

Concordance of molecular and morphological data: The example of the Acoela

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Synopsis Morphological features of the Acoela appear to be quite plastic, including those of the copulatory organs, which provide the principle characteristics used for the systematics of this group. Consequently, classification schemes of the Acoela comprise numerous polyphyletic groupings. In this review, we detail recent revisions of acoel systematics using molecular sequence data and new and reevaluated morphological characteristics. Gene trees are discordant with traditional systematic schemes but strongly concordant with new morphological characteristics obtained through the use of transmission electron microscopy and confocal laser scanning microscopy, namely, characteristics of body-wall and copulatory organ musculature, sperm, sperm ducts, sagittocysts, and immunocytochemistry of the nervous system. This merger of molecular and morphological data has led to significant changes in acoel classification, including a major emendation of the largest family of the Acoela, the Convolutidae, whereby half of its members were transferred to a newly created family, the Isodiametridae.

Introduction

The best-recognized and most commonly studied members of the Acoela are macrofaunal, epibenthic, or epiphytic worms containing photosynthetic endosymbionts and belonging to the families Convolutidae or Sagittiferidae. However, the majority of acoels are tiny, interstitial forms that receive relatively little attention except from a few acoel taxonomists (Fig. 1A). Despite the neglect of this important basal metazoan group, taxonomists, including the prolific workers Einar Westblad, Jürgen Dörjes, and Ernst Marcus, have described more than 340 Acoel species (for example, Westblad 1948; Marcus 1952; Dörjes 1968) divided among 21 families (Tyler and others 2005). Unfortunately, many of the characteristics defining these families are homoplasious, resulting in polyphyletic familial and generic groupings of convenience rather than natural groupings.

The small body size, lack of cuticular hardparts, and overall body plasticity make the identification of systematically useful characteristics in the Acoela a difficult task. Specifically, the number of characteristics visible via standard light microscopy of live animals is limited, and the differentiation of tissue types, even in semithin histological sections of epoxy-embedded specimens, can be difficult.

The reevaluation of the systematic characteristics currently used and the search for new characteristics

have been greatly facilitated by new microscopical tools such as confocal laser scanning microscopy (CLSM), which, with transmission electron microscopy (TEM) and gene sequencing, represents a suite of tools that has led to rapid progress in refining the phylogenies of several metazoan groups.

In this review, we detail how the use of 18S rDNA sequence data coupled with newly uncovered morphological characteristics obtained through the use of TEM and CLSM has led to the most substantial changes in acoel systematics in more than 30 years and, in doing so, has not only eliminated some of the polyphyletic taxonomic groupings but also provided the first inkling of the true phylogeny of the Acoela.

The gene tree

Before 2002 we had little notion of the interrelationships of the acoel families, except that the Paratomellidae were basal to all other acoels (Smith and Tyler 1985; Ehlers 1992; Raikova and others 1997). Our molecular analysis (Hooge and others 2002) used 18S rDNA gene sequences from 32 species representing 9 families of acoels. The resulting gene tree (Fig. 2), although thick with polytomies, was well supported and showed a number of interesting relationships. As expected, the Paratomellidae formed the most basal taxon. The Solenofilomorphidae formed a sister

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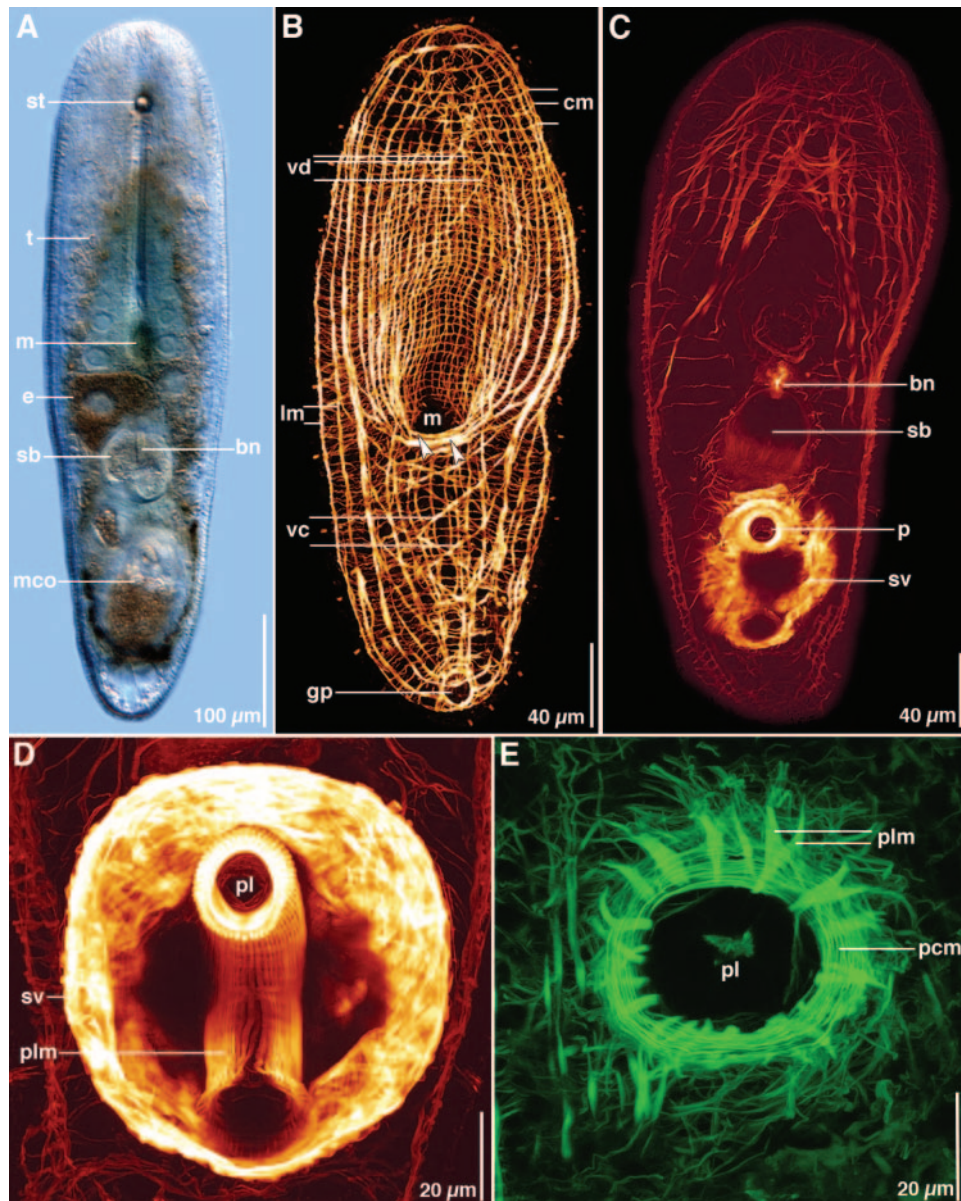


Fig. 1 (A) Ventral view of live specimen of *Isodiametra earnhardtii*. (B–E) Projections of musculature in whole-mount specimens of acoels stained with Alexa-488-labeled phalloidin and viewed using CLSM. (B) Ventral body-wall musculature of *Haplogonaria amarilla*. (C) Parenchymal musculature of *Isodiametra divae*, showing portions of copulatory organs. (D) Male copulatory organ of *I. divae*, showing musculature of seminal vesicle and invaginated penis. (E) Penis musculature of *Convoluta henseni*. bn, bursal nozzle; cm, circular muscle of body wall; e, egg; gp, gonopore; lm, longitudinal muscle of body wall; m, mouth; mco, male copulatory organ; p, penis; pcm, circular muscle of penis; pl, penis lumen; plm, longitudinal muscle of penis; sb, seminal bursa; st, statocyst; sv, seminal vesicle; t, testes; vc, ventral crossover muscle; vd, ventral diagonal muscle.

group to the Paratomellidae. The remaining taxa were grouped into a polytomy of 3 clades, with taxa clustering, for the most part, with other species from their family. The gene tree showed a few cases of familial polyphyly in the families Otocelididae, Haploposthiidae, and Convolutidae. Most strikingly, the 11 species of convolutids included in the study were grouped into 2 distinct clades: the first with

“small-bodied” members of the family that also grouped with other small-bodied acoels and the second with “large-bodied” members that, in turn, grouped with other large-bodied acoels.

Given the unexpected result of a polyphyletic Convolutidae, we (Hooge and others 2002) evaluated the tree with regard to 2 types of well-studied morphology: patterns of body-wall musculature and

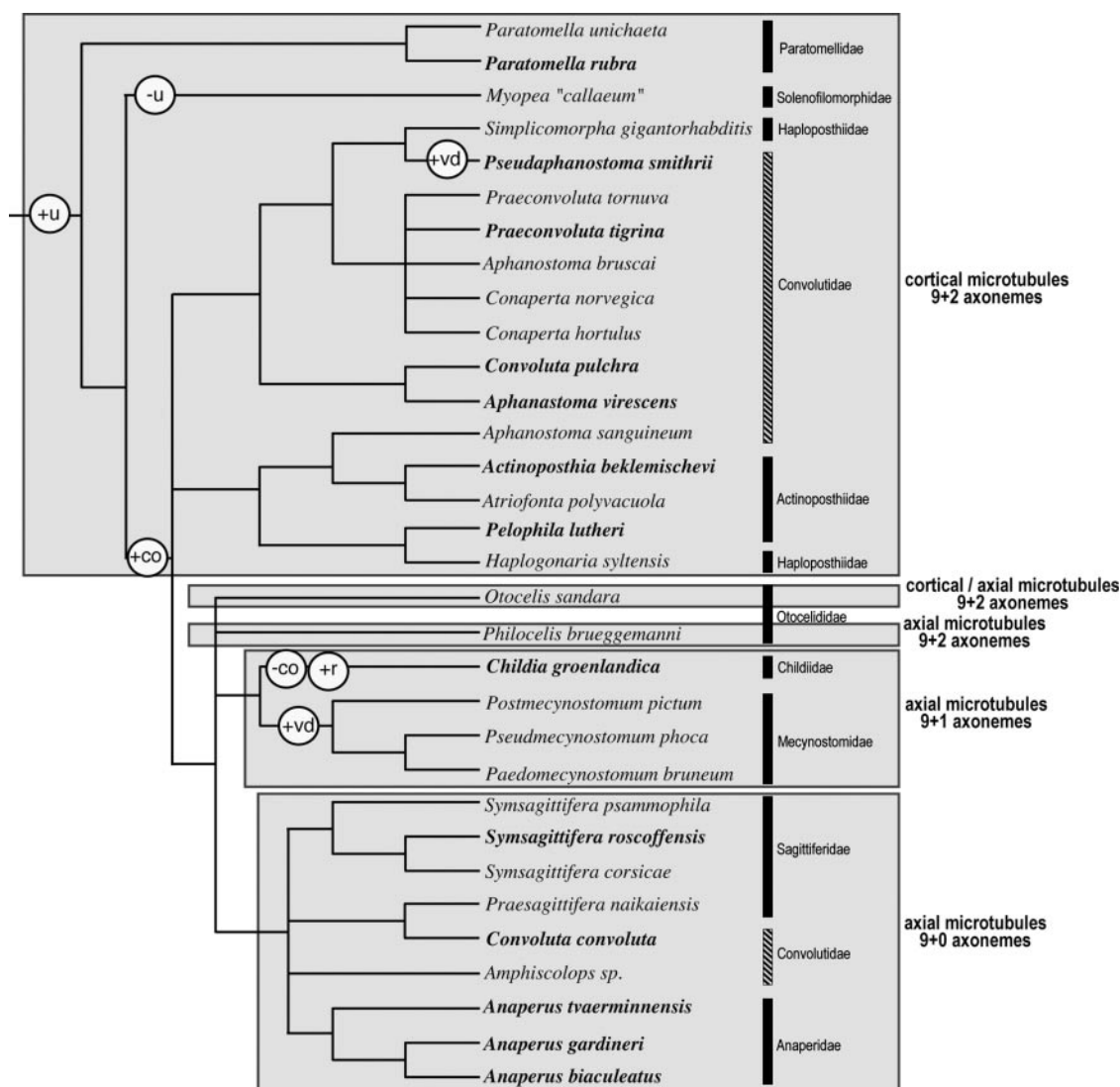


Fig. 2 The distribution of spermatozoa ultrastructure characteristics and body-wall musculature characteristics superimposed on the 18S rDNA tree of Hooge and colleagues (2002). Species names in bold denote those taxa for which sperm morphology is known. Characteristic state changes of body-wall musculature are indicated by circled codes on tree branches: +, new characteristic state; -, loss of characteristic; co, crossover muscles; r, reversal of layering of circular and longitudinal muscles; u, U-shaped muscles; vd, ventral diagonal muscles. The classification of the Convolutidae is from Dörjes (1968); in the current classification, those convolutids with 9 + 2 axonemes belong to the family Isodiametridae (Hooge and Tyler 2005). Adapted from Hooge and colleagues (2002) and Hooge and Tyler (2005).

ultrastructure of sperm (Figs. 1B and 2). Characteristics of body-wall musculature offered little support for the tree topology other than to provide autapomorphic characteristics for a few families and to support the separation of the 2 basal families, Paratomellidae and Solenofilomorphae, from the other acoel families. On the other hand, a strong correlation emerged between characteristics of sperm ultrastructure and characteristics of the gene tree. The distribution of sperm data, which until then had appeared rather enigmatic (Raikova and others 2001), supported the topology of the gene tree—including the polyphyly of the Convolutidae.

The discovery of the concordance between 18S rDNA sequence data and sperm morphology represented a significant breakthrough in organizing the Acoela into natural groupings. For the first time, we had more than just a list of taxa; we had some sense of their phylogeny. Significantly, the 18S rDNA gene tree also provided a phylogenetic tree that could be used to test the phylogenetic usefulness of newly discovered morphological characteristics.

The 18S rDNA phylogeny of the Acoela continues to be refined—most recently by Tekle and colleagues (2005), who examined the interrelationships of the family Childiidae.

Morphological characteristics

TEM and CLSM have uncovered many new characteristics for gauging phylogenetic relationships within the Acoela and have added greater depth to our understanding of known characteristics. The recent application of fluorescence-staining techniques in combination with CLSM has revealed a number of useful characteristics.

Body-wall musculature

The use of phalloidin-linked fluorophores and CLSM allows the entire musculature of a whole-mounted acoel to be visualized (Fig. 1B). The beneficial morphological information provided by this technique has made it a regular part of acoel species descriptions (for example, Hooge and Smith 2004). Whereas most taxa of soft-bodied interstitial worms have a common pattern of body-wall musculature, members of the Acoela display a number of distinct patterns (Hooge 2001).

The ventral musculature is particularly variable, apparently because the nature of the mouth seems to play a role in the configuration of musculature. Those species lacking pharynges have complex patterns of ventral body-wall musculature that compensate for this lack by allowing whole-body movements to force food into the mouth (Tyler and Rieger 1999). Those few species possessing a pharynx (such as members of the Diopisthoporidae, Solenofilomorphidae, and Proporidae) typically have simple body-wall musculature composed of a grid work of outer circular and inner longitudinal fibers. Almost all species that lack a pharynx have U-shaped muscles, muscle fibers that run longitudinally in the anterior portion of the body but then bend around the posterior rim of the mouth to form a U pattern. Also present in most acoel species are ventral crossover muscles that flank the U-shaped muscles. The ventral crossover muscles have a longitudinal orientation in the anterior portion of the body but then bend medially to cross over the ventral midline to the opposite side of the body. The possession of these 2 muscle sets, U-shaped and ventral crossovers, along with circular and longitudinal muscles characterizes the “Convolutida pattern” as defined by Hooge (2001). The majority of known acoel species possess the Convolutida pattern, including members of its namesake family, the Convolutidae.

Slight modifications of these muscle characteristics occur in some Convolutida taxa (such as U-shaped muscles modified as concentric muscles surrounding the mouth in *Convolutriloba retrogemma*; Gschwentner and others 2003), and some taxa have additional sets of taxon-specific muscles, such as special pore

muscles that radiate out from the rim of mouth and gonopores. Several taxa, including all members of the Mecynostomidae examined to date, as well as some members of the Haploposthiidae and *Pseudaphanostoma smithrii*, have diagonal muscles in the anterior portion of the body.

Examination of the body-wall musculature of acoels provides valuable morphological information that cannot be obtained through other techniques; however, some body-wall muscle characteristics originally identified as taxonomically useful (Hooge 2001; Hooge and others 2002) now seem to have questionable utility. The ventral diagonal muscles found in the anterior body wall of all members of the Mecynostomidae, some members of the Haploposthiidae, and in *P. smithrii*, now appear to be homoplasious (Fig. 2). Another characteristic, the absence of longitudinal muscles between the frontal pore and mouth, was reported by Hooge (2001) to be an autapomorphy for the Mecynostomidae, but further sampling of the family reveals that this characteristic is also absent in some species (unpublished data). Nevertheless, many taxonomically useful characteristics can be gleaned from the body-wall musculature. The pattern of musculature found in members of the genus *Paratomella* serves as an autapomorphy. Similarly, the unusual condition of body-wall musculature in the Childiidae, where longitudinal muscle fibers are positioned outside of circular fibers, is unique to this family. Tekle and colleagues (2005) found that variation of body-wall musculature within the family Childiidae is useful for phylogenetic analysis.

Penis musculature

Copulatory organ musculature, revealed through the use of phalloidin-linked fluorophores and CLSM, provides an even richer source of characteristics than body-wall musculature.

The 18S rDNA phylogeny of the Acoela produced by Hooge and colleagues (2002) revealed a polyphyletic Convolutidae that separated large-bodied members of the family (for example, *Convoluta convoluta*) from small-bodied members (for example, *Convoluta pulchra*) (Fig. 2). Although size is a surprisingly useful means of distinguishing the 2 groups, there are many exceptions, and other distinguishing characteristics also occur. For example, the 2 size groups have different patterns of microtubules in their sperm (see below), photosynthetic endosymbionts are often present in the large-bodied species but never in the small-bodied species, and when ocelli are present in the large-bodied taxa, they always contain reflective platelets (which are never present in the small-bodied taxa). Despite these

differences, it is the morphology of copulatory organs that is used as the basis for most family-level groupings in the currently accepted system established by Dörjes (1968). The large and small members of the Convolutidae were united in the same family by their mutual possession of a muscular, glandular penis that is invaginated into a muscular seminal vesicle.

To resolve this discrepancy, we (Hooge and Tyler 2005) investigated the patterns of musculature in the copulatory organs of members of the Convolutidae to search for more detailed characteristics that would reveal this apparent polyphyly (Fig. 1C and D). Our investigation revealed that the longitudinal muscles in the penes of large-bodied species have an anastomosing pattern (Fig. 1E) whereby individual longitudinal fibers cross over other longitudinal fibers. In contrast, the longitudinal penis muscles of the small-bodied convolutids were straight and never crossed over other longitudinal muscle fibers (Fig. 1D). The results of this work allowed us to correct the polyphyly of the family and segregate those taxa with nonanastomosing penis musculature into a new family, Isodiametridae; those convolutids with anastomosing musculature remain in the Convolutidae.

CLSM to visualize copulatory organ musculature is also being used to resolve the phylogeny of the Otocelididae. The diagnostic feature of the family is a characteristic of the reproductive system whereby the vagina is positioned posterior to the male copulatory organ, rather than anterior as in most other acoels. Characteristics of sperm morphology show the group to be polyphyletic (Petrov and others 2004), and our CLSM work has revealed the presence of 4 distinct types of copulatory organs (Hooge and Tyler 2005; M.D.H., unpublished data).

Microtubule arrangement of sperm

Acoel sperms are biflagellate, with the 2 axonemes of the flagella incorporated into the cell body. The arrangement of the microtubules in sperms can be visualized using TEM. Several distinct patterns of microtubules are present in the Acoela, and these patterns have proven useful for phylogenetic analyses (Raikova and others 2001; Hooge and others 2002; Petrov and others 2004).

The microtubule arrangement of axonemes in acoel sperm can be 9 + 2, as found in more basal taxa; 9 + 0, as found in derived species; or 9 + 1, as found in intermediately positioned species. Associated with the axonemes are additional microtubules that can be positioned along the cortex (cortical microtubules) or within the axis of the cell (axial microtubules). The

5 distinct patterns of sperm microtubule arrangements are concordant with molecular sequence data (Hooge and others 2002; Petrov and others 2004) and with other morphological characteristics (Hooge and Tyler 2005), and as such are an effective means of gauging the phylogenetic affinity of particular species.

Sagittocysts

Sagittocysts are complex, needle-shaped secretions of muscle-enwrapped glands that can be forcefully ejected for defense or for prey capture. The 15 species of acoels known to have sagittocysts are all grouped into the family Sagittiferidae on the basis of this synapomorphy (Achatz and others 2005).

Sagittocysts can usually be seen clearly using light microscopy for living specimens in squeeze preparations or in histological sections, but in larger sagittiferids containing numerous epithelial glands and algal endosymbionts, the sagittocysts are easily obscured. The sagittocysts of *Convolutriloba longifissura* were discovered only through viewing a phalloidin-stained specimen with CLSM (Gschwentner and others 1999). The sagittocyte that produces the sagittocysts is surrounded by a tightly spiraled muscle that contracts to eject the sagittocysts, and it is this muscle that is stained with phalloidin-linked fluorophores. The discovery of sagittocysts in *C. longifissura* was critical to understanding its phylogenetic affinities, as prior to this finding it was incorrectly assigned to the family Haploposthiidae. Further TEM work on sagittocysts by Gschwentner and colleagues (2002) revealed that the central filament of some species has a round profile in cross section whereas others are rectangular; these characteristics have potential to delineate the interrelationships of the Sagittiferidae.

Sclerotized sperm ducts

The bursal nozzle is another feature for which CLSM and TEM are providing new details to relate acoel taxa. A typical bursal nozzle is a rather prominent sclerotized spermatid duct that distributes sperm from the seminal bursa to the eggs. The substance responsible for sclerotizing the nozzle was unknown until phalloidin-treated acoels were observed to have strongly labeled bursal nozzles (Fig. 1C; Tyler and Rieger 1999; Hooge and Tyler 2001). By TEM it can be seen that the nozzles are not muscular but rather composed of stacks of cells with dense, F-actin-rich processes that form a tubular passage through which sperm pass on their way to the ovary (Petrov and others 2006).

In the current taxonomic system, the distribution of nozzles appears so haphazard as to lead to the

assumption that they had multiple independent origins. CLSM and TEM studies reveal common patterns and intermediates not easily detected by conventional histological procedures. In other words, nozzles are homologous among acoels.

An unusual type of sclerotized nozzle is found in several closely related species of the family Mecynostomidae, wherein the nozzle is positioned distally (between the bursa and the female gonopore) rather than proximally (between the bursa and the ovary). Using standard histological sectioning and TEM it is clear that this “vaginal nozzle,” despite being in a new position, has the same ultrastructure as a bursal nozzle (Petrov and others 2006). The vaginal nozzle may have arisen through a simple transformation series whereby an acoel ancestor with a bursa and bursal nozzle in their typical positions but without a female gonopore (a condition that does occur in some species of acoels) gave rise to a descendant with a gonopore connected to the distal end of the nozzle, thereby forming a secondary vagina with vaginal nozzle (Hooge and Tyler 2001; Petrov and others 2006).

Immunocytochemistry of nervous system

A variety of neurotransmitters, including 5-HT and FMRFamide, have been identified in the Acoela using immunocytochemistry. Among the 18 acoel species studied so far in this way (Raikova and others 1998, 2004; Reuter, Raikova, and Gustafsson 2001; Reuter, Raikova, Jondelius, and others 2001), these neurotransmitters show a surprising diversity in patterns, from quite simple submuscular nerve nets lacking any significant concentration that qualifies easily as a brain to more centralized systems with a well-differentiated brain sunk below the body wall. All the neurotransmitters studied so far reveal a submuscular nerve net with discernible major stems limited almost entirely to the anterior end of the body, commissures connecting these stems, and longitudinal nerve cords extending posteriorly in a variety of bilateral patterns.

Even among small groups of closely related taxa, such as species within the family Childiidae, the diversity of patterns can be high, and this diversity has been useful for gauging relationships within this family (Raikova and others 2004; Tekle and others 2005). Raikova (2004) suggests that despite a high level of homoplasy in nervous system characteristics, several hold promise for discerning relationships. Specifically, general brain shape (for example, ring-shaped, basket-shaped, barrel-shaped), as well as the number and distribution of large marker neurons immunoreactive to GYIRFamide (a flatworm-specific neurotransmitter), might provide sufficient indicators

for discerning interrelationships among higher acoel taxa.

Homology analysis

The characteristics revealed by the new microscopies are useful only to the extent that they are homologous among the taxa being compared. Before applying the characteristics to phylogenetic analysis of the Acoela, we screen them through criteria for homology, specifically those detailed by Rieger and Tyler (1979) and Tyler (1988). These criteria were vetted for applicability to microscopic features, which can have limitations in terms of holding sufficient information to constitute a phylogenetic signal.

Depending on the perceived information content and available data, certain of these criteria play more important roles for particular characteristics. For example, the copulatory organ of the Isodiametridae shows a strong probability of homology, especially by the first homology criterion: similarity in positional hierarchy of component parts. Homology of the vaginal nozzle with the standard bursal nozzle is probable not only by similarity in substructure of component parts—the actin-based sclerotization of stacked mesenchymal cells—but by the second homology criterion: connection through the transformation sequence we can perceive among several species that appear to represent stages in the evolution of the vaginal nozzle. Some characteristics that show limited information content, for example the presence of ocelli and endosymbionts in members of the Convolutidae, can still be gauged to represent probable homology by the third homology criterion: their coincidence with other homologues. The fourth homology criterion—similarity in functions of component parts of features (Tyler 1988)—can be examined using the potential for confocal microscopy in conjunction with TEM to elucidate functional morphology, for example in comparing characteristics of nozzles and of musculature.

Conclusions

Taxonomists of the Acoela have long recognized that the characteristics used to delimit supraspecific groups within this problematic taxon are not necessarily reflective of evolutionary relationships. Arriving at a better understanding of such relationships requires the discovery of new characteristics, and the newly available tools—new techniques of microscopy as well as new applications for judiciously chosen molecular sequence data—are providing some promising new characteristics. As this collection of new characteristics grows, so too does the promise for future cladistic

analyses of morphology. The Acoela is finally yielding the secrets of its relationships.

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