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Phylogenetic distribution of microRNAs supports the basal position of acoel flatworms and the polyphyly of Platyhelminthes

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SUMMARY Phylogenetic analyses based on gene sequences suggest that acoel flatworms are not members of the phylum Platyhelminthes, but instead are the most basal branch of triploblastic bilaterians. Nonetheless, this result has been called into question. An alternative test is to use qualitative molecular markers that should, in principle, exclude the possibility of convergent (homoplastic) evolution in unrelated groups. microRNAs (miRNAs), noncoding regulatory RNA molecules that are under intense stabilizing selection, are a newly discovered set of phylogenetic markers that can resolve such taxonomic disputes. The acoel *Childia* sp. has recently been shown to possess a subset of the conserved core of miRNAs found across deuterostomes and protostomes, whereas a polyclad flatworm—in addition to this core subset—possesses miRNAs restricted to just protostomes. Here, we examine another acoel, *Symsagittifera roscoffensis*, and three other platyhelminths. Our results show that the distribution of miRNAs in *S. roscoffensis* parallels that of *Childia*. In addition, two of 13 new miRNAs cloned from a triclad flatworm are also found in other lophotrochozoan protostomes, but not in ecdysozoans, deuterostomes, or in basal metazoans including acoels. The limited set of miRNAs found in acoels, intermediate between the even more reduced set in cnidarians and the larger and expanding set in the rest of bilaterians, is compelling evidence for the basal position of acoel flatworms and the polyphyly of Platyhelminthes.

INTRODUCTION

Central to virtually every theory about the early evolutionary history of animals are the flatworms (Phylum Platyhelminthes)—their simple morphology coupled with antiquated notions of progress in animal evolution has made them the perfect transitional taxon, from cnidarians to bilaterians or even from single-celled ancestors to other metazoans (Hyman 1951; Clark 1964; Salvini-Plawen 1978; Willmer 1990). However, beyond being a "flat worm," it is difficult to actually define morphologically what a flatworm is, as there are three very different lineages generally lumped under the umbrella of Platyhelminthes: Acoelomorpha, Catenulida, and Rhabditophora (Smith et al. 1986). The acoelomorphs consist of two groups, the acoels and the nemertodermatids, which conspicuously lack organs such as nephridia (i.e., kidneys), digestive glands, longitudinal nerve cords, and a true brain with neuropile (Ehlers 1984; 1985; Raikova et al. 1998; Reuter et al. 1998). The catenulids have a single dorsomedially positioned protonephridium and a true brain and nerve cords, as well as a complex reproductive system. The third group, the rhabditophorans (Ehlers 1984, 1985), which contains the most species including the parasitic forms, also has complex reproductive systems and a brain with nerve cords, as well as paired protonephridia.

There are only two characters generally regarded as Platyhelminthes autapomorphies, the possession of "neoblasts" (stem cells involved in replacement of nondividing differentiated cells) (Ehlers 1986), and the presence of a sack-like gut (i.e., lack of a proper anus). However, both of these are equivocal; the former is ill-defined and has not been properly scored in many phyla, and the latter is a likely eumetazoan plesiomorphy and has to be reassessed in other bilaterian phyla (e.g., Gnathostomulida, Gastrotricha) (reviewed in Baguñà and Riutort 2004a, b). And although recent morphological cladistic analyses weakly supported the traditional notion of Platyhelminthes monophyly (Fig. 1A) (Zrzavy et al. 1998; Peterson and Eernisse 2001), earlier analyses suggested a paraphyletic (Haszprunar 1996) or even a polyphyletic (Smith et al. 1986) Platyhelminthes. In the Peterson and Eernisse (2001) analysis, the monophyly of Platyhelminthes was supported unequivocally by the possession of "neoblasts"

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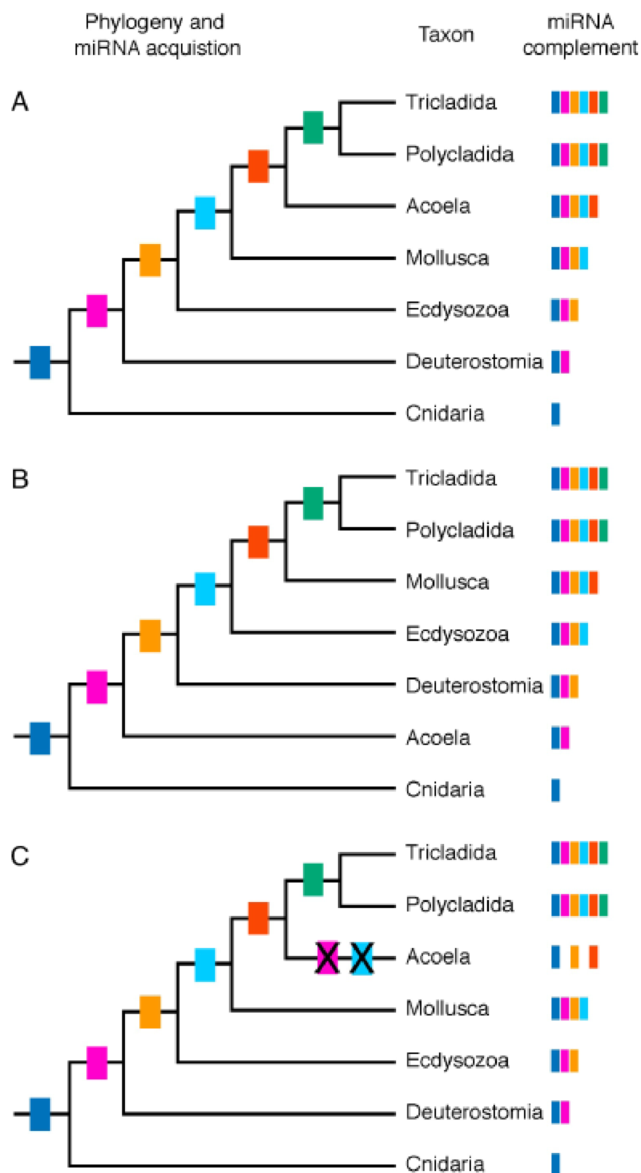


Fig. 1. Predicted complement of microRNAs (miRNAs) if acoels are members of the Platyhelminthes, as suggested by morphological cladistic analyses (A), or basal bilaterians, as suggested by 18S rDNA studies (B). The former predicts the presence of protostome- and lophotrochozoan-specific miRNAs in acoels, whereas the latter predicts the presence of only primitive miRNAs. Note that if there were secondary loss of some subset of miRNAs in acoel flatworms (C), then one would predict a mosaic pattern of miRNAs with acoels having some primitive (e.g., blue) and some (e.g., red), but not all (e.g., sky blue, indicated with an "X" on the box), derived miRNAs.

and the monophyly of Acoelomorpha+Rhabditophora to the exclusion of Catenulida was supported unequivocally by the possession of a frontal complex.

With the addition of ribosomal sequence analysis to the problem, both Peterson and Eernisse (2001) and Zrzavy et al. (1998) obtained a contrary result with respect to the morphological analysis: Platyhelminthes was polyphyletic such that acoels were basal triploblasts and Platyhelminthes *sensu stricto* consisted of Catenulida+Rhabditophora (Fig. 1B), consistent with prior analyses (Carranza et al. 1997; Littlewood et al. 1999; Ruiz-Trillo et al. 1999). Nonetheless, despite the repeatability of this result (see Baguñà et al. 2001; Jondelius et al. 2002; Telford et al. 2003; reviewed in Baguñà and Riutort 2004a, b), it has not won general acceptance because of the suspicion that it is the result of long-branch attraction (e.g., Erwin and Davidson 2002). Indeed, Peterson and Eernisse (2001) showed that not only were acoels some of the longest-branched taxa in their analysis, but that the acoels were basal even when a random sequence was used as the out-group. This experiment suggested to Peterson and Eernisse (2001) that because triploblasts were separated from diploblasts by a long internal branch, acoels were most likely basal in analyses based on ribosomal DNA for spurious reasons.

Because 18S rDNA has yet to determine unequivocally where acoels lie, additional data are needed. Phylogenetic analysis of protein sequence data, both nuclear and mitochondrial, offers a number of advantages, the most important of which is its independence from ribosomal sequence. Importantly, the basal position of acoel flatworms was supported by both a protein sequence analysis of the nuclear-encoded myosin heavy chain (Ruiz-Trillo et al. 2002), and by a phylogenetic analysis of mitochondrial genes (Ruiz-Trillo et al. 2004), suggesting that the ribosomal DNA results were not spurious, contra Peterson and Eernisse (2001).

An alternative way to test between these two competing hypotheses—acoels either lying within Platyhelminthes or lying at the base of Triploblastica—is to examine unique genetic and/or biochemical apomorphies that are relatively immune from homoplasy. Using Northern analyses, Pasquinelli et al. (2000, 2003) were able to detect the expression of the microRNA (miRNA) gene *let-7* in all triploblasts except acoel flatworms, and not in cnidarians, ctenophores, or sponges. Metazoan miRNAs (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001) are approximately 22 nucleotide regulatory RNA molecules that control gene expression by negatively regulating the stability or translation of target mRNAs by binding to their 3′ UTRs with imperfect complementarity (reviewed recently by Plasterk 2006). Sempere et al. (2006) extended this miRNA analysis to virtually all miRNAs held in common between protostomes and deuterostomes, and showed that the acoel *Childia* sp. had a subset of the miRNAs found in protostomes and deuterostomes, whereas the rhabditophoran polyclad flatworm *Stylochus zebra* had not only most of the triploblast and nephrozoan (i.e., protostomes and deuterostomes, Jondelius et al. 2002) miRNAs, it also had all of the detectable miRNAs restricted to protostomes. These data were entirely consistent with acoels

occupying a basal position among triploblasts, but given that only two taxa were analyzed (a polyclad and an acoel), these data, while compelling, need to be supplemented by examining more miRNAs and more taxa.

Because of the paleobiological ramifications stemming from a basal triploblast position including their small size and direct developing strategy (Peterson et al. 2005), we sought to further address the phylogenetic position of acoel worms. Here, we report on the examination of a second acoel taxon, *Symsagittifera roscoffensis*, and three additional rhabditophoran platyhelminth taxa, one polyclad and two triclads, for their miRNA complements using Northern analysis. We also report on the phylogenetic distribution of 13 new miRNAs cloned from an miRNA library derived from the rhabditophoran triclad *Schmidtea mediterranea* (Palakodeti et al. 2006) across a wide range of metazoans including both acoel species and all four platyhelminth species. We show that acoels are a basal group of triploblast metazoans because they possess a subset of miRNAs found across protostomes and deuterostomes, and none of the miRNAs unique to protostomes or planarians.

MATERIALS AND METHODS

To extract total RNA, animals were snap-frozen in liquid nitrogen and homogenized in Trizol (Invitrogen, Carlsbad, CA, USA) following the vendor's recommendations. Total RNA was obtained from adult whole animals, unless otherwise noted: the acoels *S. roscoffensis* and *Childia* sp., the polyclads *S. zebra* and *Discocelis tigrina*, the triclads *Girardia tigrina* and *S. mediterranea*, the annelid *Nereis vexillosa*, the mollusc *Haliotis rufescens*, the dipteran insect *Drosophila melanogaster*, the nematode *Caenorhabditis elegans* (mixed stage culture of N2 worms), the hemichordate *Ptychodera flava* (obtained from regenerating worms), the teleost fish *Danio rerio*, and the mouse *Mus musculus* (3-day-old mouse neonates).

All Northern analyses and genomic trace searches were performed as described in Sempere et al. (2006). StarFire oligonucleotide probes (IDT) were used to detect miRNAs and 5S ribosomal RNA (used as a loading control) following the vendor's recommendations; probe sequences are available upon request.

RESULTS

To determine whether the miRNA complement of *Childia* and *Stylochus* accurately reflects the miRNA repertoire of acoels and platyhelminths, we extended our miRNA expression profiling to the acoel *S. roscoffensis* and three additional rhabditophoran platyhelminths, the polyclad *D. tigrina* and the triclads *S. mediterranea* and *G. tigrina*. Sempere et al. (2006) only detected the expression of six miRNAs in the acoel *Childia* sp.: miR-31, -34, -92, -100, -124, and -219. Using Northern analysis, we detected this same set of miRNAs in the acoel *S. roscoffensis*, and did not detect any other miRNAs (Fig. 2; note that we did not examine miR-219 in this report). In contrast, we detected at least 10 of the 13 tested miRNAs in the polyclad *D. tigrina* and triclads *S. mediterranea* and *G. tigrina*, including the nephrozoan miRNAs miR-1 and -9, and the protostome miRNAs miR-8 and -87 (Fig. 2).

Recently, Palakodeti et al. (2006) reported the cloning and characterization of 71 miRNAs derived from an miRNA library from the triclad *S. mediterranea*. As expected, they cloned eumetazoan- (e.g., miR-10), triploblastic- (e.g., miR-31), nephrozoan- (e.g., miR-7), and protostome-specific (e.g., miR-8) miRNAs (see Sempere et al. 2006 for phylogenetic distribution). Also, as expected (Hertel et al. 2006; Sempere et al. 2006), they cloned novel miRNAs. These new miRNAs provide a useful set of molecular markers to further test the phylogenetic position of acoels and the monophyly of Platyhelminthes: if *S. mediterranea* is more closely related to other protostomes (e.g., annelids and molluscs) than it is to acoels, then some of these newly cloned miRNAs should be found in these other protostomes, but not in acoels. On the other hand, if the triclad and the acoel are more closely related to one another than either is to annelids or molluscs, they should share miRNAs not found elsewhere in the animal kingdom (see Fig. 1). To test this hypothesis, we examined the expression of 13 novel miRNAs cloned from *S. mediterranea* in the acoel *S. roscoffensis*, the platyhelminths *S. zebra*, *D. tigrina*, *S. mediterranea,* and *G. tigrina*, the lophotrochozoans *Haliotis rufescens* (gastropod mollusc) and *N. vexillosa* (polychaete annelid), the ecdysozoans *Caenorhabditis elegans* (nematode) and *D. melanogaster* (arthropod), and the deuterostomes *P. flava* (hemichordate), *D. rerio* (teleost fish), and *M. musculus* (mouse). These selected miRNAs did not belong to any previously described miRNA family or there were more than four mismatches between the new miRNAs and the miRNA founding member (see Palakodeti et al. 2006). Importantly, none of the *Schmidtea mediterranea* miRNAs were detected in the acoel *S. roscoffensis* (Fig. 2). Three of the 13 miRNAs (miR-751, -752, and -755) were detected only in *S. mediterranea* but not in the triclad *G. tigrina* or any other taxon (note: miR-755 has been detected in the triclad *Dugesia tigrina*; see Palakodeti et al. 2006). Two of the 13 miRNAs were detected in both triclads but not in the polyclads, and six of the 13 miRNAs were detected in both the triclads and the polyclads; none of these 11 miRNAs were detected outside Platyhelminthes (Figs. 2 and 3).

Of the 13 miRNAs examined, two miRNAs, miR-745, and miR-750, were also detected in both the annelid and mollusc (Fig. 2B), and were also found in the genomic traces of the annelid *Capitella* sp. and gastropod mollusc *Lottia gigantea* (Fig. 2C). These two miRNAs are not present in ecdysozoans or deuterostomes, and are not found or detected in basal metazoans including the acoels. This taxonomic

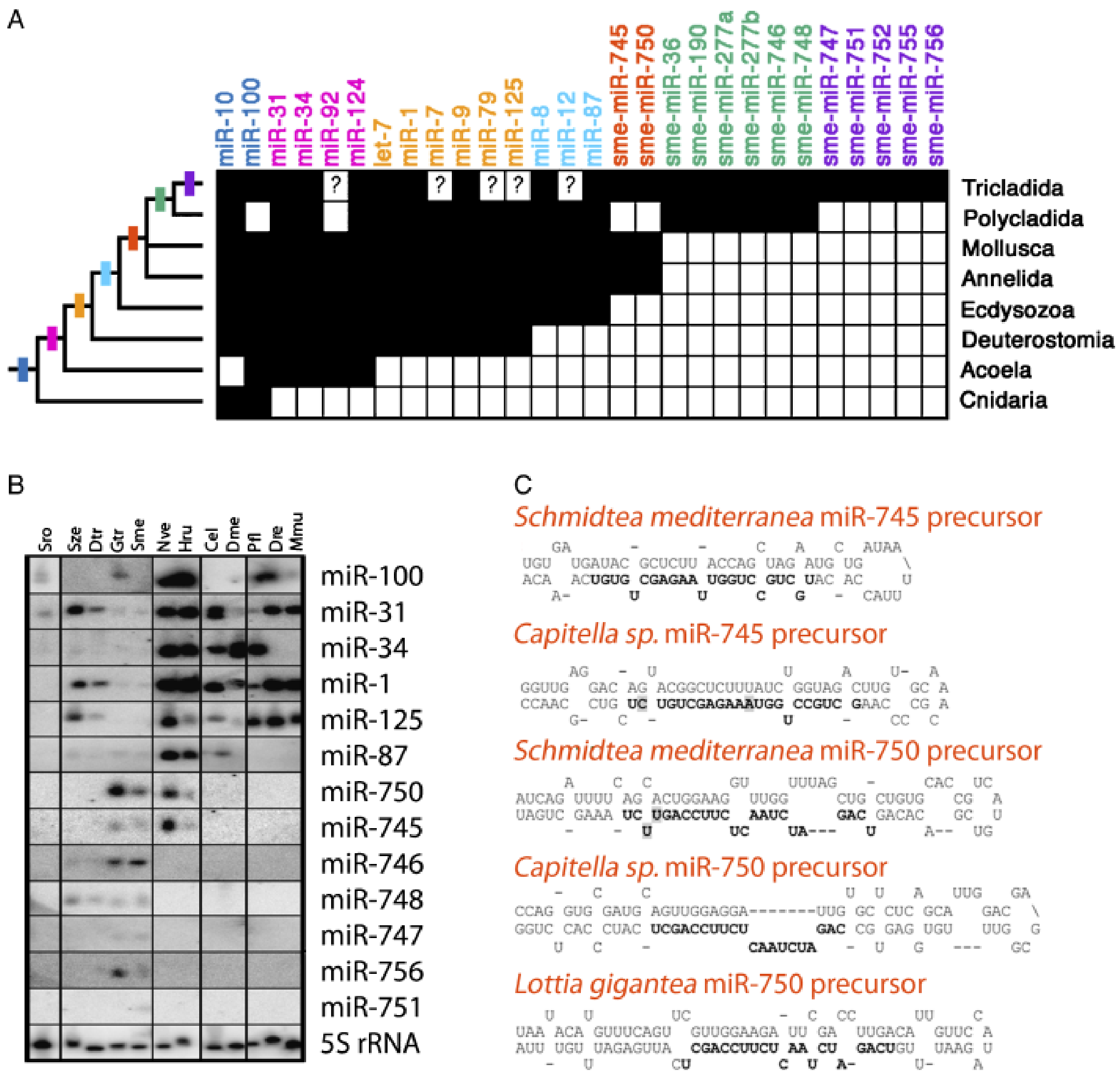
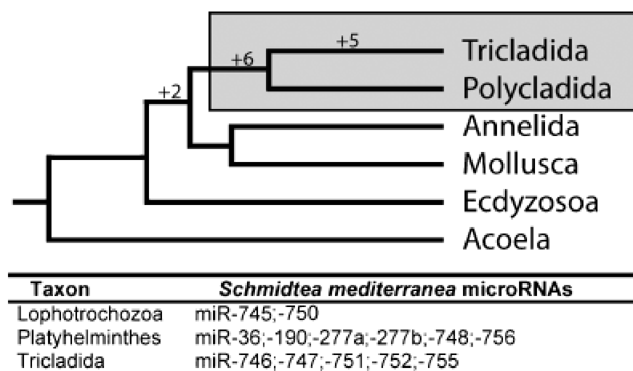


Fig. 2. Expression of microRNAs (miRNAs) as detected via Northern analysis for a variety of metazoans including acoel flatworms. (A) Summary for all Northern data for all major metazoan taxa analyzed. Only five miRNAs were detected in *Symsagittifera roscoffensis*, the same five detected by Sempere et al. (2006) in *Childia* sp.; neither acoel species expressed miR-10 in the adult stage, in contrast to all other taxa analyzed thus far. The flatworms possess most of the eumetazoan (blue), triploblast (magenta), nephrozoan (orange), and protostome (sky blue) miRNAs as expected (Sempere et al. 2006). Triclads, but not polyclads, also express two miRNAs, miR-745, and miR-750, only detected (B) and found (C) in total RNA and genomes, respectively, of annelids and molluscs (red). Finally, a set of the novel miRNAs cloned by Palakodeti et al. (2006) are only detected in the flatworms analyzed (blue-green), and a subset of these only in triclads (purple). Boxes with question marks indicate that a degenerate miRNA, with a sequence that differs by >3 mismatches to cognate miRNA complementary to indicated probe, might be expressed and/or was cloned from *Schmidtea mediterranea* by Palakodeti et al. (2006). (B) Examples of the Northern results. 5S rRNA is used as a loading control. Sro: *S. roscoffensis* (acoel flatworm); Sze: *Stylochus zebra* (polyclad flatworm); Dtr: *Discocelis tigrina* (polyclad flatworm); Gtr: *Girardia tigrina* (triclad flatworm); Sme—*S. mediterranea* (triclad flatworm); Nve: *Nereis vexillosa* (polychaete annelid); Hru: *Haliotis rufescens* (gastropod mollusc); Cel: *Caenorhabditis elegans* (nematode worm); Dme: *Drosophila melanogaster* (dipteran insect); Pfl: *Ptychodera flava* (enteropneust hemichordate); Dre: *Danio rerio* (teleost fish); and Mmu: *Mus musculus* (mouse). (C) Sequences and putative secondary structures for miR-745 (top) and miR-750 (bottom) for flatworms (*S. mediterranea*), annelids (*Capitella* sp.), and molluscs (*Lottia gigantea*). The mature sequence is shown in bold, and mismatches between the mature and star arms are shown in gray. An unequivocal miR-745 sequence was not found in the genomic traces of *L. gigantea*, but note that transcripts are clearly detected in the gastropod *H. rufescens* (B).



Taxon	*Schmidtea mediterranea* microRNAs
Lophotrochozoa	miR-745;-750
Platyhelminthes	miR-36;-190;-277a;-277b;-748;-756
Tricladida	miR-746;-747;-751;-752;-755

Fig. 3. Summary of the phylogenetic distribution of the 13 new miRNAs cloned by Palakodeti et al. (2006). Five of the 13 are restricted to triclads, six are restricted to Platyhelminthes *sensu stricto*, and two are present in other lophotrochozoans. None of the 13 miRNAs are detected in acoels.

distribution suggests that miR-745 and -750 are lophotrochozoan-specific miRNAs (Fig. 3). Curiously, though, neither is detected in either polyclad examined—determining whether these have been secondarily lost or simply not expressed at detectable levels in adult worms will have to await genomic sequences. Nonetheless, these results strengthen the conclusion from our previous report (Sempere et al. 2006) that Acoela is a basal taxon of triploblastic metazoans, and strongly suggests that Platyhelminthes, as classically defined, is polyphyletic.

DISCUSSION

Here, we have shown that the miRNA complement of acoel flatworms is entirely consistent with the molecular results demonstrating that Acoela is, in fact, a basal bilaterian taxon and not part of the Nephrozoa. Importantly, some of miRNAs absent in acoels (e.g., miR-1) are involved in the development of the very organs that are not found outside of the Nephrozoa (e.g., heart), suggesting that acquisition of these miRNAs was crucial for the evolution of complex body plans. In addition, we provide further support for the notion that Platyhelminthes *sensu stricto* (i.e., Catenulida+Rhabditophora; reviewed in Baguñà and Riutort, 2004a) is allied with the annelids and molluscs into the super-clade Lophotrochozoa (Eernisse and Peterson 2004; Halanych 2004; Telford 2006), as only these taxa possess miR-745 and miR-750. Finally, we have demonstrated the utility of miRNAs for unraveling the phylogenetic tree of metazoans.

Arguably one of the most important insights that molecular phylogenetic analysis has given to our understanding of metazoan evolution, in addition to the paraphyly of "Porifera" (Peterson and Butterfield 2005; Sperling et al. 2007), is the polyphyly of Platyhelminthes (Baguñà and Riutort 2004a, b; Peterson et al. 2005). A basal position of acoelomorphs among triploblasts allows for the polarization of several key characters including the nephridium, the brain, the coelom, and the possession of a primary larval stage. Here, we confirm the hypothesis that Platyhelminthes is polyphyletic (Littlewood et al. 1999; Ruiz-Trillo et al. 1999, 2002, 2004; Jondelius et al. 2002; Pasquinelli et al. 2003; Telford et al. 2003) by showing that only a subset of the miRNAs detected in both protostomes and deuterostomes are detected in the acoels, in contrast to the polyclads and triclads, which express a wide variety of miRNAs including a set restricted to just protostomes and, at least for the triclad, two miRNAs found exclusively in other lophotrochozoans.

The limited set of miRNAs found in acoels, intermediate between the even more reduced set in cnidarians and the larger and expanded set in the rest of the bilaterians, is very powerful evidence for the basal position of acoel flatworms, and the polyphyly of Platyhelminthes. If acoels were, in fact, members of the Platyhelminthes, and simply had a reduced number of miRNAs due to secondary loss, then one would expect a mosaic or "salt-and-pepper" pattern of miRNAs such that a few triploblast-, nephrozoan-, and protostome-specific miRNAs would be detected (Fig. 1C). Instead, only a subset of the miRNAs detected in protostomes *and* deuterostomes is detected, and one of them, miR-100, is also detected in cnidarians (Sempere et al. 2006) (Fig. 1B). Furthermore, the nature of the expression patterns of the miRNAs found in acoels is also consistent with our arguments of primitiveness rather than secondary reduction. In the fish, many of these miRNAs are either expressed in the nervous system (miR-100, -34, -124, -219) or are expressed ubiquitously (miR-31, -92) (http://www.exiqon.com/SEEEMS/4519.asp); none of the "organ-associated" miRNAs are detected in acoels (or more basal taxa for that matter) including miR-1 (heart) and miR-9 (brain) (see also Sempere et al. 2006).

The finding that acoels have only a subset of the nephrozoan miRNAs, and none of these subsets are associated with the development of any particular organ (but only with certain cell types like neurons) is of particular interest, given that ctenophores, cnidarians, and acoel flatworms, aside from occasional sensory structures like statocysts, and eyes, are just axially organized sheets of cells. That is, they lack organs such as the kidneys, hearts, brains, or digestive glands like livers. The possession of *Hox* genes and signaling systems in these axially organized but essentially "organ-less" animals may reflect an intermediate stage of animal evolution between the evolution of simple collections of cells like sponges and the evolution of complex and integrated organ systems like that of protostomes and deuterostomes. We hypothesize that this final stage of animal evolution was predicated upon the elaboration of miRNAs, and suggest that they are a *key* innovation underlying the evolution of deuterostome and protostome organs and organ systems.

The only miRNA not detected that was expected to be present is miR-10. miR-10 is an unusual miRNA—not only is it the only phylogenetically conserved miRNA embedded in the *Hox* cluster, it is also the only miRNA that appears to have a role in body plan patterning as opposed to tissue and organ specification (reviewed in Pearson et al. 2005). One could argue that the apparent loss of *Hox4* in acoels (Cook et al. 2004; see Cameron et al. 2006 for phylogenetic analysis) may have resulted in the loss of miR-10 as well, given that miR-10 lies between *Hox4* and *Hox5* (Pearson et al. 2005). However, *Hox4* has also been lost in echinoid echinoderms, but miR-10 is still present (Cameron et al. 2006). Indeed, the entire zebrafish *hoxDb* cluster has been lost, *except* for miR-10 (Woltering and Durston 2006). These data suggest that miR-10 has not been lost, and we predict that miR-10 is present, but transcripts are restricted to embryonic stages, which we have yet to examine. Of course, without genomic sequences from an acoel, it is not possible to make any definitive claims about the presence or absence of miR-10, but the available comparative information suggests that a secondary loss would be surprising.

Contrary to the acoels, polyclad, and triclad flatworms possess not only most of the nephrozoan miRNAs but also possess protostome-specific miRNAs, consistent with the possession of internal organs such as nephridia and a true brain. Indeed, we show here that the triclads possess two miRNAs not found outside of the Lophotrochozoa: miR-745 and miR-750. These data are entirely consistent with a placement of Platyhelminthes *sensu stricto* within lophotrochozoans as suggested by numerous phylogenetic studies (recently reviewed by Baguñà and Riutort 2004a). As expected from the studies of Hertel et al. (2006) and Sempere et al. (2006), new miRNAs appear to be continuously added to the metazoan genome, and thus the discovery of lophotrochozoan- and triclad-specific miRNAs is not surprising. Indeed, we expect that further studies will show that most nodes in the metazoan tree will be supported by the unique acquisition of at least one new miRNA. Of course, therein lies their power—the unique ability to unequivocally resolve a great deal of the metazoan tree, from species to super-phyla, using a set of genes that are under intense purifying selection and intimately involved with the development of the very body plans whose phylogeny is in question (Sempere et al. 2006). Resolution of the Platyhelminthes polyphyly problem, as demonstrated herein, is only the tip of the iceberg.

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