

Validating Livanow: Molecular Data Agree That Leeches, Branchiobdellidans, and *Acanthobdella peledina* Form a Monophyletic Group of Oligochaetes

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To investigate the phylogenetic relationships of leeches, branchiobdellidans, and acanthobdellidans, whole nuclear 18S rDNA and over 650 bp of mitochondrial cytochrome *c* oxidase subunit I were acquired from 101 annelids, including 36 leeches, 18 branchiobdellidans, *Acanthobdella peledina*, as well as 28 oligochaetes and combined with homologous data for 17 polychaete outgroup taxa. Parsimony analysis of the combined aligned dataset supported monophyly of leeches, branchiobdellidans, and acanthobdellidans in 100% of jackknife replicates. Monophyly of the oligochaete order Lumbriculida with Acanthobdellida, Branchiobdellida, and Hirudinea was supported in 84% of jackknife replicates. These results provide support for the hypotheses that leeches and branchiobdellidans are sister groups, that acanthobdellidans are sister to them, and that together with the family Lumbriculidae they all constitute a clade within Oligochaeta. Results support synonymy of the classes Clitellata and the more commonly used Oligochaeta. Leeches, branchiobdellidans, and acanthobdellidans should be regarded as orders equal to their closest relatives, the order Lumbriculida. © 2001 Elsevier Science

INTRODUCTION

Personal experiences with leeches can be as benign as a summer-time swimming inconvenience or as horrifying as attacks from the hordes of terrestrial leeches that are the scourge of tropical jungles. The past 15 years, however, have seen a dramatic increase in the biomedical utility of leeches (Sawyer, 1986), most notable being the promise of finding powerful anticoagulants and tumor-inhibiting antistasisins in leech sali-

vary secretions (Markwardt, 1985; Brankamp *et al.*, 1990). Already leeches are enjoying a renaissance in their application (quite literally) to postoperative hematomas. Their use routinely has led to faster healing and better prognosis following the reattachment of tissues with microsurgical procedures (Otto *et al.*, 1999; Cho and Ahn, 1999). It is surprising that until recently (Apakupakul *et al.*, 1999) there has not been a better understanding of the evolutionary origin of leeches concomitant with this renewed biomedical interest. Already it has been demonstrated that the ancestral leech was a blood feeder (Apakupakul *et al.*, 1999), suggesting that the closest relatives of leeches might be expected to have powerful anticoagulants of their own if the common ancestor was preadapted.

The origin of leeches and other symbiotic annelids has been and remains a hotly debated issue in annelid systematics. Leeches have a posterior sucker used for attachment to hosts, but leeches are not the only annelid worms with this attachment organ. The tiny branchiobdellidans also are equipped with a caudal sucker. Rather than feeding on vertebrate blood, branchiobdellidans are found predominantly on freshwater crayfish. Another attribute that indicates the relatedness of the leeches, oligochaetes, and branchiobdellidans is their possession of a clitellum, an organ involved in egg-laying that is found about one-third of the way down the body. Moreover, they are all hermaphrodites. What has been controversial is their relative position in the class Clitellata. Odier (1823) first proposed that branchiobdellidans were closely related to leeches. Livanow (1931) agreed and also suggested that the unusual ectoparasite of salmon, *Acanthobdella peledina*, shared a recent common ancestor with branchiobdellidans and leeches. Various attempts to resolve this question using morphological data have suggested independent origins for these groups (Brinkhurst and Gelder, 1989; Holt, 1989), maintain-

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ing the traditional notions of Oligochaeta, Hirudinea, and Branchiobdellida. Two separate assessments of this question with contemporary cladistic methods applied to morphological data (Brinkhurst, 1994; Purschke *et al.*, 1993) both suggested that leeches and branchiobdellidans are each other's closest relatives, but derived from lumbriculid oligochaetes. Remarkably, when faced with this result, in both instances the authors rejected their own findings, asserting that the result was erroneous and due to a high degree of convergence among animals adapted to an ectosymbiotic way of life. Since then, the debate between those arguing in favor of the evidence (Siddall and Bureson, 1996) and those arguing against (Brinkhurst, 1999) has been a fixture at international symposia.

MATERIALS AND METHODS

Many of the 18S rDNA and COI sequences used in this study have been reported elsewhere for phylogenetic analyses of leeches (Siddall and Bureson, 1998; Apakupakul *et al.*, 1999; Trontelj *et al.*, 1999), branchiobdellidans (Gelder and Siddall, 2001), and oligochaetes (Christensen and Theisen, 1998; Nylander *et al.*, 1999; Erséus *et al.*, 2000; Martin *et al.*, 2000) or were otherwise available in GenBank (U67324, U50972, X79873, U67143, U67323, U67142).

Taxa new to this study are the polychaetes *Eurythoe complanata* (AY040685), *Notomastus latericeus* (AY040697), *Eunice pennata* (AY040684), *Nainereis laevigata* (AY040696), *Armandia maculata* (AY040681), *Marphysa sanguinea* (AY040695, AY040708), *Pectinaria regalis* (AY040698), *Loimia medusa* (AY040690, AY040704), as well as a yet to be identified maldanid and cirratulid (AY040682, AY040694, AY040707), the oligochaetes *Grania americana* (AY040686), *Heterodrilus keenani* (AY040688, AY040703), *Monopylephorus rubroniveus* (AY040709), *Uncinails uncinata* (AY040700), *Spirosperma ferox* (AY040699), *Limnodriloides lateroporus* (AY040689), *Ecliplidrilus frigidus* (AY040692 AY040706), a species of *Trichidrilus* (AY040691, AY040705), an *Enchytraeus albidus*-like species from Bermuda (AY040683), and the leech *Haemopsis caeca* (AY040687, AY040702). Additionally, sequences for one or the other of the two genes used are new. Significantly, new sequences were obtained for 18S rDNA and COI for *A. peledina* and *Lumbriculus variegatus* (AY040680, AY040701, AY040693) following our own determinations that accession numbers AF003257, AF003264, and AF115978 were contaminants not recognized by us earlier (Siddall and Bureson, 1998; Apakupakul *et al.*, 1999). For all of the foregoing, DNA extractions were accomplished with the DNeasy tissue kit (QiaGen Inc., Valencia, CA) or through standard proteinase K, phenol-chloroform extractions. PCR amplification and sequencing of 18S rDNA and COI were performed as

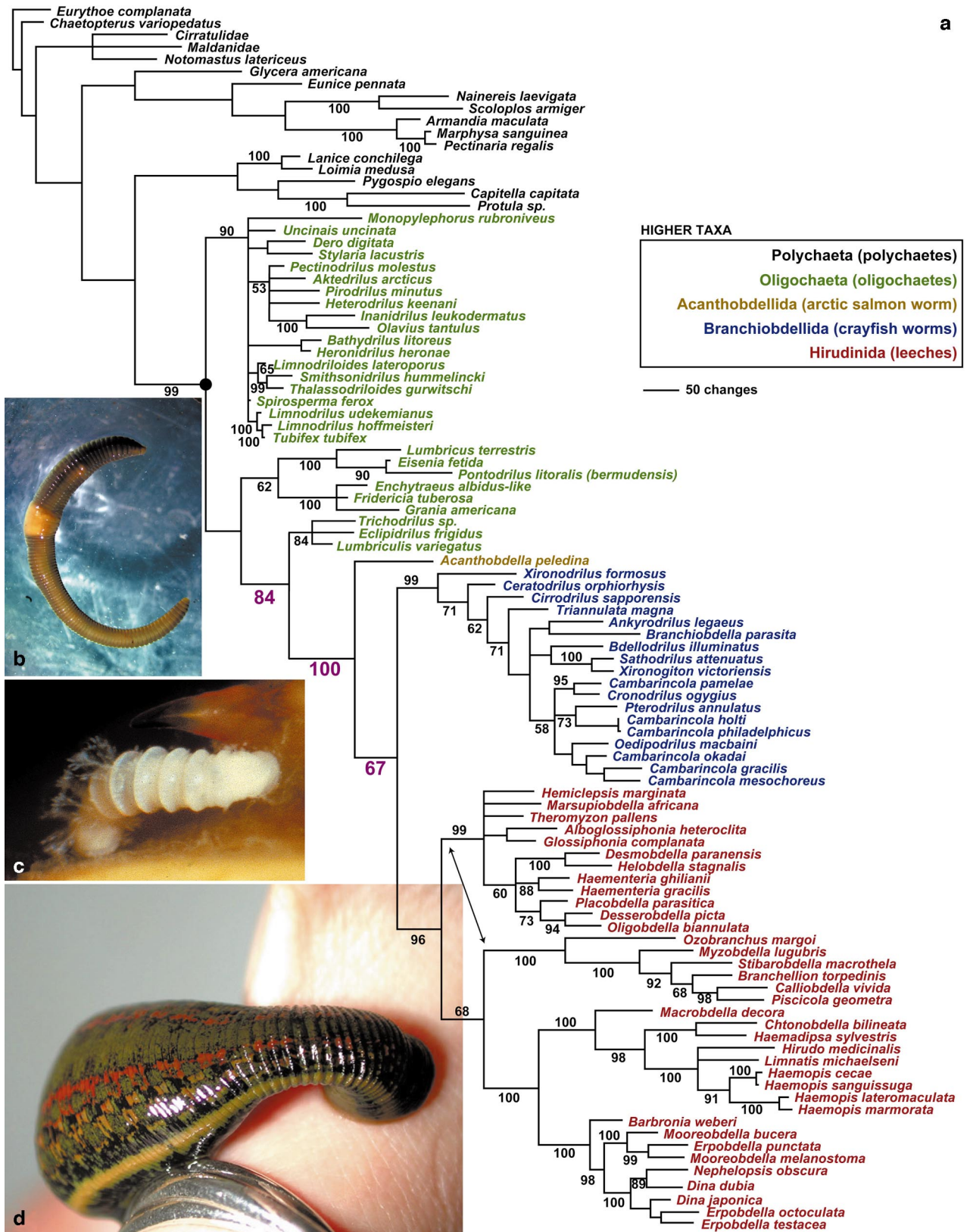
described elsewhere (Apakupakul *et al.*, 1999). Amplification products were sequenced in both directions and reconciled with Sequence Navigator (Applied Biosystems).

Faced with this taxonomically large data set of nuclear 18S rDNA, we used the American Museum of Natural History's parallel computing cluster to search for optimal alignments for phylogenetic analyses. This cluster of 256 Pentium III processors operates in Linux using Parallel Virtual Machine. A parallel version of the multiple alignment software Malign (Wheeler and Gladstein, 1996) was employed with a battery of alignment parameters varying transition:transversion:indel cost ratios and choosing that alignment which returned the shortest number of steps. Multiple alignments of 18S rDNA then were combined with cytochrome *c* oxidase subunit I data (for which there were no indels) for available taxa.

Phylogenetic analyses employed 20 replicates of the simulated annealing "ratchet" (Nixon, 1999) in the software package Nona (Goloboff, 1999). Sensitivity of parsimony estimates to character information was assessed with parsimony jackknife analyses using 200 replicates of each of 5 random additions with branch swapping (i.e., "200*/5") conducted with Xac (Farris, 1999). Likelihood analyses and Kishino-Hasegawa tests (Kishino and Hasegawa, 1989) were conducted in PAUP* (Swofford, 1999) using a Jukes-Cantor model since overall base compositions were approximately equal and the transition:transversion ratio was only 1.2. All sequences and the alignment are available at <http://research.amnh.org/~siddall/>.

RESULTS

In the face of arguments that morphological data may be subject to selective constraints and convergence to a symbiotic lifestyle (Purschke *et al.*, 1993; Brinkhurst, 1994, 1999), we acquired whole nuclear 18S rDNA and over 650 bp of mitochondrial cytochrome *c* oxidase subunit 1 from 101 annelids, including 36 leeches, 18 branchiobdellidans, *A. peledina*, and 28 oligochaetes and combined this with homologous data for 17 polychaete outgroup taxa. Use of 18S rDNA alone yielded >5000 equally parsimonious trees of length 4957 and retention index 0.72. Use of COI alone resulted in 24 equally parsimonious trees (length = 5191, retention index = 0.46). Parsimony analysis of the combined aligned dataset resulted in 60 equally optimal trees with 10,353 steps and a retention index of 0.60. The consensus (Fig. 1a) places the Branchiobdellida (Jac = 99) and Hirudinea (Jac = 96) together in a monophyletic group (Jac = 67). Monophyly of leeches, branchiobdellidans, and *A. peledina* was supported in 100% of parsimony jackknife replicates. Monophyly of Lumbriculidae with Acanthobdellida, Branchiobdellida, and Hirudinea was supported in 84% of parsi-



mony jackknife replicates. The least well-resolved clitellate group was that comprising the 19 naidids and tubificids. Enchytraeidae and Lumbricidae (each with three taxa) were each other's closest relatives (Jac = 62). Use of maximum likelihood with a Jukes–Cantor model yielded one tree with a negative log likelihood of 59,323.27. This tree differed from the consensus of most parsimonious trees only in the relative placement of the leech families Piscicolidae and Glossiphoniidae (Fig. 1a), but was not significantly different from the most parsimonious tree topologies (difference in negative log likelihood was less than 37 in comparisons against the 60 most parsimonious trees, $P > 0.20$ in Kishino–Hasegawa tests).

DISCUSSION

The implications of this phylogenetic analysis are far-reaching. Our results suggest that the systematics of clitellate annelids should be revised. The class Clitellata clearly is synonymous with the more commonly used class Oligochaeta. We believe that leeches, branchiobdellidans, and acanthobdellids should be regarded as orders (Hirudinida, Branchiobdellida, and Acanthobdellida, respectively) within whichever of those class names survives and on equal footing with their closest relatives, the order Lumbriculida (so long as the latter remains monophyletic).

The close relationship between branchiobdellidans and leeches is not surprising, nor is the relationship for *A. peledina* nor that for the lumbriculids as sister to these. Each of these has been argued on morphological grounds for quite some time (Ocier, 1823; Livanow, 1906, 1931; Sawyer, 1986; Siddall and Bureson, 1996; Martin *et al.*, 2000). In particular, Livanow (1931) postulated that branchiobdellidans are sister to leeches, and *Acanthobdella* sister to those, precisely as determined in our analyses. Moreover, phylogenetic analyses using morphological characters alone have supported these views (Purschke *et al.*, 1993; Brinkhurst, 1994; Siddall and Bureson, 1996). However, both Purschke *et al.* (1993) and Brinkhurst (1994) repudiated the results of their *own* phylogenetic analyses. That is, all of the morphological synapomorphies uniting *Acanthobdella*, Branchiobdellida, and the Hirudinea in these analyses were rejected by those authors as spurious and due solely to convergent morphological changes resulting from massive parallelism in light of the ectosymbiotic life-style of these three taxa.

The view contrary to monophyly of leeches, branchiobdellidans, and *Acanthobdella* was outlined first by Michaelsen (1919), then by Holt (1965), and eventually more vehemently by Holt (1989) and by Brinkhurst and Gelder (1989). The arguments for convergence were premised on the lack of absolute correspondence among the various morphological structures proposed as synapomorphies. Nonetheless, these same arguments implicitly deny the notion that ectosymbiosis may have been inherited from a common ancestor. Putative synapomorphies for leeches, branchiobdellidans, and *Acanthobdella* include the constant number of body somites, reduction or loss of chaetae, reduction of the coelom, and, most strikingly, the possession of a caudal sucker for attachment. However, it has been argued (Holt, 1989; Brinkhurst and Gelder, 1989) that the number of somites differs. There are 34 in the hirudinids, 27 in *Acanthobdella*, and only 15 in branchiobdellidans. Chaetae, though absent in branchiobdellidans, are still present in *Acanthobdella* in which there are 5 cephalic sets used for attachment (Dahm, 1962). Although the coelom is reduced both in *Acanthobdella* and in leeches, the former retains septa; and as for the posterior sucker, it has been argued that this structure in branchiobdellidans is not a sucker at all but rather an “attachment disc” or a “duo-adhesive organ” (Weigl, 1980; Gelder and Rowe, 1988) distinct from the muscular attachment organ in leeches (Holt, 1989; Brinkhurst and Gelder, 1989; Purschke *et al.*, 1993; Brinkhurst, 1994, 1999). Mitigating against these interpretations is the strong correspondence between histological microscopic observations of Weigl (1994) on the caudal attachment organ of branchiobdellidans and those of Appy and Cone (1982) for a piscicolid leech (see also Sawyer, 1986). Both have a similar muscular structure and both have two types of subepidermal secretory cells, one being fuchsinophilic and the other with strongly PAS-positive granules. As well, though *Acanthobdella* is unlike most leeches in having intersomatic septae, so do leeches in the genus *Ozobranchus* (cf., MacCallum and MacCallum, 1918: Plate 38, Fig. 1). Holt (1965) suggested that all three of these ectosymbiotic annelids were independently derived, though from what in particular he did not specify.

We excluded morphological data from our analyses because of stipulations that *Acanthobdella*, branchiobdellidans, and leeches would only group together because of convergence. We are in no sense adverse to the

difference in the optimal likelihood solution was a swapping of the placement of the glossiphoniid leeches and the piscicolids (arrows). This solution indicates paraphyly of Oligochaeta from within which are derived as an ectocommensalistic monophyletic group, the branchiobdellidans, the acanthobdellidans, and the leeches. This supports the notion that an oligochaete like the earthworm (in b), and in particular like the lumbriculid mud-worm, was ancestral to branchiobdellidans like *Xironogiton victoriensis* (c) seen feeding from the surface of a crayfish and of leeches like *Hirudo medicinalis* (d) here feeding on human blood. Numbers at nodes are the frequencies with which each clade was found in 1000 parsimony jackknife replicates (200 iterations of 5 random additions with branch swapping).

inclusion of these data, and elsewhere some of us have argued strongly that morphology had solved the matter long ago (e.g., Siddall and Bureson, 1996). Rather, we used molecular data alone because it would be difficult for others to suggest that 18S rDNA or COI would exhibit convergence as a result of ectosymbiotic lifestyles.

In addition to the morphological synapomorphies outlined (but denied) by Purschke *et al.* (1993) and others, there has been growing corroboration from sperm ultrastructure characters of a close relationship for *Acanthobdella*, leeches, and branchiobdellidans, and then these too with lumbriculids. Jamieson *et al.* (1987) were perhaps the first to note that sperm ultrastructure placed leeches within Oligochaeta. Franzén (1991) noted a correspondence between leeches and *A. peledina* in their possession of a long helical nucleus and a corkscrew-shaped acrosome with an anterior extension (see also Westheide and Purschke, 1996). Ferraguti and Lanzavecchia (1977) already had noted the twisted acrosome in lumbriculids, as then did Ferraguti *et al.* (1999) and Ferraguti and Erséus (1999). In addition to the foregoing, Ferraguti and Erséus (1999) pointed to the coiled nuclear fibers, the single mitochondrion, and the elongation of the midpiece as shared derived characters for *Acanthobdella* and leeches. Their phylogenetic analysis of sperm ultrastructure supported a close relationship for the branchiobdellidans as well, but they admitted this was complicated by the highly derived nature of their spermatozoa (Ferraguti and Erséus, 1999). Later, Ferraguti (2000) added the synapomorphy of a "multiple start" corkscrew nucleus, thereby distinguishing these taxa from oligochaetes with twisting symmetries.

A prior molecular analyses of the Clitellata (Martin *et al.*, 2000) visited the question of leech, branchiobdellidan, acanthobdellid, and lumbriculid relationships. Martin *et al.* (2000) suggested that their results from 18S rDNA were confounded by rapidly evolving lineages in the Clitellata. This analysis, however, only included 15 taxa, 2 of which were leeches and 2 branchiobdellidans. Although their analyses of COI included 49 taxa and did provide some support for monophyly of the ectosymbiotic worms, they arrived at the unusual hypothesis of monophyly of *Acanthobdella* and the branchiobdellidans. Part of the difficulty in these previous analyses was due to some of us (Siddall and Bureson, 1998; Apakupakul *et al.*, 1999) having placed incorrectly identified sequences in GenBank for *A. peledina* and for *Lumbriculus variegatus*. With the revised sequences from fresh material and 83 ingroup taxa which includes critically important representatives from the Lumbriculida, the earlier misgivings of Martin *et al.* (2000) appear unwarranted. Rather than being indicative of poor resolution due to long branches or rapid radiation, those earlier difficulties appear to have arisen from low or uneven taxon sampling, a

critical issue in optimizing ancestral states in phylogenetic analyses (Graybeal, 1998).

The combined molecular data of the nuclear 18S ribosomal DNA gene and the mitochondrial cytochrome *c* oxidase I gene, independently reflecting evolutionary history, provide robust support for the hypotheses that leeches (Hirudinida) and Branchiobdellida indeed are sister groups and that these together with *A. peledina* and the order Lumbriculida constitute a clade within the Oligochaeta. The same data also support, even more strongly, that Acanthobdellida is sister to Hirudinida + Branchiobdellida—a view proposed already by Livanov (1931). Additional taxon sampling will reveal whether Lumbriculida is monophyletic or paraphyletic, which would then suggest that the ancestor of the ectosymbiotic worms was itself a lumbriculid oligochaete.

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