Supporting Information

Bely and Sikes 10.1073/pnas.0907931107

SI Materials and Methods

Animal Identification and Culture. Naidines were identified using a standard key (1), and cultures were established for most species. Species of *Allonais*, *Dero*, *Nais*, *Pristina*, and *Stylaria* were cultured at room temperature in bowls of artificial spring water (1% artificial seawater) with brown paper towel as substrate and powdered "Spirulina" (the cyanobacterium *Arthrospira platensis*) or cracked wheat grains as food. The carnivorous species *Chaetogaster diaphanus* was fed cut pieces of other worms (typically *Allonais paraguayensis*). Species of *Amphichaeta* (*A. "raptisae"* C), *Arcteonais*, *Monopylephorus*, *Paranais*, and *Tubifex* were cultured at 15°C in sand (*Tubifex*) or mud (other species; mud previously sieved and frozen) in artificial spring water (*Monopylephorus*, *Tubifex*), ~10% (3 parts per thousand [ppt]) artificial seawater (*Arcteonais*), or ~30% (10 ppt) artificial seawater (*Amphichaeta*, *Paranais*).

Comparative Regeneration Experiments. Specimens were obtained from actively growing laboratory cultures or, in the few cases where field-collected animals had to be used, within 2 days of collection. For anterior amputation, we removed all head (cephalic) segments, which are the segments formed during each round of fission. For posterior amputation, we amputated one or two segments anterior to the anterior-most fission zone, ensuring the removal of fission zones that could interfere with regenerative processes. If no fission zone was visible in a particular individual, we cut two segments anterior to the anterior-most fission zone characteristic of that species. For *Allonais paraguayensis*, which does not form a visible fission zone, and the outgroups *Tubifex tubifex* and *Lumbriculus variegatus*, we amputated the head segments or the posterior third of the animal. Two replicate experiments, performed at different times and by different experimenters, were performed for nearly all species. Regeneration progress was monitored at least every 1–2 days for a minimum of 10 days, roughly twice the time required for full regeneration in most naidines.

Worms were amputated with a scalpel after brief anesthetization (1–5 min in 0.05–0.10 mM nicotine in culture water); control uncut worms were anesthetized for comparable periods. Worms were maintained individually, unfed, in ~2 mL culture water at room temperature for the duration of the trial, with partial water changes every 2–4 days. Wound healing was scored as complete when the wound appeared sealed by an intact epithelium rather than by muscular contraction. The blastema stage was recognized by the formation of a visible, clear mass at the wound site. Regeneration was scored as complete once chaetae were seen emerging from the new segments.

The absence of head regeneration in *Pa. litoralis*, *C. diaphanus*, and *A. "raptisae"* C was confirmed through additional trials performed under a broad set of conditions, including trials in which only young worms without fission zones were used, worms were amputated without anesthesia, amputees were maintained at a lower temperature (15°C), cuts were made at a range of body locations, or worms were maintained until death.

Phylogenetic Analysis: Naidine Phylogeny. For both Bayesian and maximum likelihood analyses, we partitioned our data into five subsets (COI, 28S, 12S, 16S, and 18S) and used MrModeltest 2.3 (2), a modified version of Modeltest 3.6 (3), to select a best-fit nucleotide substitution model for each subset. For COI, 28S, and 16S, both the Akaike information criterion (AIC) and hierarchical likelihood-ratio tests (hLRTs) indicated as the best fit a general time reversible (GTR) substitution model with a γ-distribution of rates of change for evolving sites (G) and a fraction of invariant sites (I). For 12S, both AIC and hLRTs indicated GTR + G as the best-fit model; for 18S, AIC indicated GTR + G + I, but hLRTs indicated the slightly simpler model SYM (4) + G + I as the best fit. Given the established advantages of AIC over hLRTs for phylogenetics (5, 6), in our analyses we used the GTR + G model for 12S and GTR + G + I for the remaining four partitions.

For our Bayesian analysis, we used MrBayes 3.1.2 (7, 8), a program that implements a Markov chain Monte Carlo (MCMC) approach to approximate the posterior probabilities of phylogenetic trees and clades. Each run included one cold chain along with three heated chains (to facilitate a more efficient search of tree space), with a tree sampled every 100 generations. We ran two simultaneous, completely independent analyses starting from different random trees. We discarded as burn-in all generations preceding the convergence of these two independent runs. Convergence was determined as the point at which the standard deviation of split frequencies dropped below 0.01. As further assurance that our runs had reached stationarity, we also verified that the potential scale reduction factor was reasonably close to 1.0 for all parameters.

For our partitioned maximum likelihood analysis, we used a prerelease version of GARLI, which implements a genetic algorithm for rapid likelihood inference, provided by Derrick Zwickl (version 0.96 revision 323, 2008) (9). We used the same basic substitution models for each partition as in the Bayesian analysis, with six search replicates (to minimize the possibility of the search being trapped at local optima); other settings were left at default values.

Voucher specimens were preserved from the same asexual strain or the same sampling effort as specimens used for DNA extraction.

Phylogenetic Analysis: *nanos*. *Paranais litoralis* and *Pr. leidyi* sequences were aligned to other *nanos* sequences (obtained from GenBank) using Clustal X (v. 1.83) (10). We analyzed the zinc-finger domain (54 amino acids) with Mr. Bayes (v. 3.1.2) (7, 8), using MCMC model estimation (posterior probabilities: Vt model, 0.989; Dayhoff, 0.009; WAG, 0.002). The burn-in phase was determined by the convergence of two simultaneous independent runs, and these burn-in samples were excluded. The consensus tree was displayed in FigTree (11).

BrdU-Labeling. Following head amputation, individuals were incubated in BrdU (0.1 mg/mL; 18-h pulse) on day 1 [1–19 h post amputation (hpa)], day 2 (24–42 hpa), or day 3 (48–66 hpa). During FZ-regeneration, individuals were incubated in BrdU (0.1 mg/mL; 18-h pulse) on day 4. Immediately following the BrdU pulse, worms were fixed 30 min in 4% formaldehyde in PBS, washed in PBS and then in PBTx (PBS + 0.1% Triton-X), incubated in 75% HCl (37°C, 30 min), washed in PBTx, blocked in 10% normal goat serum (NGS) in PBTx (1 h, room temperature), and incubated in mouse anti-BrdU monoclonal antibody (Sigma) at 1:100 in 10% NGS/0.9× PBTx overnight at 4°C. Samples were washed repeatedly in PBTx over 1 h, incubated in an HRP-conjugated goat anti-mouse antibody (Jackson Immunoresearch) at 1:200 in 10% NGS/0.9× PBTx overnight at 4°C, and washed multiple times in PBTx and finally in PBS. Samples were mounted in 70% glycerol.

Tubulin/Serotonin/Phalloidin-Labeling. Worms were triple labeled for acetylated tubulin (which labels cilia and the peripheral

nervous system), for serotonin (which labels parts of the central nervous system), and with phalloidin (which labels F-actin of muscle fibers). Specimens were relaxed 10 min in cold (4°C) relaxant solution (12), fixed 30 min in 4% formaldehyde in PBS, and washed in PBS. Fixed specimens were permeabilized in PBTx, blocked 1 h in 10% NGS in PBTx, and incubated at 4°C for 15–20 h with mouse anti-acetylated α-tubulin monoclonal (Sigma) and rabbit anti-serotonin polyclonal antibodies (Sigma) diluted 1:100 in blocking solution. Specimens were washed in PBTx and incubated at room temperature for 3–8 h in blocking solution containing FITC-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch) diluted 1:200, Alexa-Fluor-546-conjugated goat anti-rabbit antibodies (Invitrogen) diluted 1:200, and 66 nM Alexa-Fluor-647-conjugated phalloidin (Invitrogen). Specimens were then washed with PBTx and then with PBS, transferred through graded glycerol series (33%, 50%, and 75% glycerol in PBS), and mounted in Fluoromount-G.

Confocal Imaging of Tubulin/Serotonin/Phalloidin-Labeled Specimens. Three-channel imaging was performed using a Zeiss LSM-510 confocal microscope, with sequential acquisition of images using 488 nm (Ar), 543 nm (HeNe), and 633 nm (HeNe) laser lines. Maximum-intensity Z-projections were created from stacks for each channel and subsequently merged using ImageJ. To obtain a true lateral view of the FZ-regenerated head (Fig. 4*H*), the stack for this image was 3D-rotated 5° using ImageJ before Z-stack projection. The *inset* of the chaetal bundle in Fig. 4*H* was generated by making a maximum projection including only optical sections containing this chaetal bundle. Image stitching and level curve optimization for each channel were performed in Adobe Photoshop.

1. Kathman RD, Brinkhurst RO (1998) *Guide to the Freshwater Oligochaetes of North America* (Aquatic Resources Center, College Grove, TN).
2. Nylander JAA (2004) *MrModeltest v2 Program* (Evolutionary Biology Center, Uppsala University). Program distributed by the author.
3. Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–818.
4. Zharkikh A (1994) Estimation of evolutionary distances between nucleotide sequences. *J Mol Evol* 39:315–329.
5. Posada D, Buckley TR (2004) Model selection and model averaging in phylogenetics: Advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. *Syst Biol* 53:793–808.
6. Pol D (2004) Empirical problems of the hierarchical likelihood ratio test for model selection. *Syst Biol* 53:949–962.
7. Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754–755.
8. Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574.
9. Zwickl DJ (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. PhD dissertation. The University of Texas, Austin.
10. Larkin MA, et al. (2007) ClustalW and ClustalX v 2.0. *Bioinformatics* 23:2947–2948.
11. Rambaut A (2009) *FigTree v1.2.2* (Institute of Evolutionary Biology, University of Edinburgh).
12. Bely AE, Wray GA (2001) Evolution of regeneration and fission in annelids: insights from *engrailed*- and *orthodenticle*-class gene expression. *Development* 128: 2781–2791.

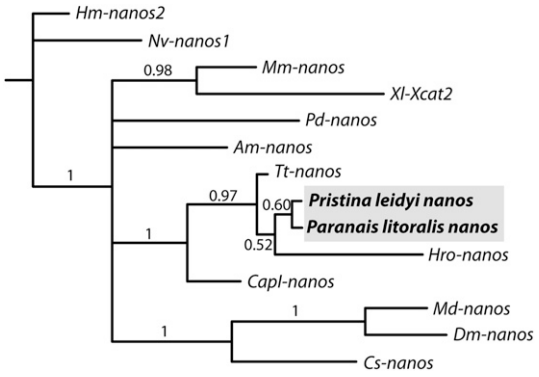

Fig. S1. Phylogenetic position of *Pr. leidyi* and *Pa. litoralis nanos* sequences. The tree is from a Bayesian analysis of the zinc-finger domain; numbers above nodes are posterior probabilities. GenBank sequences are from the annelids *Tubifex tubifex* (*Tt-nanos*, BAD90110), *Helobdella robusta* (*Hro-nanos*, AAB63111), *Capitella* sp. I (*CapI-nanos*, DAA06318), and *Platynereis dumerilii* (*Pd-nanos*, CAJ28985); the arthropods *Drosophila melanogaster* (*Dm-nanos*, NP-476658), *Chironomus samoensis* (*Cs-nanos*, AAA87459), *Apis mellifera* (*Am-nanos*, NP-001035321), and *Musca domestica* (*Md-nanos*, AAA87461); the vertebrates *Xenopus laevis* (*Xl-Xcat*, NP-001081503) and *Mus musculus* (*Mm-nanos*, NP-848508); and the cnidarians *Hydra magnipapillata* (*Hm-nanos2*, BAB01492) and *Nematostella vectensis* (*Nv-nanos1*, AAW29070).

Table S1. Collection information for species included in comparative regeneration experiments

Species	Collection site or source*
Naidines	
Allonais paraguayensis	Ward's Natural Science (sold as *Stylaria*)
Amphichaeta "raptisae" B	Paint Branch, University of Maryland, College Park, MD
Amphichaeta "raptisae" C	Rhode River, Smithsonian Environmental Research Center, Edgewater, MD
Arcteonais lomondi	Constitution Marsh, Hudson River, Cold Spring, NY
Chaetogaster diaphanus	Edwards Lake, University of Maryland, College Park, MD
Chaetogaster diastrophus	Paint Branch, University of Maryland, College Park, MD
Dero sp. 1	Edwards Lake, University of Maryland, College Park, MD
Dero furcata	Connecticut Valley Biological Supply
Nais communis	Fort Defiance State Park, IA
Nais elinguis	Paint Branch, University of Maryland, College Park, MD
Paranais frici	Rhode River, Smithsonian Environmental Research Center, Edgewater, MD
Paranais litoralis	Rhode River, Smithsonian Environmental Research Center, Edgewater, MD
Piguetiella michiganensis	Goose Creek, Middleburg, VA
Pristina aequiseta	Paint Branch, University of Maryland, College Park, MD
Pristina leidyi	Carolina Biological Supply (sold as *Stylaria*)
Slavina appendiculata	Paint Branch, University of Maryland, College Park, MD
Specaria josinae	Goose Creek, Middleburg, VA
Stylaria lacustris	Paint Branch, University of Maryland, College Park, MD
Vejdovskyella sp. 1	Constitution Marsh, Hudson River, Cold Spring, NY
Outgroups	
Tubifex tubifex	Western Fisheries Research Center, USGS, Sand Point, Lake Washington, WA (supplied by C. Rasmussen)
Lumbriculus variegatus	Carolina Biological Supply

*All field collections were made by A.E.B. or J.M.S. unless otherwise noted.

Table S2. Amputation positions and number of regenerated segments in comparative regeneration experiments

Species	Anterior regeneration		Posterior regeneration	
	No. of segments removed*	No. of segments regenerated†	No. of segments retained‡	Maximum no. of segments regenerated
Naidines				
Allonais paraguayensis	4	4	~12	13
Amphichaeta "raptisae" B	2	0	~7	0
Amphichaeta "raptisae" C	2	0	~7	2
Arcteonais lomondi	4	4	~20	13
Chaetogaster diaphanus	4	0	~8	12
Chaetogaster diastrophus	4	0	~5	0
Dero sp. 1	4	4	~24	10
Dero furcata	3	3	~17	15
Nais communis	4	4	~12	4
Nais elinguis	4	4	~13	10
Paranais frici	3	0	~9	10
Paranais litoralis	3	0	~12	8
Piguetiella michiganensis	4	4	~19	6
Pristina aequiseta	6	4	~10	7
Pristina leidyi	6	4	~12	7
Slavina appendiculata	4	4	~10	13
Specaria josinae	4	4	~14	15
Stylaria lacustris	4	4	~13	5
Vejdovskyella sp. 1	2	2	~10	1
Outgroups				
Tubifex tubifex	3	1	~32	3
Lumbriculus variegatus	8	8	~28	16

*The number of anterior segments removed is the number of head segments in that species.

†Species typically regenerated a fixed number of head segments (as is common for annelids), and this number is reported here. For nearly all species, this typical number matched the number of segments removed. However, in both *Pristina* species the number of head segments that typically regenerated (4) was less than the number of head segments (6). In addition, a small fraction of individuals in four species regenerated one or two head segments more than the typical number: *D. furcata* (four or five segments), *Nais communis* (five segments), *Pristina aequiseta* (five or six segments), and *Pristina leidyi* (five segments).

‡Posterior amputation locations in naidines varied slightly across individuals because cuts were made relative to the fission zones, which can vary in position.

Table S3. Comparative regeneration experiment sample sizes and outcomes

Species	Anterior regeneration				Posterior regeneration			
	No. cut	No. with blastema*	No. completely regenerated†	Median days to death‡	No. cut	No. with blastema*	No. completely regenerated†	Median days to death‡
Naidines								
Allonais paraguayensis	20	20	20	—	20	20	20	—
Amphichaeta "raptisae" B	10	0	0	2	10	0	0	3
Amphichaeta "raptisae" C	10	0	0	3	10	1	1	7
Arcteonais lomondi	5	5	5	—	5	5	5	—
Chaetogaster diaphanus	22	0	0	—	22	3	3	—
Chaetogaster diastrophus	12	0	0	4	12	0	0	7
Dero sp. 1	15	15	15	—	15	15	15	—
Dero furcata	12	12	12	—	12	10	10	—
Nais communis	16	12	12	—	16	12	10	—
Nais elinguis	16	9	8	6	16	12	12	8
Paranais frici	16	0	0	5	16	5	5	7
Paranais litoralis	23	0	0	6	23	12	12	6
Piguetiella michiganensis	9	9	7	7	9	7	5	8
Pristina aequiseta	10	8	8	—	10	10	10	—
Pristina leidyi	20	20	20	—	20	20	20	—
Slavina appendiculata	10	10	10	—	10	10	10	—
Specaria josinae	7	7	7	—	7	7	7	—
Stylaria lacustris	10	10	10	—	10	10	10	8
Vejdovskyella sp. 1	7	3	3	4	7	4	2	6
Outgroups								
Tubifex tubifex	12	3	3	3	12	12	12	—
Lumbriculus variegatus	10	10	10	—	10	10	10	—

*This number is the total number of individuals that formed a detectable blastema during the course of the 10-d experiment, as evaluated by live observations of specimens using a dissecting microscope (×63 magnification). Individuals that were cut but that did not form a blastema either died or remained alive but without progressing beyond wound healing. Some individuals that formed a blastema went on to regenerate completely ("No. completely regenerated"); the remainder either died or stalled in the regeneration process.

†The total number of individuals that regenerated all missing structures (including segments) by the end of the experiment (10 dpa), as evaluated by live observations of specimens using a dissecting microscope (×63 magnification).

‡No median is reported (indicated as "—") if more than half of the individuals were alive at the end of the experiment (10 dpa).

Table S4. Species and GenBank accession numbers for the five-gene data matrix used in the phylogenetic analysis

Species*	COI	12S	16S	18S	28S
Allonais paraguayensis	AF534828	GQ355380	GQ355399	GQ355423	GQ355439
Amphichaeta "raptisae" A	AF534829	—	GQ355400	GQ355438	GQ355440
Amphichaeta "raptisae" B	GQ355365	—	GQ355402	GQ355424	GQ355442
Amphichaeta "raptisae" C	—	—	GQ355401	—	GQ355441
Arcteonais lomondi	AF534830	—	—	—	—
Chaetogaster diaphanus	GQ355366	GQ355381	GQ355403	GQ355425	GQ355443
Chaetogaster diastrophus	GQ355367	—	GQ355404	—	GQ355444
Chaetogaster limnaei	AF534834	GQ355382	GQ355405	—	—
Dero furcata	AF534837	GQ355383	GQ355406	—	GQ355445
Dero sp. 1	GQ355368	GQ355384	GQ355407	GQ355426	GQ355446
Nais bretscheri	AF534843	—	—	—	GQ355447
Nais communis	AF534845	GQ355385	GQ355408	GQ355427	GQ355448
Nais elinguis	GQ355369	GQ355386	GQ355409	GQ355428	GQ355449
Nais variabilis A	AF534844	—	—	—	GQ355450
Nais variabilis B	GQ355370	GQ355387	GQ355410	—	—
Ophidonais serpentina	AF534846	GQ355388	GQ355411	GQ355429	GQ355451
Paranais frici	GQ355371	GQ355389	GQ355412	—	GQ355452
Paranais litoralis	GQ355372	GQ355390	GQ355413	GQ355430	GQ355453
Piguetiella michiganensis	GQ355373	GQ355391	GQ355414	GQ355431	GQ355454
Pristina aequiseta	GQ355374	GQ355392	GQ355415	GQ355432	GQ355455
Pristina leidyi	AF534853	GQ355393	GQ355416	GQ355433	GQ355456
Pristina osborni	AF534855	—	GQ355417	—	GQ355457
Ripistes parasita	AF534856	—	—	—	GQ355458
Slavina appendiculata†	GQ355375	GQ355394	GQ355418	GQ355434	GQ355459
Specaria josinae	GQ355376	GQ355395	GQ355419	—	GQ355460
Stylaria lacustris†	AF534861	GQ355396	GQ355420	GQ355435	GQ355461
Vejdovskyella sp. 1	GQ355377	GQ355397	GQ355421	—	GQ355462
Vejdovskyella sp. 2	GQ355378	—	—	—	GQ355463
Monopylephorus rubroniveus	GQ355379	—	GQ355422	GQ355436	GQ355464
Tubifex tubifex†	AF534866	GQ355398	AF326001	GQ355437	GQ355465
Branchiura sowerbyi†	AF534864	DQ459924	DQ459957	DQ459985	—
Lumbriculus variegatus†	AY519464	DQ459885	AY885578	AF209457	GQ355466

*Species collection localities are provided in GenBank files in the "Country" field.
†Not all gene sequences for this species originate from the same isolate.

Table S5. FZ-regeneration success in *Pa. litoralis* during two replicate 14-day experiments

	Structures regenerated (day first detected)				
	Blastema*	Prostomium†	Mouth†	Chaetae†	Death (day)‡
Experiment 1					
Individual 1	—	—	—	—	8
Individual 2	5	8	9	9	—
Individual 3	5	7	9	9	—
Individual 4	—	—	—	—	—
Individual 5	5	9	12	—	—
Individual 6	3	4	4	5	—
Individual 7	—	—	—	—	—
Individual 8	7	11	13	—	—
Individual 9	—	—	—	—	—
Individual 10	3	6	9	11	—
Individual 11	3	3	7	—	—
Individual 12	4	6	7	8	—
Experiment 2					
Individual 13	3	4	5	—	12
Individual 14	—	—	—	—	3
Individual 15	3	3	—	—	12
Individual 16	3	4	—	—	7
Individual 17	3	5	—	6	—
Individual 18	3	6	6	—	7
Individual 19	—	—	—	—	7
Individual 20	4	5	5	—	—
Individual 21	4	6	6	6	12
Individual 22	3	3	4	4	—

*The first day on which a blastema was detected in live animals scored using a dissecting microscope at ×63 magnification. A blastema was considered to have formed if a zone of clear cells developed at the tip of the animal *and* this clear zone subsequently grew in size. "—" indicates that the individual did not form a blastema during the 14 days of the experiment.

†The first day on which a prostomium, mouth, or chaetae were detected in live animals scored using a dissecting microscope at ×63 magnification. A prostomium was scored as having formed if the dorsal region of the blastema formed the pointed shape characteristic of early prostomium development. A mouth was scored as having formed if an unambiguous opening below the prostomium developed. Chaetae were scored as having formed if one or more chaetae were seen emerging from the blastema region. "—" indicates that the animal did not form the structure during the 14 days of the experiment.

‡The day of death is noted for individuals that died before the end of the experiment on day 14.