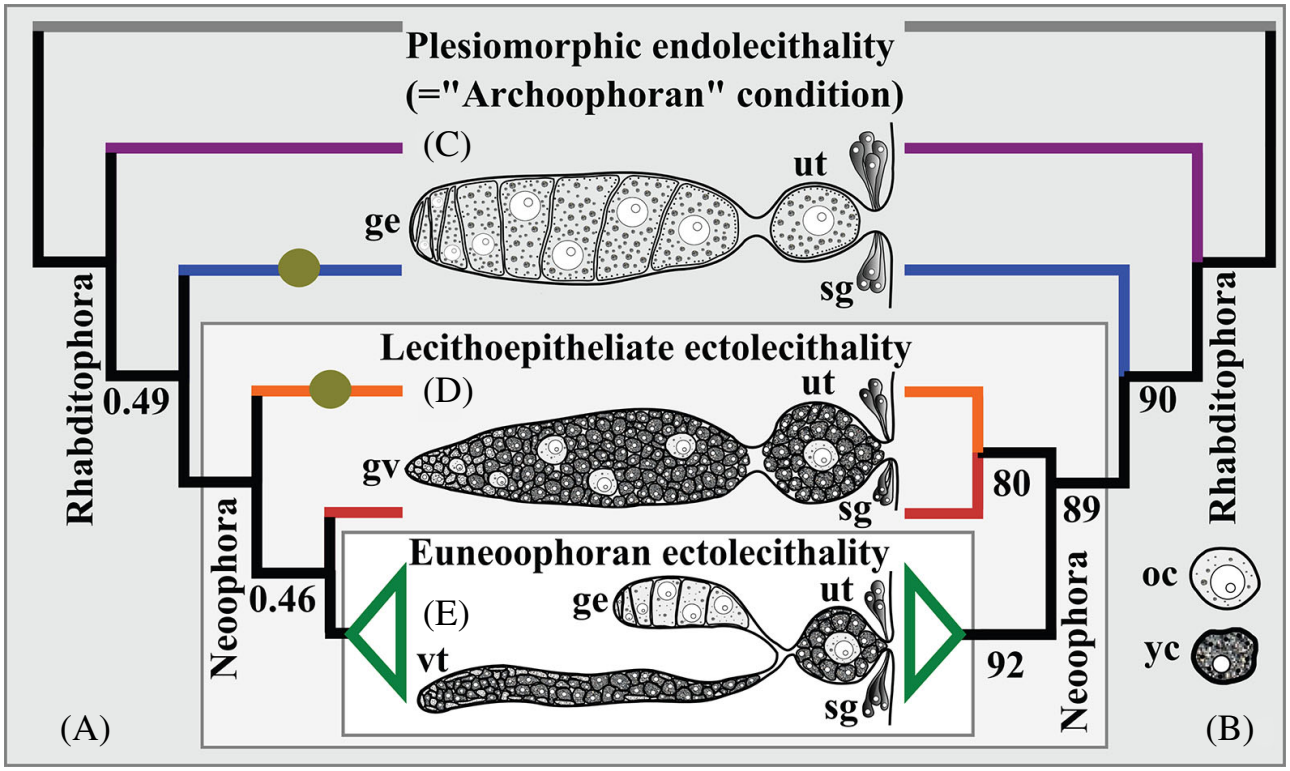

Figure 1. Proposed phylogenetic relationships between endolecithal, lecithoepitheliate, and euneoophoran ectolecithal higher platyhelminth taxa (branches coloured as in Fig. 3). A, summary of Bayesian mixture model analyses of Matrix B. Nodal support values below 1 are given as posterior probabilities from a reversible-jump Markov chain Monte Carlo run for 5.747×10^7 generations, with the first 25% discarded as burn-in. Olive dots represent branches for which two lengths were fit to model heterotachy, with posterior probability > 0.9. B, summary of implied weights parsimony ($k = 3$) analyses of Matrix B. Nodal support values below 100 are given as the proportion of 100 symmetric resampling replicates supporting each node. C, D, E, schematic illustrations of the female reproductive system of (C) an endolecithal, (D) a gnosonesimid lecithoepitheliate, abstracted into unipartite form, and (E) a euneoophoran platyhelminth, oriented with germinal areas to the left, and the female gonopore to the right. ge, germarium (ovary); gv, germovitellarium (compound ovary/yolk gland); oc, oocyte; sg, shell glands; ut, uterus; vc, vitellocyte (yolk cell); vt, vitellarium (yolk gland)

almost or entirely yolkless oocytes and separate, specialized yolk-bearing cells (sometimes called vitellocytes; Gremigni, 1983) (Fig. 1D, E). Indeed, only three platyhelminth higher taxa (Catenulida, Macrostomorpha, and Polycladida) retain a plesiomorphic (endolecithal) condition, producing a single major cell type during oogenesis, as in most Metazoa (Fig. 1C) (Rieger *et al*., 1991). Ectolecithality is also a functional division: in the species that possess them, vitellocytes are largely or entirely responsible for the synthesis and storage of yolk and the formation of a protective shell by marginal granules and shell gland secretions (Gremigni, 1988; Swiderski & Xylander, 2000), which are tasks usually accomplished by the oocyte and/or its accessory cells in other metazoa (Shinn, 1993).

In further contrast to the accessory cell types (e.g. nurse cells or follicle cells) known from other metazoa, flatworm vitellocytes play an active role in embryonic development. During oviposition, ectolecithal flatworms deposit hundreds of mature vitellocytes into thick-shelled egg capsules alongside one or several fertilized zygotes (Shinn, 1993). Development then proceeds with the small, almost yolkless embryo surrounded by these yolk cells. Although at least polyclads progress through a recognizable spiralian cleavage program (Surface, 1907; Boyer, Henry & Martindale, 1998; Rawlinson, 2010), early development in many other flatworm taxa shows little similarity to other metazoans (Martín-Durán & Egger, 2012). In ectolecithal flatworms in particular, such developmental modifications may be especially profound: most taxa form a 'hull membrane', a transient structure used to envelop and secure vitellocytes within or against the developing embryo, and many groups (including planarian model systems; Sánchez Alvarado, 2003) display no trace of spiral cleavage; instead, they undergo a dispersive cleavage and 'blas-

tomere anarchy' in which blastomeres ephemerally lose physical contact and develop adrift in a matrix of external, syncytial yolk. Although almost nothing is known about the developmental mechanisms underlying these processes (in large part because of difficulties in observing and manipulating ectolecithal embryos within their capsules), these modifications have been explained in evolutionary terms as embryonic adaptations to the presence of external yolk (Thomas, 1986; Martín-Durán & Egger, 2012). Ectolecithality is also associated with increased functional compartmentalization in the adult reproductive morphology of most taxa: among the major lineage of parasitic Platyhelminthes (Neodermata) in particular, the presence of ectolecithality has allowed a spectacular anatomical specialization of female reproductive organs (exemplified by the compound assembly of an egg capsule in the digenean ootype; Ehlers, 1985; Coil, Harrison & Bogitsh, 1991; Fried & Haseeb, 1991), which is possibly an adaptation to the complex life cycles and vast fecundity required of such obligate parasites (Whittington, 1997). It has also been suggested that the developmental mechanism of epidermal turnover within the hull membrane used to secure vitellocytes in egg capsules (a clear adaptation to ectolecithality) may have been exapted during the evolution of the neodermis (Tyler & Tyler, 1997). Ectolecithality should thus be seen as a deeply consequential developmental innovation, unique to Platyhelminthes.

Despite its widespread phylogenetic distribution and pervasive functional and developmental significance, relatively little empirical work has been undertaken concerning the evolutionary origins of this phenomenon. One of the oldest and most influential hypotheses in flatworm systematics is the common ancestry of all ectolecithal taxa, which are thus grouped into a clade, Neoophora (Karling, 1974). This hypothesis implies homology of vitellocytes and the organs that produce them (vitellaria, also sometimes called yolk glands) across the wide anatomical variety of female gonads in the groups comprising Neoophora (Hyman, 1951; Gremigni & Falleni, 1998). However, precisely because of this heterogeneity, several studies have proposed multiple origins of ectolecithality (Hyman, 1951; Ax, 1961), as well as the possibility of reversals to an entolecithal state (Karling, 1967).

Central to historical discussion on the evolution of ectolecithality has been the female reproductive morphology of the taxon Lecithoepitheliata (Figs 1D, 2D, E, F; Reisinger, 1926; Karling, 1974; Timoshkin, 1991b). Usually regarded as a minor order within the phylum, lecithoepitheliates comprise two distinct groups: Prorhynchida, a freshwater and terrestrial taxon of 29 valid species in three genera (Fig. 2E, F; Timoshkin, 1991a), and Gnosonesimida, a rare meiobenthic group of six species presently classified in the single genus *Gnosonesima*, known from shallow (intertidal to 350 m) marine sediments (Fig. 2D) (Karling, 1968). The sole hypothesized synapomorphy of these taxa is the structure of their female gonads, which are organized as chains of follicles, each consisting of a layer of vitellocytes more or less tightly enveloping single oocytes, thus organized as the consequence of a common germinative zone for vitellocytes and oocytes in this taxon (Fig. 1D) (Rieger *et al*., 1991). Historically, the tetrapartite germovitellaria of *Gnosonesima* was proposed to represent the primitive condition from which other ectolecithal taxa diverged (Reisinger, 1926); later systematists have followed this convention, placing a monophyletic Lecithoepitheliata as the sister group to the remaining Neoophora, a hypothesis that persists to the present (Martín-Durán & Egger, 2012). This early-branching position within Neoophora is further substantiated by observations of a spiralian-like (hence, plesiomorphic) alternation of the orientation of mitotic spindles in the early cleavages of Prorhynchida (Martín-Durán & Egger, 2012), in contrast to the strongly modified cleavage of most other ectolecithal taxa.

However, this conventional systematic placement of the lecithoepitheliate taxa has been met with much controversy. Despite the gross anatomical (Rohde & Watson, 1991) similarity of their germovitellaria, on the histological (Karling, 1968) and ultrastructural levels (Bogolyubov & Timoshkin, 1993; Gremigni, 1997) in this and other characters Prorhynchida and Gnosonesimida show few correspondences. Hence, several prominent specialists on this taxon (Karling, 1968, 1974; Timoshkin, 1991b) have implicitly or explicitly considered the possibility that 'Lecithoepitheliata' is a polyphyletic assemblage. Indeed, simply on the basis of comparative morphology alone, few flatworm taxa have seen as great a diversity of proposed sister groups: Prorhynchida in particular have been considered as relatives of Prolecithophora (Timoshkin, 1991a, b), Rhabdocoela (Rohde & Watson, 1991), Polycladida (Reisinger, 1968), and even Hofsteniidae (Steinböck, 1966; Tyler, 1976; Falleni, 1997), a taxon today recognized as an early-diverging family of Acoela (Hooge *et al*., 2007; Jondelius *et al*., 2011). (By contrast, although the putative non-monophyly of Gnosonesimida and Prorhynchida has frequently been advanced, few alternative positions of Gnosonesimida appear to have been considered in the literature.) Unfortunately, to our knowledge, all modern phylogenetic analyses of Platyhelminthes employing morphological character data have simply assumed the monophyly of Lecithoepitheliata (Ehlers, 1985; Zrzavý *et al*., 1998; Littlewood *et al*., 1999a;

Figure 2. Life habitus of selected problematic platyhelminth lineages and lecithoepitheliate taxa so far lacking molecular data. A, *Acanthiella* sp., adults typically 0.25–0.5 mm. B, *Bothrioplana semperi*, active adults range approximately 1–5 mm. C, *Acholades asteris* encysted (left) on the tube foot of the sea star *Coscinasterias calamaria* (right, scale bar = 1 mm). D, *Gnosonesima* sp. IV (scale bar = 0.1 mm). E, *Xenoprorhynchus* sp. II, active adult worms are 2–3 mm long. F, *Prorhynchus alpinus*, anterior view (scale bar = 0.1 mm).

Littlewood, Rohde & Clough, 1999b), precluding explicit evaluation of alternative hypotheses on the evolution of ectolecithality.

The relationships between lecithoepitheliates and other ectolecithal flatworms have also largely eluded molecular phylogeneticists. Despite early (and equivocal) evidence for their polyphyly (Rohde *et al*., 1993, 1995; Katayama, Nishioka & Yamamoto, 1996; Campos *et al*., 1998; Litvaitis & Rohde, 1999), most analyses of 18S rRNA, sometimes supplemented with 28S rRNA, have validated the monophyly of at least the non-lecithoepithelate Neoophora (Littlewood *et al*., 1999a, b; Norén & Jondelius, 1999, 2002; Baguñà *et al*., 2001; Joffe & Kornakova, 2001; Littlewood & Olson, 2001; Lockyer, Olson & Littlewood, 2003; Willems *et al*., 2006; as reviewed by Baguñà & Riutort, 2004). However, with few exceptions (Rohde *et al*., 1993), nucleic acid sequences (largely 18S rRNA data) from lecithoepitheliate flatworms have been available only for a few species of the prorhynchid genus *Geocentrophora*. Given this limited gene and taxon sampling, it is perhaps unsurprising that the sister group of prorhynchids has proven highly unstable across phylogenetic analyses. Indeed, in molecular phylogenetics, as in discussions based on comparative morphology, few groups have seen such a great diversity of proposed placements: the sampled *Geocentrophora* have been placed in various analyses (albeit always with poor support) as the sister group to polyclads (Jondelius, Norén & Hendelberg, 2001; Lockyer *et al*., 2003), macrostomids (Campos *et al*., 1998; Jondelius, 1998), a clade of both endolecithal rhabditophorans (Lockyer *et al*., 2003), a clade of triclads and fecampiids (Lockyer *et al*., 2003), a clade of all other ectolecithal flatworms (Littlewood *et al*., 1999b; Baguñà *et al*., 2001; Littlewood & Olson, 2001), all other rhabditophorans (Littlewood & Olson, 2001) or, frequently, as sister to

haplopharyngids [(Littlewood *et al*., 1999b; Norén & Jondelius, 2002; Willems *et al*., 2006); contradicting the morphologically well-validated clade Macrostomorpha (Rieger, 2001)]. Lecithoepitheliata thus remains the highest taxon *incertae sedis* within the flatworms, leaving the monophyly of Neoophora an open question as well.

To overcome these issues, and with the express goal of determining the origins of ectolecithality within the phylum, we present a large-scale molecular phylogeny of Platyhelminthes using almost complete 18S and 28S rRNA sequences and mitochondrial (mt)DNA fragments from 83 terminal species, 54 of which have been newly sequenced; most other sequences (including all Neodermata analyzed in the present study) were drawn from Lockyer *et al*. (2003). To minimize the possibility of long-branch attraction artefacts, we aimed to encompass a rich sample of the taxonomic diversity of each free-living major taxon, also including a number of outstanding systematically problematic lineages (Fig. 2A, B, C). To position the lecithoepitheliate taxa in particular, we include sequences from 17 prorhynchid species in all known genera. Additionally, we have generated sequence data from Gnosonesimida (the rare marine lecithoepitheliates), the only major classical platyhelminth lineage still lacking molecular data; we include three representatives. Employing both conventional [unweighted parsimony and maximum likelihood (ML)] and more sophisticated analytical models (character weighting schemes and mixture models incorporating the possibility of heterotachy), we provide an updated, explicit analysis of the deepest splits within the rhabditophoran Platyhelminthes, focused on determining the relationships between endo- and ectolecithal groups, and including for the first time a representational sample of species from all free-living higher flatworm taxa.

MATERIAL AND METHODS

Specimen collection and identification

We sought specimens for genomic DNA extraction from species completely spanning the diversity of each major clade of free-living Platyhelminthes, using the present Linnean higher taxonomy (Tyler *et al*., 2012) or, where available, published molecular phylogenies (Baguñà *et al*., 2001; Norén & Jondelius, 2002; Willems *et al*., 2006; Larsson & Jondelius, 2008; Curini-Galletti *et al*., 2010) to guide our selection. Marine specimens represented largely interstitial meiofauna, which were retrieved using a $MgCl_2$ extraction approach (Schockaert, 1996), retaining the fauna on a 62-μm sieve. Freshwater, terrestrial, and brackish water organisms, by contrast, were largely extracted from environmental samples using an oxygen depletion technique (Schockaert, 1996). Specimens were studied alive in semi-squeezed preparations, and identified to the lowest rank possible with the aid of recent synoptic guides to free-living flatworms (Cannon, 1986; Ax, 2008), the Turbellarian Taxonomic Database (Tyler *et al*., 2012) or, when necessary, consultation with original descriptions and/or appropriate specialists. Squeezed specimens were then directly subjected to DNA extraction or preserved at –80 °C or –20 °C in RNA*later* (Ambion, Inc.) or 95–100% EtOH. Taxonomic names and gene matrix occupancy for all specimens used in the present study are provided in Table 1; further specimen data, including locality information and microphotograph vouchers where available, are searchable by accession number in MCZbase (http://mczbase.mcz.harvard.edu/).

Nucleic acid extraction, amplification, and sequencing

Genomic DNA was extracted from live or preserved individual specimens using a DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA), eluting in 100–200 μL of Buffer AE. From each extraction, we amplified and directly sequenced approximately 1.76 kb of 18S rRNA in three overlapping amplicons, approximately 3.8 kb of 28S rRNA in five overlapping amplicons, approximately 440 bp of 16S rRNA, and approximately 390 bp of cytochrome *b* (cyt *b*) (both single amplicons), yielding a total of approximately 6.4 kb per specimen. Primer sequences for all markers are available in the Supporting information (see Supporting information, Table S1). Ribosomal genes were amplified using AmpliTaq (Applied Biosystems) in 25-μL reactions with 1.5 mM $MgCl_2$, at annealing temperatures ranging between 43 and 51 °C. Cytochrome *b* was amplified at annealing temperatures between 41–45 °C, with 2.5 mM $MgCl_2$. Polymerase chain reactions (PCRs) were purified using MultiScreen filter plates (Millipore, Inc.), labelled with BigDye Terminator, version 3.1 (Applied Biosystems), and sequenced on an ABI 3730*xl* DNA Analyzer (Applied Biosystems) in accordance with the manufacturer's instructions. Each amplicon was sequenced with forward and reverse reads using the same primers as those employed during the PCR, with the exception of cyt *b*, for which M13 universal primers were used (Regier & Shi, 2005). Reads were assembled into contigs in SEQUENCHER, version 4.7 (Genecodes, Inc.) or GENEIOUS, version 5.6.3 (Biomatters Ltd), and all contigs were screened for contamination by BLASTN to NCBI nucleotide collection prior to downstream analysis. Certain amplicons proved recalcitrant, with the mitochondrial markers

Table 1. Taxon names, MCZ accession numbers, and GenBank accession numbers for all taxa involved in the present study

Taxon	MCZ accession number	18S	28S	16S	Cytochrome *b*
Acanthiella sp.	DNA106019	**KC869786**	**KC869839**	**KC869747**	–
Acanthomacrostomum sp.	DNA105907	**KC869788**	**KC869841**	**KC869748**	**KC869889**
Acholades asteris	DNA105961	**KC869826**	**KC869879**	**KC869774**	**KC869901**
Baicalellia canadensis	DNA105932	**KC869833**	**KC869886**	**KC869779**	**KC869903**
Bdelloura candida	–	Z99947	AY157154	–	–
Boninia divae	DNA105955	**KC869793**	**KC869846**	**KC869751**	**KC869925**
Bothrioplana semperi	DNA105909	**KC869822**	**KC869875**	**KC869773**	–
Bresslauilla relicta	DNA105927	**KC869832**	**KC869885**	–	–
Caryophyllaeus laticeps	–	AJ287488	AY157180	–	–
Castrella pinguis/*truncata*	DNA105930	AY775777	**KC869887**	**KC869780**	**KC869890**
Catenula sp./*Catenula turgida*	–	FJ384798.1	AY157152	–	–
Cavernicola **sp. nov.**	DNA105956	**KC869823**	**KC869876**	–	**KC869904**
Chromoplana **sp. nov.**	DNA105908	**KC869794**	**KC869847**	–	**KC869920**
Chromyella **sp. nov.**	DNA105964	**KC869795**	**KC869848**	–	**KC869926**
Diclidophora denticulata	–	AJ228779	AY157169	–	–
Dictyocotyle coeliaca	–	AJ228778	AY157171	–	–
Didymorchis sp.	–	AY157182	AY157163	–	–
Geocentrophora applanata	DNA104925	**KC869809**	**KC869857**	**KC869763**	**KC869908**
Geocentrophora baltica	DNA104971	**KC869810**	**KC869854**	**KC869764**	**KC869907**
Geocentrophora marcusi	DNA104883	**KC869811**	**KC869856**	**KC869765**	**KC869909**
Geocentrophora sphyrocephala	DNA104884	**KC869808**	**KC869855**	**KC869762**	**KC869906**
Geocentrophora wagini	–	AJ012509	AY157156	–	–
Gigantolina magna	–	AJ243681	AY157179	–	–
Girardia tigrina	–	AF013157	U78718	–	–
Gnosonesima cf. *mediterranea*	IZ 29192	**KC869785**	**KC869838**	**KC869746**	–
Gnosonesima sp. I	DNA105896	**KC869784**	**KC869837**	**KC869744**	–
Gnosonesima sp. IV	DNA106020	**KC869783**	**KC869836**	**KC869745**	–
Gyratrix hermaphroditus	DNA105905	**KC869827**	**KC869880**	**KC869775**	**KC869902**
Gyrocotyle urna	–	AJ228782	AY157178	–	–
Haplopharynx sp.	DNA106018	**KC869787**	**KC869840**	–	–
Hoploplana californica	DNA106152	**KC869797**	**KC869850**	**KC869753**	**KC869923**
Hymenolepis diminuta	–	AF124475	AY157181	–	–
Kronborgia isopodicola	–	AJ012513	AY157168	–	–
Kytorhynchus **sp. nov.**	DNA105910	**KC869829**	**KC869882**	**KC869777**	–
Lepidophyllum steenstrupi	–	AJ287530	AY157175	–	–
Lithophora **gen. and sp. nov.**	DNA105922	**KC869817**	**KC869870**	**KC869769**	**KC869896**
Lobatostoma manteri	–	L16911	AY157177	–	–
Macrostomum rubrocinctum	DNA105928	**KC869789**	**KC869842**	**KC869749**	**KC869900**
Macrostomum sp.	DNA106151	**KC869790**	**KC869843**	–	**KC869899**
Maricola **gen. and sp. nov.**	DNA105963	**KC869825**	**KC869878**	–	–
Microstomum lineare	DNA105906	**KC869791**	**KC869844**	**KC869750**	–
Monocelis fusca	DNA105934	**KC869814**	**KC869867**	**KC869767**	**KC869894**
Monocelis lineata	–	U45961	AY157159	–	–
Nematoplana sp.	–	AJ270160	AY157160	–	–
Notocaryoplana arctica	DNA105923	**KC869816**	**KC869869**	**KC869768**	**KC869892**
Notoplana australis	–	AJ228786	AY157153	–	–
Paracatenula sp.	IZ 29193	**KC869782**	**KC869835**	**KC869743**	**KC869895**
Paramalostomum fusculum	–	AJ012531	AY157155	–	–
Paraplanocera oligoglena	IZ 29194	**KC869796**	**KC869849**	**KC869752**	**KC869922**
Plagiostomum stellatum	DNA105937	**KC869819**	**KC869872**	**KC869770**	–
Plagiostomum whitmani	DNA105929	**KC869818**	**KC869871**	–	**KC869893**
Plicastoma cuticulata	–	AF065422	AY157158	–	–
Polystomoides malayi	–	AJ228792	AY157170	–	–

Table 1. *Continued*

Taxon	MCZ accession number	18S	28S	16S	Cytochrome *b*
Polystyliphora karlingi	DNA106017	**KC869815**	**KC869868**	–	**KC869921**
Procotyla fluviatilis	DNA105938	**KC869824**	**KC869877**	–	–
Prolecithophora gen. undet. (cf. *Euxinia*)	DNA106016	**KC869821**	**KC869874**	**KC869772**	–
Promesostoma cochleare	DNA106013	**KC869831**	**KC869884**	**KC869778**	**KC869891**
Prorhynchus alpinus	DNA105902	**KC869806**	**KC869858**	–	**KC869910**
Prorhynchus fontinalis	DNA105548	**KC869807**	**KC869853**	–	–
Prorhynchus haswelli	DNA105898	**KC869804**	**KC869862**	**KC869760**	**KC869913**
Prorhynchus putealis	DNA105608B/609	**KC869805**	**KC869863**	**KC869761**	**KC869917**
Prorhynchus sp. I	DNA104880	**KC869799**	**KC869865**	**KC869755**	**KC869912**
Prorhynchus sp. II	DNA104885	**KC869800**	**KC869859**	**KC869756**	**KC869916**
Prorhynchus sp. III	DNA104974	**KC869801**	**KC869860**	**KC869757**	**KC869915**
Prorhynchus sp. IV	DNA105899	**KC869802**	**KC869861**	**KC869758**	**KC869914**
Prorhynchus stagnalis	DNA104882	**KC869798**	**KC869866**	**KC869754**	**KC869911**
Prorhynchus tasmaniensis	DNA105897	**KC869803**	**KC869864**	**KC869759**	**KC869918**
Protomonotresidae **sp. nov.**	DNA105901	**KC869820**	**KC869873**	**KC869771**	**KC869897**
Pterastericola australis	–	AJ012518	AY157161	–	–
Reisingeria hexaoculata	–	AF065426	AY157157	–	–
Rugogaster hydrolagi	–	AJ287573	AY157176	–	–
Sasala nolani	–	AY157184	AY157174	–	–
Schistosoma mansoni	–	U65657	AY157173	–	–
Stenostomum leucops	–	AJ012519	AY157151	–	–
Strongylostoma elongatum spinosum	DNA106014	**KC869830**	**KC869883**	–	–
Stylochus zebra	–	AF342801	AF342800	–	–
Temnocephala fasciata	DNA105957	**KC869834**	**KC869888**	**KC869781**	**KC869919**
Temnosewellia minor	–	AY157183	AY157164	–	–
Theama sp.	DNA105960	**KC869792**	**KC869845**	–	**KC869924**
Toia ycia	DNA106015	**KC869828**	**KC869881**	**KC869776**	**KC869898**
Udonella caligorum	–	AJ228796	AY157172	–	–
Xenoprorhynchus sp. I	DNA105900	**KC869813**	**KC869852**	**KC869766**	**KC869905**
Xenoprorhynchus sp. II	DNA105926	**KC869812**	**KC869851**	–	–

Further locality information, photographic vouchers where available, and ancillary specimen information are available at: http://mczbase.mcz.harvard.edu/.
Bold accession numbers represent sequences newly generated for the present study.

being particularly elusive across the diversity of Platyhelminthes; hence, for 16S rRNA, 28% (15/54), and, for cyt *b*, 30% (16/54) of the newly-sequenced taxa are missing data. However, with few exceptions (notably, cyt *b* in Gnosonesimida), almost no higher free-living taxa are missing mtDNA markers completely. Individual nucleotide sequences are accessible in GenBank under accession numbers KC869743–KC869926 (Table 1).

Matrix construction and multiple sequence alignment

For all available markers, we aimed to align bases according to the structure of their gene products in a transparent, repeatable manner. Because platyhelminth rRNA sequences, particularly in parasitic taxa, frequently contain large insertions (Lockyer *et al*., 2003), similarity-based alignments that do not incorporate structural information risk spuriously homologizing bases from the insertions with bases in flanking regions. Hence, after an initial MAFFT (E-INS-i) alignment, 18S and 28S rRNA sequences were each aligned using RNASALSA (Stocsits *et al*., 2009), comprising software written to automate structural alignments of large rRNA molecules at deep phylogenetic scales. RNASALSA estimates secondary structures for each sequence, combining information from thermodynamic folding models, comparative evi-

dence, and an initial constraint (in this case, the provided protostome structural data from *Anopheles albimanus*); these secondary structure models are then used to guide a final sequence-similarity-based alignment. For the 16S rRNA gene, we used the *x*-INS-i algorithm of MAFFT (using the MXSCARNA option for pairwise structural alignment) because this algorithm does not require an input structural constraint and is appropriate for more divergent ncRNA sequences. For cyt *b*, we aligned nucleotides in the TranslatorX web server (Abascal, Zardoya & Telford, 2010) according to a MUSCLE alignment of their amino acid translations (using the rhabditophoran mitochondrial genetic code). For all rRNA markers, we performed a sequence masking step to minimize nonphylogenetic signal in poorly aligned regions near indel-prone areas, particularly as a result of taxon-specific insertions, using the Gblocks web server (allowing for both less strict flanking positions and for gap positions within the final blocks; Castresana, 2000). This sequence masking removed 56% of bases from the initial 18S rRNA alignment (1313 of 2996 positions retained), 58% of bases from the initial 28S rRNA alignment (2466 of 5919 positions retained), and 43% of bases from the 16S rRNA alignment (332 of 558 positions retained). Data from each marker were then concatenated using SEQUENCEMATRIX (Vaidya, Lohman & Meier, 2011) to create a matrix of 4513 bp, incorporating evidence from all available markers for 83 taxa (Matrix A).

Even with a structure-aware alignment, the presence of taxon-specific insertions may necessitate a higher degree of stringency in sequence masking than is strictly optimal, causing the removal of insert-adjacent regions that retain salient phylogenetic signal. In addition, because, for certain taxa, regions of the 18S and 28S rRNA markers failed to amplify, and because of the high level of missing data for the fast-evolving mtDNA markers, we were concerned about the possible effects of such matrix incompleteness on our ability to detect ancient phylogenetic signals within our data (Roure, Baurain & Philippe, 2013). For these reasons, we constructed a second *ad hoc* matrix (Matrix B) to account for these potential sources of error, excluding all mitochondrial data, as well as insert-rich (particularly, all parasitic) taxa, and also several other species with missing rRNA data; no lecithoepitheliates were excluded. Alignment and sequence masking were performed as before, except with all 'less stringent' Gblocks server settings enabled. Matrix B consists of 63 taxa, and 4387 bp of nucleotide data. Although smaller than the complete dataset, Matrix B includes 608 more bases of rRNA data than Matrix A, as a result of the exclusion of parasitic taxa with long inserts and the subsequently more lenient sequence masking. Phylogenetic analyses were carried out in an identical manner on both matrices except where noted, selecting Catenulida as the outgroup to Rhabditophora (Larsson & Jondelius, 2008). All alignments generated during the present study are available for download in NEXUS format from the Dryad Data Repository (Laumer & Giribet, 2014).

Phylogenetic analysis

Parsimony

For each matrix, a strict consensus of the shortest trees was found under the criterion of unweighted maximum parsimony (MP) using a serial, Linux64 distribution of TNT (Goloboff, Farris & Nixon, 2008; version current as of 19 May 2011), searching from 100 starting Wagner trees followed by tree bisection–reconnection (TBR) branch swapping; identical MP trees were found when tree drifting and parsimony ratchet procedures were explored. Trees from the unweighted parsimony analyses (see Supporting information, Fig. S1) show poor symmetric resampling support for basal relationships, and the shortest trees differ considerably from other analyses in the branching order of higher taxa (e.g. with Matrix B yielding Gnosonesimida as the earliest-diverging rhabditophoran clade). It is possible that these effects result from a predominance of homoplastic characters overwhelming the limited subset of characters retaining signals of more ancient relationships. We therefore aimed to control the effects of homoplasy by using the TNT implementation of Goloboff's concavity (implied weighting; IW) function (Goloboff, 1993), which assigns characters differential weights during the tree search process according to their implied homoplasy. To observe the sensitivity of our results to the strength of the weighting function, we performed identical searches (with 20 starting Wagner trees and TBR branch swapping) for all k values between 1 and 30 (higher values of which result in less pronounced weighting); we show trees (Fig. 1C; see also Supporting information, Fig. S2) for the default value of $k = 3$. For all tree searches, we assessed support using symmetric resampling (with probability = 0.33), a support measure that, unlike bootstrapping or jackknifing, is not distorted by character weighting procedures (Goloboff *et al*., 2003). The frequency of each clade in the strict consensus of shortest trees was calculated using SUMTREES, version 2.0.2 (in the DENDROPY, version 3.2.1, Python library Sukumaran & Holder, 2010) in a set of resampled trees (1000 for unweighted searches, 100 for all implied weighting searches) output by TNT. The effects of k on particular topological hypotheses relevant to the origin of ectolecithality (and support for each hypothesis) are summarized in the Supporting information (Fig. S3).

Table 2. Results (*p*-values) of nonparametric topological hypothesis tests, comparing unconstrained trees to various topologies in which the monophyly of Neoophora is constrained

Matrix A (83 taxa, all data)	Approximately unbiased	Shimodaira–Hasegawa
Unconstrained	0.556	0.631
Neoophora w/Gnosonesima + Euneoophora	0.497	0.675
Neoophora w/Prorhynchida + Euneoophora	0.487	0.693
Neoophora w/Lecithoepitheliata + Euneoophora	0.227	0.513
Matrix B (63 taxa, rRNA only)		
Unconstrained (Neoophora w/Gnosonesima + Euneoophora)	0.505	0.738
Neoophora w/Prorhynchida + Euneoophora	0.477	0.739
Neoophora w/Lecithoepitheliata + Euneoophora	0.479	0.512

Tests were performed in CONSEL (Shimodaira & Hasegawa, 2001) on site-likelihoods output from GARLI searches.

Likelihood

Trees computed under the ML criterion were identified using GARLI, version 2.0 (Zwickl, 2006), chosen for its rapid search functions and versatile model implementation and partitioning options. Tree searches were largely carried out with default configuration options, although with searchreps = 20 and genthreshfortopoterm = 20 000. We explored a variety of partitioning schemes (by-gene, nuclear versus mtDNA, and others), selecting appropriate models for each partition from among 88 possibilities using JMODELTEST (Posada, 2008). The optimal partitioning strategy was then chosen using GARLI via an *ad hoc* Akaike information criterion procedure described by Zwickl (2012). Tree searches proceeded under a single GTR+I+G model for all sites in Matrix A and separate GTR+I+G models for each rRNA in Matrix B (with branch lengths unlinked between partitions). To assess support for ML trees, clade frequencies from among 1000 bootstrap replicates (with searchreps = 1 and genthreshfortopoterm = 10000) were plotted on the single ML tree from each search using SUMTREES. Nonparametric hypothesis testing (approximately unbiased, Shimodaira–Hasegawa) to determine the significance of various topological constraints relevant to the origin of ectolecithality was performed using CONSEL (Shimodaira & Hasegawa, 2001) on site-likelihoods output from GARLI searches (Table 2). Note that CONSEL does not allow for consideration of separate partitions; site likelihoods for the Matrix B tests were therefore calculated using a reanalysis of these data under a single GTR+I+G model.

Bayesian mixture model

We employed a parallelized version of BayesPhylogenies, version 2.0 (beta) with openMPI; Pagel & Meade, 2004, 2008) to implement Bayesian phylogenetic analyses, sampling posterior probability distributions under a nucleotide mixture model using a reversible jump Markov chain Monte Carlo (rjMCMC). Each rjMCMC was run as an unfixed-degree mixture of GTR+G rate matrices (but with a single base frequency matrix), using BayesPhylogenies' reversible jump procedure to choose the number of patterns required. In addition to this standard mixture model, we also employed BayesPhylogenies' model of heterotachy (Pagel & Meade, 2008), allowing reversible jumps between trees with up to two distinct branch lengths per node. For each matrix, we ran six independent heated chains across eight to 24 CPUs (usually 2.3 Ghz Xeon E5410 dual quad-cores) on the Harvard Odyssey Cluster, for a minimum of 20 million iterations each, cooling from 150 degrees over the first million iterations, and sampling every 10^3 (Matrix A) or 10^4 (Matrix B) iterations. In Matrix B analyses, to ensure stationarity had been reached, two chains in each analysis were also run much longer (approximately 57 and 100 million iterations). In several instances during these analyses, individual chains were interrupted as a result of technical failures; searches were continued from the last sample using the 'append' command, although this resulted in a transient disruption to apparent stationarity, leading us to discard the 200 000 iterations following each disruption, in addition to the first 25% of iterations that were discarded as burn-in. Majority-rule consensus trees were built from each posterior sample using BayesTrees, version 1.3 (Meade, 2012). Because most phylogenetic tree manipulation software (e.g. FIGTREE, version 1.4.0; http://tree.bio.ed.ac.uk/software/figtree/) cannot manipulate the tree files with two lengths per node that BayesPhylogenies generates under its heterotachy model, to generate Figure 3, we modified the trees file output by BayesPhylogenies in a custom PYTHON script, averaging both lengths at each branch; the tree samples were then summarized in a consensus using SUMTREES. We emphasize that

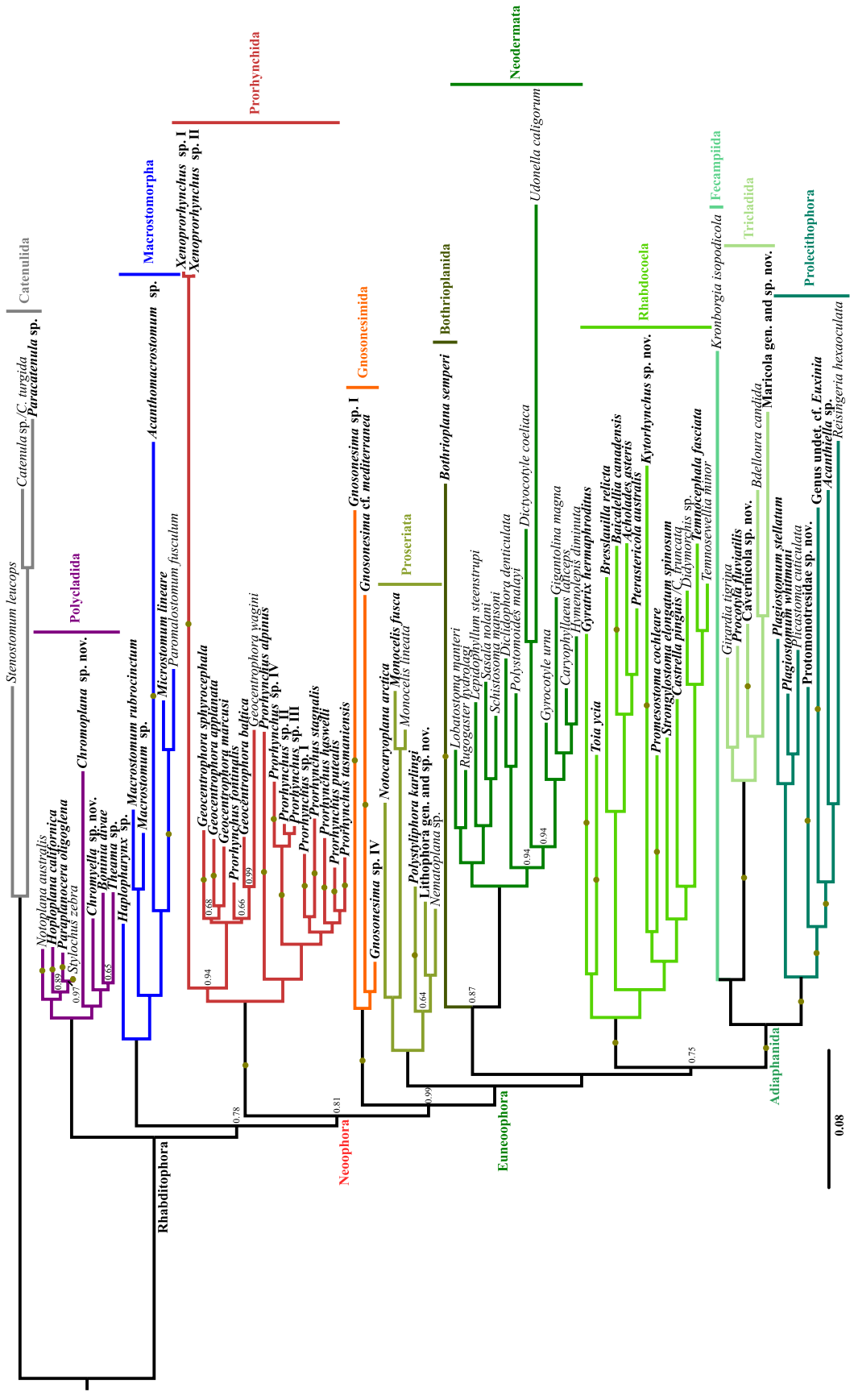

Figure 3. Phylogenetic relationships of rhabditophoran Platyhelminthes, summarized as a majority-rule consensus phylogram from a Bayesian reversible-jump Markov chain Monte Carlo analysis of Matrix A, sampled every 10^3 generations under an unfixed-degree mixture of GTR+Γ rate matrices, with up to two lengths fit per branch. The chain chosen for display was run for 3.1884×10^7 generations, with the first 25% removed as burn-in. For clarity, lengths at each branch were averaged in a custom PYTHON script. Catenulida was selected as the outgroup taxon. Well-supported classical higher taxa are labelled and coloured individually. Species newly sequenced for the present study are shown in bold. Branches with olive dots represent those for which the posterior probability of two lengths fit to that branch exceeded 0.9 (we also include *Bothrioplana semperi*, which has a posterior probability of two branch lengths = 0.88). Nodal values represent clade posterior probability ($pp = 1$ not shown).

the branch lengths presented in Figure 3 may be for this reason somewhat distorted by the influence of heterotachous sites; indeed, some taxa that display long-branches in Figure 3 (e.g. *Bothrioplana semperi*, *Gnosonesima* sp. I) are assigned short branches in other model-based analyses (see Supporting information, Fig. S4). To ensure that stationarity had been reached, we inspected plots and mean values of parameter estimates for each chain; all chains converged on similar harmonic mean likelihoods and numbers of patterns chosen (seven for Matrix A and five for Matrix B), although estimates of some other model parameters appeared to differ from chain to chain, possibly indicating difficulties in parameter identifiability or convergence. However, all chains yielded identical majority-rule consensus topologies, and comparable branch length estimates and nodal posterior probabilities, consistent with accurate Bayesian estimation of at least these aspects of our analyses.

RESULTS

Monophyly of all free-living higher taxa

We were able, for the first time, to represent in a molecular phylogeny essentially all known lineages within each major free-living platyhelminth clade, allowing for an explicit evaluation of the monophyly of these clades using the largest nucleic acid matrix yet available. Such an evaluation is relevant because previous phylogenetic analyses have failed to demonstrate the monophyly of certain taxa, particularly Macrostomida (Littlewood *et al*., 1999a, b; Norén & Jondelius, 2002) and Proseriata (Littlewood, Curini-Galletti, & Herniou, 2000), whereas other taxa (e.g. Polycladida, Prorhynchida) have remained relatively poorly sampled in most phylogenetic studies to date. In all optimal trees from our analyses of both data matrices, all major higher taxa (as defined in Fig. 3) are recovered as monophyletic. Support for these clades is uniformly strong (symmetric resampling proportion > 90%, bootstrap > 95%, *pp* = 1.0) under all optimality criteria, with the exception of our unweighted parsimony analyses (in which support for Proseriata is somewhat lessened; see Supporting information, Fig. S1). Notably, in our analyses Macrostomorpha (Macrostomida + *Haplopharynx*; Rieger, 2001) was also strongly supported; in no case was a *Haplopharynx* + Prorhynchida clade recovered (as in several previous studies: Littlewood *et al*., 1999b; Norén & Jondelius, 2002; Willems *et al*., 2006). We therefore interpret this clade from previous studies as being artefactual, perhaps as a result of the poor sampling of Prorhynchida.

Although it was not the primary focus of the present study, pending more targeted research, our taxon sampling also provides glimpses at aspects of the internal phylogeny of several groups that have received comparatively little recent phylogenetic attention; for example, within Polycladida, we see no support for the monophyly of Cotylea and Acotylea, and, within Tricladida, our inclusion of a putative Cavernicola shows a sister-group relation between this species and our representatives of the marine taxon Maricola (Fig. 3). It is also notable that, in contrast to the results of the study producing the sequence data for the Neodermata that we reanalyzed in the present study (Lockyer *et al*., 2003), as well as those of a more recent study based on mitogenomic data (Perkins *et al*., 2010), in none of our analyses (Fig. 3; see also Supporting information, Figs S1–S3) do we find strong support for a clade of Cestoda + Trematoda; indeed, both our ML and Bayesian mixture model analyses (Fig. 3; see also Supporting information, Fig. S3A) suggest (although, we emphasize, with mediocre posterior probability) a relationship between the Monopisthocotylea (Monogenea) and Cestoda (consistent with the historical 'Cercomer hypothesis' Lockyer *et al*., 2003), with Polyopisthocotylea as the sister group to this clade (indicating paraphyly of Monogenea, as also seen in Perkins *et al*., 2010). Because we designed our gene-sampling and analytical strategy to resolve relationships between free-living taxa only, however, our sample of Neodermata precisely mirrors that used by Lockyer *et al*. (2003), including longer-branched taxa (e.g. *Udonella*). The contrast of the two sets of results must therefore be attributable to analytical parameters: perhaps application of a mixture model more accurately discerns phylogenetic signal in these data or, equally plausibly, perhaps the automated structural alignment we used inaccurately models secondary structure in taxa showing large insertions in rRNA such as Neodermata. Further research on the internal relationships of Neodermata appears warranted in either case.

Phylogenetic placement of problematic species

We have included in our taxon sample a number of morphologically aberrant species of uncertain phylogenetic placement within Platyhelminthes (Rieger *et al*., 1991) (Fig. 2A, B, C). *Acanthiella* is an interstitial flatworm with an unusual combination of characters: a subepidermal matrix of calcareous spicules, apparent gonochory, the presence of a *pharynx simplex* (ostensibly a platyhelminth symplesiomorphy) and, most notably, a fully ectolecithal germovitellarium (Rieger & Sterrer, 1975). In our trees, it appears firmly nested within Prolecithophora, in a clade with our representative Cylindriostomidae and Pseudostomidae (Fig. 3), a placement in accordance

with the relative positions of mouth and genital openings (Rieger & Sterrer, 1975) and the characteristic prolecithophoran-like ultrastructure of its spermatozoa (Ehlers, 1988). We have also been able to sample *Acholades asteris*, an enigmatic rhabdocoel-like worm symbiotic on the sea star *Coscinasterias calamaria*, lacking gut, pharynx, and mouth (Hickman & Olsen, 1955). *Acholades* is presently the sole member of the rhabdocoel taxon Endoaxonemata (Jondelius & Thollesson, 1993; Tyler *et al*., 2012), a taxon originally erected on the basis of a presumed spermatozoan synapomorphy, encompassing Neodermata, Fecampiida *s.l.* (= the INUK clade of Baguñà & Riutort, 2004), and a clade of 'dalyelloid' rhabdocoels symbiotic on echinoderms (Pterastericolidae). Molecular phylogenies have refuted the existence of such a clade (Baguñà & Riutort, 2004) but have not yet included *Acholades asteris*. Our analyses clearly group *A. asteris* as the sister taxon of a pterastericolid rhabdocoel within the larger clade Neodalyellida (Willems *et al*., 2006; Van Steenkiste *et al*., 2013); thus, there is no phylogenetic justification for Endoaxonemata. Finally, our data suggest a putative systematic placement of the monospecific freshwater taxon Bothrioplanida, historically considered a relative of Proseriata and Tricladida in the now-defunct clade Seriata (Sopott-Ehlers, 1985; Baguñà & Riutort, 2004). In all analyses, *B. semperi* appears with strong support in a clade with all other non-lecithoepitheliate ectolecithal flatworms (see below); however, in Matrix A, its precise position within this clade was somewhat unstable to analytical treatment (Fig. 3; see also Supporting information, Figs S1, S2, S3, S4). Remarkably, in the consensus tree from our Bayesian mixture model analysis of this matrix (Fig. 3), *B. semperi* is recovered as the sister group of Neodermata, a result that would have profound implications for understanding the origin of obligate vertebrate parasitism in Platyhelminthes; however, this clade clearly lacks credibility ($pp = 0.87$). Interestingly, this topology was also recovered in the ML analysis of Baguñà *et al*. (2001), albeit apparently without any attempt to assess support. By contrast, under all Matrix B analyses, *B. semperi* occurred as the sister group to Proseriata, with modest support in likelihood and unweighted parsimony, and strong support under IW parsimony and mixture model analyses (see Supporting information, Figs S2, S4). To our knowledge, the present study is the first to recover this (or any) sister-group relationship for *B. semperi* with strong support; we note that this position is compatible with the original synapomorphies for which Seriata was erected (Sopott-Ehlers, 1985), as well as by more recently discovered ultrastructural correspondences (Kornakova & Joffe, 1996; Kornakova, 2010).

Euneoophora, a clade with specialized ectolecithality

Under almost all analytical conditions we explored (except in unweighted parsimony; see Supporting information, Fig. S1), we saw strong or unequivocal support for a clade of all non-lecithoepitheliate ectolecithal flatworms. Indeed, several other recent studies on the deep phylogeny of Platyhelminthes incorporating 28S rRNA in addition to the usual 18S rRNA data have also recovered this node (Norén & Jondelius, 2002; Lockyer *et al*., 2003) with strong resampling support. Notably, all flatworms in this clade share a unique form of ectolecithality relative to the lecithoepitheliate taxa, with functionally and spatially divided oogenesis not only at the cellular level (yielding separate oocytes and yolk cells), but also at the tissue level, with spatially separate oocyte and yolk-cell generating organs (called germaria and vitellaria, respectively), each possessing a distinct population of putative stem cell precursors (Bunke, 1981; Gremigni, 1983, 1988; Rieger *et al*., 1991) (Fig. 1E). Even in the few species of this clade in which these organs are fused together into a single tunic-bound organ (called a germovitellarium, as in some Prolecithophora and Rhabdocoela), there are always spatially distinct oogenetic zones, in marked contrast to the germovitellaria of the lecithoepitheliate taxa, in which oocytes and yolk cells alike appear to arise from a single population of putative stem cell precursors (Hyman, 1951; Rieger *et al*., 1991; Falleni, Lucchesi & Gremigni, 1995; Falleni, 1997) (Fig. 1D). To recognize the distinctive female reproductive morphology and advanced ectolecithality of this well-supported clade of flatworms relative to the lecithoepitheliate taxa, we propose the name Euneoophora **new taxon** (a new higher taxon, to which we avoid assigning a Linnean rank), inclusive of all descendants from the common ancestor of Proseriata and Rhabdocoela in Figure 3.

Monophyly of Neoophora

Neoophora, classically understood, would consist of a monophyletic Lecithoepitheliata sister to the clade that we have called Euneoophora (Ehlers, 1985). We recover precisely this topology, which appears robust to symmetric resampling, in our IW parsimony analyses of both matrices (although support is strongest in Matrix B; Fig. 1B; see also Supporting information, Fig. S2). This topology is not strongly dependent on the strength of the implied weighting function (controlled by the concavity term, k) because it was recovered for all k values from 1–10, although with declining support as k increased (see Supporting information, Fig. S3). Furthermore, Neoophora (although with different placements of the lecitho-

epitheliate taxa at high k) was recovered for all k explored in our analyses (up to 30). To validate this result, we also sought to recover Neoophora using a model-based optimality criterion. In our relatively simply partitioned ML analyses, the lecithoepitheliates are not recovered as sister taxa, with Polycladida instead as sister group to Prorhynchida, rendering Neoophora polyphyletic (see Supporting information, Fig. S4). However, bootstrap support for this topology is poor (< 50%). Using nonparametric tests of tree topology (approximately unbiased, Shimodaira–Hasegawa), we compared the ML topology with results obtained from searches in which the monophyly of Neoophora was constrained in several ways, including the classical hypothesis (monophyletic Lecithoepitheliata + Euneoophora). The ML topology was not found to have a significantly higher likelihood than any of the constrained topologies; in other words, trees in which Neoophora is monophyletic are statistically indistinguishable from the ML tree (Table 2). We also ran a more sophisticated model-based analysis using a Bayesian reversible-jump MCMC to sample models with unfixed-degree mixtures of GTR+Γ rate matrices, also allowing the option to assign more than one length to each branch. Such mixture models have been argued to more accurately account for heterogeneous (and, when incorporating multiple branch lengths, heterotachous) molecular evolutionary processes, and in a less subjective manner than even well-justified (e.g. stem/loop) a priori partitioning schemes (Pagel & Meade, 2004, 2008). Remarkably, such analyses support the monophyly of Euneoophora with at least one (in Matrix A) (Fig. 3) or indeed, both (in Matrix B) (Fig. 1A; see also Supporting information, Fig. S5) of the lecithoepitheliate taxa, with ≥ 0.99 posterior probability. However, the consensus topologies of these Bayesian analyses also imply, albeit without support, the paraphyly of Lecithoepitheliata, with Prorhynchida as sister group to Gnosonesimida + Euneoophora in Matrix A (Fig. 3), or with Prorhynchida as the immediate sister group to Euneoophora in Matrix B (see Supporting information, Fig. S5). It is also noteworthy that, in our analyses of Matrix B, in 90% or more of the rjMCMC samples, the branches leading to Macrostomida, Proseriata + Bothrioplanida, and Gnosonesimida were assigned more than one branch length (Fig. 1A; see also Supporting information, Fig. S5), suggesting that heterotachy may indeed be a relevant problem for rRNA analyses of deep platyhelminth relationships, particularly for these historically difficult nodes.

DISCUSSION

Lecithoepitheliates have been traditionally viewed as early-branching members of a monophyletic Neoophora because this phylogenetic hypothesis invites a simple, compelling explanation for the origin of ectolecithality: if we accept the lecithoepitheliate germovitellarium as primitive in structure, with its apparent common cell lineage for yolk cells and oocytes (Fig. 1D), this implies that yolk cells may have had a germ line origin within all Neoophora, originating within a lecithoepitheliate-like ancestral lineage as essentially specialized, sterile eggs (Hyman, 1951; Gremigni, 1983). In all non-lecithoepitheliate ectolecithal flatworms (the clade we have called Euneoophora), this serial homology is less obvious because Euneoophora present a different and perhaps more refined manifestation of ectolecithality, in which functional and spatial division does not occur only at the cellular level; in Euneoophora, yolk cells and oocytes are generated in distinct organs (Fig. 1E). Are these two manifestations of ectolecithality homologous?

Using the most phyletically inclusive (though not the largest) sample of free-living Platyhelminthes to date, including for the first time molecular data from the rare marine lecithoepitheliate taxon Gnosonesimida, we addressed this question phylogenetically, by discerning the relationships between all major flatworm groups, with particular emphasis on sampling the previously poorly-represented lecithoepitheliate and endolecithal taxa. Under almost all analytical conditions that we explored, we saw a robust signal of monophyly for Euneoophora (Figs 1, 3; see also Supporting information, Figs S2, S3, S4, S5). Furthermore, using both weighted parsimony and Bayesian inference under a sophisticated mixture model (two very different optimality criteria), we found, to our knowledge for the first time, support for the common ancestry of not only Euneoophora, but also of all ectolecithal taxa (Neoophora), with Lecithoepitheliata either mono- or paraphyletic (Fig. 1A, B). Either topology is thus consistent with the traditional view that lecithoepitheliates retain a form of ectolecithality similar to that of the earliest Neoophora, thus appearing to narrow the gap between the primitive, endolecithal oogenesis of the early-branching Rhabditophora (Macrostomorpha and Polycladida) and the highly organized ectolecithality (in all its cellular, anatomical, and developmental aspects) of taxa such as Neodermata and Rhabdocoela (Karling, 1974). Our demonstration of the common ancestry of lecithoepitheliates and Euneoophora is therefore fully consistent with the classical idea that the vitellocytes of these taxa are indeed homologous, and may have arisen through exaptation of an ancestral oocyte differentiation pathway (Hyman, 1951; Shinn, 1993). However, to test this hypothesis on the origin of vitellocytes rigorously, developmental investigations are clearly required. Ultrastructural and histochemical observations should be able to reveal the

existence of chromatoid bodies ('nuage' structures) in vitellocyte precursors, and the existence of maternal RNA storage in euneoophoran vitellocytes, as in those of prorhynchids (Reisinger, 1975). Although much more technically difficult, the availability of cell lineage tracing methods within adult flatworms would permit observation of the stem cells (neoblasts?) serving vitellaria, germaria, or both. Finally, and perhaps most tractably, further systematic characterization and expression screening of genes specifying female germ cell identity (perhaps using the planarian regenerative model system *Schmidtea mediterranea*; Sánchez Alvarado, 2003; Chong *et al*., 2011) may uncover developmental markers shared in common by vitellocyte and oocyte precursors.

Although our data validate the monophyly of both Euneoophora and, at least under IW parsimony and a Bayesian mixture model, Neoophora, they are less decisive regarding the longstanding question of the monophyly of Lecithoepitheliata; these analyses suggest that lecithoepitheliates may either be monophyletic (as seen under IW parsimony) or may represent a grade at the base of Neoophora (as seen under the Bayesian mixture model). Both of these scenarios are consistent with the plesiomorphic nature of lecithoepitheliate ectolecithality, and therefore the homology of lecithoepitheliate and euneoophoran vitellocytes; indeed, if lecithoepitheliates represent a paraphyletic assemblage, this interpretation of character state polarity is bolstered (Fig. 1). However, under this interpretation, the structure of the lecithoepitheliate female gonad, with its apparently common generative zone for oocytes and vitellocytes alike, becomes irrelevant to the question of lecithoepitheliate monophyly because it represents a symplesiomorphy of Neoophora, and not a synapomorphy of Lecithoepitheliata. Further molecular phylogenetic work is thus needed to bring resolution to this node. In addition, continued ultrastructural and embryological research, particularly focused on the rare Gnosonesimida, may bring to light the real shared derived characters of both of the lecithoepitheliate taxa with other flatworm groups or potentially, each other. Even in the case of lecithoepitheliate monophyly, however (an assertion, we emphasize, for which there remains no convincing morphological evidence at present), the freshwater Prorhynchida and marine Gnosonesimida represent such structurally and ecologically disparate organisms that we propose to refer to these higher taxa individually *sensu* Karling (Karling, 1974), rather than invoking the less specific (and possibly non-monophyletic) taxon Lecithoepitheliata.

Ectolecithality serves diverse adaptive functions within Neoophora. Fundamentally, it is a dissemination of the nutritive and protective functions of the oocyte into many smaller cells, with concurrent reduction in oocyte size (Rieger *et al*., 1991) (Fig. 1C, D, E). This dissemination vastly increases the functional 'oolemma' surface area to volume ratio for the egg capsule's contents (vitellocytes + oocytes). Hence, Neoophora may have the capacity to synthesize yolk at a higher rate than their endolecithal ancestors because yolk precursors (often originating from gut tissues adjacent to vitelline follicles Karling, 1967) may be transferred into many small maturing vitellocytes more efficiently than into a single oocyte of equivalent volume (Ruppert, Fox & Barnes, 2003), yielding an increase in the theoretical rate of egg capsule production. Production of marginal granules (and hence eggshell material) by vitellocytes may conceivably benefit from the same effect. Additionally, because ectolecithal oocytes are much smaller, it is common for Neoophora to include several zygotes into a single egg capsule by various mechanisms, whose embryos then partition (and possibly, compete for; Cardona, Hartenstein & Romero, 2006) a common pool of vitellocytes (Martín-Durán & Egger, 2012). As in adelphophagic animals (recently understood to include some Platyhelminthes; Harrath *et al*., 2009), the death of a single embryo does not then entail loss of the resources assigned to that individual (although, in adelphophagic flatworms, it is the vitellocytes of lost siblings, and not only embryonic tissues *per se*, that are cannibalized). Although such increases in fecundity would be of inherent evolutionary advantage, they may also represent important preadaptations to parasitic lifestyles, where fecundity is required to effectively disperse to new hosts (Whittington, 1997). Indeed, there is a strong functional analogy between ectolecithal development in Platyhelminthes and oophagic/adelphophagic development in gastropods and other marine invertebrates, and so, similar adaptive advantages (e.g. concerning developmental time, variability in offspring size, etc.; Collin & Spangler, 2012) may apply to both developmental modes. In at least some Neoophora, ectolecithality may be of non-embryonic ecological benefit because external yolk may be stored in the gut for post-hatching digestion (Martín-Durán & Romero, 2011). However, it is also clear that the presence of vitellocytes has intimate functional importance during the embryogenesis of Neoophora: a variety of transient embryonic structures and mechanisms exist for uptaking vitellocytes into the developing gut, and, furthermore, in several taxa, vitellocytes themselves apparently participate in the formation of such epithelial structures (Martín-Durán & Egger, 2012). Finally, especially (although not necessarily exclusively) in prorhynchids, for which there is evidence for maternal RNA storage in vitellocytes (Reisinger,

1975), and extensive intercellular connections between vitellocytes and oocytes (Bogolyubov & Timoshkin, 1993; Falleni *et al*., 1995; Falleni, 1997), it should be considered that vitellocytes themselves may play poorly understood roles in early embryonic specification.

Evidently, ectolecithality itself has diversified within Neoophora, and hence some of these biological functions may be apomorphic. Although a thorough understanding of this diversification requires much more comparative functional research on vitellocyte biology, the establishment of a well-resolved phylogeny of Platyhelminthes is necessary to both polarize these character state changes and validate their homology. Our evidence for the monophyly of both Euneoophora and Neoophora suggests that lecithoepitheliates in particular should be considered as important model systems for understanding the original functional roles and developmental mechanisms responsible for the advent of ectolecithality in Platyhelminthes.

Conclusions

With broadly expanded sampling of free-living taxa, and the novel application of well-justified methods of phylogenetic inference, the deepest phylogenetic splits within Platyhelminthes continue to gain resolution. Our results support both the monophyly of Neoophora and the classical hypothesis for the sister group relationship of the lecithoepitheliate flatworms with other members of this clade, hence providing a framework for future research on the evolution of vitellocytes and embryonic development more generally within Neoophora. In addition, we have been able to sequence and phylogenetically position several morphologically unusual lineages of uncertain affinity. However, several of the deepest splits within the phylum differ in topology between methods of analysis, and continue to show poor nodal support even in the face of thorough taxon sampling; of particular importance is the question of the monophyly of Lecithoepitheliata, as well as the identity of the nearest outgroup of Neoophora within the endolecithal Rhabditophora (Polycladida or Macrostomorpha, or a clade of both). These remaining uncertainties in our analyses and those of others suggest that the ability of the familiar nuclear rRNA markers to resolve deep relationships in Platyhelminthes has been effectively exhausted. To address these and other remaining problems, and to test the established but almost entirely rRNA-based molecular phylogeny of the phylum, methods of rapidly collecting and analysing large-scale phylogenetic data from across the nuclear genome should now be directed towards Platyhelminthes, as has recently been successfully carried out for several other metazoan phyla (Kocot *et al*., 2011; Smith *et al*., 2011; Oakley *et al*., 2013).

ACKNOWLEDGEMENTS

We thank the Willi Hennig Society for their sponsorship allowing free use of the software TNT, as well as Andrew Meade (University of Reading) and the Harvard Research Computing staff for providing and supporting a beta, MPI-enabled version of BayesPhylogenies, version 2.0. Tom Artois and Nathalie Revis are thanked for donating preserved specimens of *Gnosonesima* cf. *mediterranea*. Tom Artois, Marco Curini-Galletti, Miquel Vila-Farré, and Marian Litvaitis provided help with specimen identification. Numerous individuals and institutions provided aid with the fieldwork and specimen collection; Sal Genovese, Katharina Jörger, Paul Davison, Karen Johnson, Duncan Gray, Valentina Delogu, and Marco Curini-Galletti were particularly integral. Many specimens used in the present study were collected during the 2010 STRI Meiofauna workshop in Bocas del Toro organized by Jon Norenburg and Rachel Collin and supported by an Encyclopedia of Life Biodiversity Synthesis grant. C.E.L. was supported by a Harvard University Alexander Agassiz Fellowship, by an NSF EAPSI grant (Award 1015273), and by the Harvard University Department of Organismic and Evolutionary Biology. This research was supported by internal funds from the Museum of Comparative Zoology. Cassandra Extavour, three anonymous reviewers, and the editor provided valuable comments that improved the initial manuscript.

REFERENCES

Abascal F, Zardoya R, Telford MJ. 2010. TranslatorX: multiple alignment of nucleotide sequences guided by amino acid translations. *Nucleic Acids Research* **38:** W7–W13.

Ax P. 1961. Verwandtschaftsbeziehungen und Phylogenie der Turbellarien. *Ergebnisse der Biologie* **24:** 1–68.

Ax P. 2008. *Plathelminthes aus Brackgewässern der Nordhalbkugel*. Mainz: Akademie der Wissenschaften und der Literatur; Steiner.

Baguñà J, Carranza S, Paps J, Ruiz-Trillo I, Riutort M. 2001. Molecular taxonomy and phylogeny of the Tricladida. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor and Francis, 49–56.

Baguñà J, Riutort M. 2004. Molecular phylogeny of the Platyhelminthes. *Canadian Journal of Zoology* **82:** 168–193.

Bogolyubov D, Timoshkin O. 1993. Comparative characterization of the female gonads of the Lecithoepitheliata (Plathelminthes) with remarks on the taxonomy of the order. *Zoologichesky Zhournal* **72:** 17–26.

Boyer BC, Henry JJ, Martindale MQ. 1998. The cell lineage of a polyclad turbellarian embryo reveals close similarity to coelomate spiralians. *Developmental Biology* **204:** 111–123.

Bunke D. 1981. Ultrastruktur-Untersuchungen an Vitellocyten von *Microdalyellia fairchildi* (Turbellaria, Neorhabdocoela). *Zoomorphology* **99:** 71–86.

Caira JN, Littlewood DTJ. 2013. Worms, Platyhelminthes. In: Levin SA, ed. *Encyclopedia of biodiversity*, 2nd edn. Waltham, MA: Academic Press, 437–469.

Campos A, Cummings MP, Reyes JL, Laclette JP. 1998. Phylogenetic relationships of platyhelminthes based on 18S ribosomal gene sequences. *Molecular Phylogenetics and Evolution* **10:** 1–10.

Cannon LRG. 1986. *Turbellaria of the world: a guide to families and genera*. Brisbane: Queensland Museum.

Cardona A, Hartenstein V, Romero R. 2006. Early embryogenesis of planaria: a cryptic larva feeding on maternal resources. *Development Genes and Evolution* **216:** 667–681.

Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17:** 540–552.

Chong T, Stary JM, Wang Y, Newmark PA. 2011. Molecular markers to characterize the hermaphroditic reproductive system of the planarian *Schmidtea mediterranea*. *BMC Developmental Biology* **11:** 69.

Coil WH, Harrison FW, Bogitsh BJ. 1991. Platyhelminthes: Cestoidea. *Microscopic anatomy of invertebrates. Volume 3, Platyhelminthes and Nemertinea*. New York, NY: Wiley-Liss, 211–283.

Collin R, Spangler A. 2012. Impacts of adelphophagic development on variation in offspring size, duration of development, and temperature-mediated plasticity. *The Biological Bulletin* **223:** 268–277.

Conn DB. 2000. *Atlas of invertebrate reproduction and development*. New York, NY: Wiley.

Curini-Galletti M, Webster BL, Huyse T, Casu M, Schockaert E, Artois T, Littlewood DTJ. 2010. New insights on the phylogenetic relationships of the Proseriata (Platyhelminthes), with proposal of a new genus of the family Coelogynoporidae. *Zootaxa* **2537:** 1–18.

Ehlers U. 1985. *Das phylogenetische System der Plathelminthes*. Stuttgart: Gustav Fischer.

Ehlers U. 1988. The Prolecithophora – a monophyletic taxon of the Plathelminthes. *Fortschritte der Zoologie* **36:** 359–365.

Falleni A. 1997. Ultrastructural aspects of the germovitellarium of two prorhynchids (Platyhelminthes, Lecithoepitheliata). *Invertebrate Reproduction and Development* **31:** 285–296.

Falleni A, Lucchesi P, Gremigni V. 1995. Ultrastructural and cytochemical studies of the female gonad of *Prorhynchus* sp. (Platyhelminthes, Lecithoepitheliata). *Hydrobiologia* **305:** 199–206.

Fried B, Haseeb MA. 1991. Platyhelminthes: Aspidogastrea, Monogenea, and Digenea. *Microscopic anatomy of invertebrates. Volume 3, Platyhelminthes and Nemertinea*. New York, NY: Wiley-Liss, 141–210.

Goloboff PA. 1993. Estimating character weights during tree search. *Cladistics* **9:** 83–91.

Goloboff PA, Farris JS, Källersjö M, Oxelman B, Ramírez MJ, Szumik CA. 2003. Improvements to resampling measures of group support. *Cladistics* **19:** 324–332.

Goloboff PA, Farris JS, Nixon KC. 2008. TNT, a free program for phylogenetic analysis. *Cladistics* **24:** 774–786.

Gremigni V. 1983. Platyhelminthes-Turbellaria. *Reproductive Biology of Invertebrates, Vol. 1. Oogenesis, oviposition, and oosorption*. New York, NY: Wiley, 67–107.

Gremigni V. 1988. A comparative ultrastructural study of homocellular and heterocellular female gonads in free living Platyhelminthes-Turbellaria. *Fortschritte der Zoologie* **36:** 245–261.

Gremigni V. 1997. The evolution of the female gonad in Platyhelminthes-Turbellaria: ultrastructural investigations. *Invertebrate Reproduction and Development* **31:** 325–330.

Gremigni V, Falleni A. 1998. Characters of the female gonad and the phylogeny of Platyhelminthes. *Hydrobiologia* **383:** 235–242.

Harrath AH, Sluys R, Zghal F, Tekaya S. 2009. First report of adelphophagy in flatworms during the embryonic development of the planarian *Schmidtea mediterranea* (Benazzi, Baguñà, Ballester, Puccinelli & Del Papa, 1975) (Platyhelminthes, Tricladida). *Invertebrate Reproduction and Development* **53:** 117–124.

Hickman VV, Olsen AM. 1955. A new turbellarian parasitic in the sea-star, *Coscinasterias calamaria* (Gray). *Papers and Proceedings of the Royal Society of Tasmania* **89:** 55–64.

Hooge M, Wallberg A, Todt C, Maloy A, Jondelius U, Tyler S. 2007. A revision of the systematics of panther worms (*Hofstenia* spp., Acoela), with notes on color variation and genetic variation within the genus. *Hydrobiologia* **592:** 439–454.

Hyman LH. 1951. *The invertebrates, Vol. 2: Platyhelminthes and Rhynchocoela: the acoelomate Bilateria*. New York, NY: McGraw-Hill.

Joffe BI, Kornakova EE. 2001. Flatworm phylogeneticist: between molecular hammer and morphological anvil. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor and Francis, 279–291.

Jondelius U. 1998. Flatworm phylogeny from partial 18S rDNA sequences. *Hydrobiologia* **383:** 147–154.

Jondelius U, Norén M, Hendelberg J. 2001. The Prolecithophora. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor and Francis, 74–80.

Jondelius U, Thollesson M. 1993. Phylogeny of the Rhabdocoela (Platyhelminthes): a working hypothesis. *Canadian Journal of Zoology* **71:** 298–308.

Jondelius U, Wallberg A, Hooge M, Raikova OI. 2011. How the worm got its pharynx: phylogeny, classification and Bayesian assessment of character evolution in Acoela. *Systematic Biology* **60:** 845–871.

Karling TG. 1967. Zur Frage von dem systematischen Wert der Kategorien Archoophora und Neoophora (Turbellaria). *Commentationes Biologicae / Societas Scientiarum Fennica* **30:** 1–11.

Karling TG. 1968. On the genus *Gnosonesima* Reisinger (Turbellaria). *Sarsia* **33:** 81–108.

Karling TG. 1974. On the anatomy and affinities of the turbellarian orders. In: Riser NW, Morse MP, eds. *Biology of the Turbellaria*. New York, NY: McGraw-Hill, 1–16.

Katayama T, Nishioka M, Yamamoto M. 1996. Phylogenetic relationships among turbellarian orders inferred from 18S rDNA sequences. *Zoological Science* **13:** 747–756.

Kocot KM, Cannon JT, Todt C, Citarella MR, Kohn AB, Meyer A, Santos SR, Schander C, Moroz LL, Lieb B, Halanych K. 2011. Phylogenomics reveals deep molluscan relationships. *Nature* **477:** 452–456.

Kornakova EE. 2010. Ultrastructure of excretory system in *Bothrioplana semperi* (Platyhelminthes, Turbellaria). *Journal of Evolutionary Biochemistry and Physiology* **46:** 407–413.

Kornakova EE, Joffe BI. 1996. Sensory receptors in the head of *Bothrioplana semperi* (Platyhelminthes, Seriata). *Journal of Submicroscopic Cytology and Pathology* **28:** 313–317.

Larsson K, Jondelius U. 2008. Phylogeny of Catenulida and support for Platyhelminthes. *Organisms, Diversity and Evolution* **8:** 378–387.

Laumer CE, Giribet G. 2014. Data from: Inclusive taxon sampling suggests a single, stepwise origin of ectolecithality in Platyhelminthes. *Dryad Digital Repository*. doi: 10.5061/dryad.nh31f

Littlewood DTJ, Curini-Galletti M, Herniou EA. 2000. The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. *Molecular Phylogenetics and Evolution* **16:** 449–466.

Littlewood DTJ, Olson PD. 2001. Small subunit rDNA and the Platyhelminthes: signal, noise, conflict and compromise. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor and Francis, 262–278.

Littlewood DTJ, Rohde K, Bray RA, Herniou EA. 1999a. Phylogeny of the Platyhelminthes and the evolution of parasitism. *Biological Journal of the Linnean Society* **68:** 257–287.

Littlewood DTJ, Rohde K, Clough KA. 1999b. The interrelationships of all major groups of Platyhelminthes: phylogenetic evidence from morphology and molecules. *Biological Journal of the Linnean Society* **66:** 75–114.

Litvaitis MK, Rohde K. 1999. A molecular test of Platyhelminth phylogeny: inferences from partial 28S rDNA sequences. *Invertebrate Biology* **118:** 42–56.

Lockyer AE, Olson PD, Littlewood DTJ. 2003. Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory. *Biological Journal of the Linnean Society* **78:** 155–171.

Martens PM, Schockaert ER. 1986. The importance of turbellarians in the marine meiobenthos: a review. *Hydrobiologia* **132:** 295–303.

Martín-Durán JM, Egger B. 2012. Developmental diversity in free-living flatworms. *EvoDevo* **3:** 7.

Martín-Durán JM, Romero R. 2011. Evolutionary implications of morphogenesis and molecular patterning of the blind gut in the planarian *Schmidtea polychroa*. *Developmental Biology* **352:** 164–176.

Meade A. 2012. *BayesTrees*, Version 1.3. Available at: http://www.evolution.reading.ac.uk/BayesTrees.html.

Norén M, Jondelius U. 1999. Phylogeny of the Prolecithophora (Platyhelminthes) Inferred from 18S rDNA Sequences. *Cladistics* **15:** 103–112.

Norén M, Jondelius U. 2002. The phylogenetic position of the Prolecithophora (Rhabditophora, 'Platyhelminthes'). *Zoologica Scripta* **31:** 403–414.

Oakley TH, Wolfe JM, Lindgren AR, Zaharoff AK. 2013. Phylotranscriptomics to bring the understudied into the fold: monophyletic Ostracoda, fossil placement, and pancrustacean phylogeny. *Molecular Biology and Evolution* **30:** 215–233.

Pagel M, Meade A. 2004. A phylogenetic mixture model for detecting pattern-heterogeneity in gene sequence or character-state data. *Systematic Biology* **53:** 571–581.

Pagel M, Meade A. 2008. Modelling heterotachy in phylogenetic inference by reversible-jump Markov chain Monte Carlo. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences* **363:** 3955–3964.

Perkins EM, Donnellan SC, Bertozzi T, Whittington ID. 2010. Closing the mitochondrial circle on paraphyly of the Monogenea (Platyhelminthes) infers evolution in the diet of parasitic flatworms. *International Journal for Parasitology* **40:** 1237–1245.

Posada D. 2008. jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* **25:** 1253–1256.

Poulin R, Morand S. 2000. The diversity of parasites. *The Quarterly Review of Biology* **75:** 277–293.

Rawlinson KA. 2010. Embryonic and post-embryonic development of the polyclad flatworm *Maritigrella crozieri*; implications for the evolution of spiralian life history traits. *Frontiers in Zoology* **7:** 1–25.

Regier JC, Shi D. 2005. Increased yield of PCR product from degenerate primers with nondegenerate, nonhomologous 5′ tails. *BioTechniques* **38:** 34, 36, 38.

Reisinger E. 1926. *Zur Turbellarienfauna der Antarktis. Deutsche Südpolar-Expedition 1901–1903*. Berlin: W. De Gruyter und Company, 417–462.

Reisinger E. 1968. *Xenoprorhynchus*, ein Modellfall für progressiven Funktionswechsel. *Journal of Zoological Systematics and Evolutionary Research* **6:** 1–55.

Reisinger VE. 1975. *Geocentrophora applanata* (Kennel), ein Modellfall für gekoppelte Differenzierung und Reduktion zweier Organsysteme. *Zeitschrift für zoologische Systematik und Evolutionsforschung* **13:** 184–206.

Rieger RM. 2001. Phylogenetic systematics of the Macrostomorpha. In: Littlewood DTJ, Bray RA, eds. *Interrelationships of the Platyhelminthes*. London: Taylor and Francis, 28–38.

Rieger RM, Sterrer W. 1975. New spicular skeletons in Turbellaria, and the occurrence of spicules in marine meiofauna. *Journal of Zoological Systematics and Evolutionary Research* **13:** 207–278.

Rieger RM, Tyler S, Smith III JPS, Rieger GE. 1991. Platyhelminthes: turbellaria. *Microscopic anatomy of invertebrates. Volume 3, Platyhelminthes and Nemertinea*. New York, NY: Wiley-Liss, 7–140.

Rohde K, Hefford C, Ellis JT, Baverstock PR, Johnson AM, Watson NA, Dittmann S. 1993. Contributions to the phylogeny of Platyhelminthes based on partial sequencing of 18S ribosomal DNA. *International Journal for Parasitology* **23:** 705–724.

Rohde K, Johnson AM, Baverstock PR, Watson NA. 1995. Aspects of the phylogeny of Platyhelminthes based on 18S ribosomal DNA and protonephridial ultrastructure. *Hydrobiologia* **305:** 27–35.

Rohde K, Watson N. 1991. Ultrastructure of the flame bulbs and protonephridial capillaries of *Prorhynchus* (Lecithoepitheliata, Prorhynchidae, Turbellaria). *Zoologica Scripta* **20:** 99–106.

Roure B, Baurain D, Philippe H. 2013. Impact of missing data on phylogenies inferred from empirical phylogenomic data sets. *Molecular Biology and Evolution* **30:** 197–214.

Ruppert EE, Fox RS, Barnes RD. 2003. *Invertebrate zoology: a functional evolutionary approach*. Belmont, CA: Brooks Cole Thomson.

Sánchez Alvarado A. 2003. The freshwater planarian *Schmidtea mediterranea*: embryogenesis, stem cells and regeneration. *Current Opinion in Genetics and Development* **13:** 438–444.

Schockaert ER. 1996. Turbellarians. In: Hall GS, ed. *Methods for the examination of organismal diversity in soils and sediments*. Wallingford: CAB International, 211–225.

Shimodaira H, Hasegawa M. 2001. CONSEL: for assessing the confidence of phylogenetic tree selection. *Bioinformatics* **17:** 1246–1247.

Shinn GL. 1993. Formation of egg capsules by flatworms (Phylum Platyhelminthes). *Transactions of the American Microscopical Society* **112:** 18–34.

Smith SA, Wilson NG, Goetz FE, Feehery C, Andrade SCS, Rouse GW, Giribet G, Dunn CW. 2011. Resolving the evolutionary relationships of molluscs with phylogenomic tools. *Nature* **480:** 364–367.

Sopott-Ehlers B. 1985. *The phylogenetic relationships within the Seriata (Platyhelminthes)*. In: Conway Morris S, George DJ, Gibson R, Platt HM, eds. *The origins and relationships of lower invertebrates*. Oxford: Oxford University Press, 159–167.

Steinböck VO. 1966. Die Hofsteniiden (Turbellaria Acoela): Grundsätzliches zur Evolution der Turbellarien. *Zeitschrift für Zoologische Systematik und Evolutionsforschung* **4:** 58–195.

Stocsits RR, Letsch H, Hertel J, Misof B, Stadler PF. 2009. Accurate and efficient reconstruction of deep phylogenies from structured RNAs. *Nucleic Acids Research* **37:** 6184–6193.

Sukumaran J, Holder MT. 2010. DendroPy: a Python library for phylogenetic computing. *Bioinformatics* **26:** 1569–1571.

Surface FM. 1907. The early development of a polyclad, *Planocera inquilina* Wh. *Proceedings of the Academy of Natural Sciences of Philadelphia* **59:** 514–559.

Swiderski Z, Xylander WER. 2000. Vitellocytes and vitellogenesis in cestodes in relation to embryonic development, egg production and life cycle. *International Journal for Parasitology* **30:** 805–817.

Thomas MB. 1986. Embryology of the Turbellaria and its phylogenetic significance. *Hydrobiologia* **132:** 105–115.

Timoshkin OA. 1991a. Turbellaria of Lake Baikal. 1. Family Prorhynchidae: morphology, systematics and phylogeny (Lecithoepitheliata). In: Linevich AA, Afanaseva EL, eds. *Morphologija i Evolutsija Bespozvonochnih. Fauna Baikal serija osnovana*. Novosibirsk: Nauka, 63–185.

Timoshkin OA. 1991b. Turbellaria Lecithoepitheliata: morphology, systematics, phylogeny. *Hydrobiologia* **227:** 323–332.

Tyler S. 1976. Comparative ultrastructure of adhesive systems in the Turbellaria. *Zoomorphology* **84:** 1–76.

Tyler S, Schilling S, Hooge M, Bush L. 2012. *Turbellarian taxonomic database*, *Version 1.7* Available at: http://turbellaria.umaine.edu/.

Tyler S, Tyler MS. 1997. Origin of the epidermis in parasitic platyhelminths. *International Journal for Parasitology* **27:** 715–738.

Vaidya G, Lohman DJ, Meier R. 2011. SequenceMatrix: concatenation software for the fast assembly of multi-gene datasets with character set and codon information. *Cladistics* **27:** 171–180.

Van Steenkiste N, Tessens B, Willems W, Backeljau T, Jondelius U, Artois T. 2013. A comprehensive molecular phylogeny of Dalytyphloplanida (Platyhelminthes: Rhabdocoela) reveals multiple escapes from the marine environment and origins of symbiotic relationships. *PLoS ONE* **8:** e59917.

Whittington ID. 1997. Reproduction and host-location among the parasitic Platyhelminthes. *International Journal for Parasitology* **27:** 705–714.

Willems WR, Wallberg A, Jondelius U, Littlewood DTJ, Backeljau T, Schockaert ER, Artois TJ. 2006. Filling a gap in the phylogeny of flatworms: relationships within the Rhabdocoela (Platyhelminthes), inferred from 18S ribosomal DNA sequences. *Zoologica Scripta* **35:** 1–17.

Zrzavý J, Mihulka S, Kepka P, Bezděk A, Tietz D. 1998. Phylogeny of the Metazoa based on morphological and 18S ribosomal DNA evidence. *Cladistics* **14:** 249–285.

Zwickl DJ. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Unpublished PhD Thesis, University of Texas-Austin.

Zwickl DJ. 2012. *Using partitioned models*. Available at: https://www.nescent.org/wg_garli/Using_partitioned_models.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Unweighted parsimony analyses. Nodal values represent the proportion of 1000 symmetric resampling replicates supporting each node. A, strict consensus of two shortest trees from a nucleotide search of Matrix A at 20 224 steps. B, single shortest tree from nucleotide search of Matrix B at 17 237 steps.
Figure S2. Implied weighting parsimony analyses, shown for $k = 3$. Nodal values represent proportion of 100 symmetric resampling replicates supporting each node. A, fittest topology from searches on Matrix A, with total fit = 1247.31737. B, fittest topology from searches on Matrix B, with total fit = 1102.49173.
Figure S3. Heat map depicting the sensitivity of the implied weighting parsimony analyses fittest topology and symmetric resampling support to variation in the implied weighting concavity parameter (for all k 1–30), plotted for seven topological hypotheses relevant to the origin of ectolecithality. Lecithoepitheliata paraphyletic = (Prorhynchida,(Gnosonesimida,Euneoophora)). Trepaxonemata = (Macrostomorpha,(Polycladida,Neoophora)).
Figure S4. A, maximum likelihood topology ($-\ln L = -85399.491$) from unpartitioned GARLI analysis of Matrix A. B, maximum likelihood topology ($-\ln L = -71284.249$) from partitioned GARLI analysis of Matrix B. Nodal values represent the percentage of 1000 bootstrap replicates supporting each node.
Figure S5. Consensus phylogram from a BayesPhylogenies chain of 57.47×10^6 generations run on Matrix B. Colours of higher taxa are arbitrarily chosen and have no relation with the colours in Fig. 3.
Table S1. List of primer sequences used for amplification and sequencing with original references of the primer sequences.

ARCHIVED DATA

Data deposited at Dryad (Laumer & Giribet, 2014).