A phylum-wide survey reveals multiple independent gains of head regeneration in Nemertea

Eduardo E. Zattara[1,2,5], Fernando A. Fernández-Álvarez[3], Terra C. Hiebert[4], Alexandra E. Bely[2] and Jon L. Norenburg[1]

[1] Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC, USA
[2] Department of Biology, University of Maryland, College Park, MD, USA
[3] Institut de Ciències del Mar, CSIC, Barcelona, Spain
[4] Institute of Ecology and Evolution, University of Oregon, Eugene, OR, USA
[5] Instituto de Investigaciones en Biodiversidad y Medioambiente, Consejo Nacional de Investigaciones Científicas y Técnicas, Bariloche, RN, Argentina

Corresponding author: E.E. Zattara, ezattara@gmail.com

Electronic Supplementary Materials

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Supporting Tables

Table S1: Family and species of specimens collected during this survey, along with site collection code, locality, collector and month and year of collection.

Group	Species	Site code	Locality	Collector(s)	Date
PALAEONEMERTEA	*Tubulanus ruber*	coos13	Charleston, OR, USA	JLN, TCH	May 2013
	Tubulanus sexlineatus	coos13	Charleston, OR, USA	JLN, TCH	May 2013
	Cephalothrix rufifrons	nzsi2	Ruahine Corner, Invercargill, South Island, New Zealand	JLN	March 2013
	Cephalothrix spiralis	port	Charleston, OR, USA	S. Maslakova	January 2012
HOPLONEMERTEA	*Prosorhochmus sp.* JLN1554	nz13	Kaikoura, South Island, New Zealand	JLN	March 2013
	Prostoma cf *eilhardi*	blin	Bloomington, IN, USA	EEZ	March 2015
	Prostoma cf *eilhardi*	bost	Boston, MA, USA	C. Laumer	October 2012
	Nemertopsis bivittata	vkfl	Virginia Keys, FL, USA	EEZ, JLN	February 2013
	Nemertopsis cf *bivittata*	mcba	Mar Chiquita, Buenos Aires, Argentina	EEZ	May 2013
	Prosadenoporus californiensis	coos13	Charleston, OR, USA	JLN	May 2013
	Paranemertes sanjuanensis	coos13	Charleston, OR, USA	JLN	May 2013
	Zygonemertes albida	fpfl	Fort Pierce, FL, USA	EEZ, JLN	February 2013
	Poseidonemertes sp.	fpfl	Fort Pierce, FL, USA	EEZ, JLN	February 2013
PILIDIOPHORA	*Hubrechtella* sp.883	fpfl	Fort Pierce, FL, USA	EEZ, JLN	February 2013
	Baseodiscus delineatus	vkfl	Virginia Keys, FL, USA	EEZ, JLN	February 2013
	Siphonenteron bilineatum	ases	Villaviciosa Estuary, Asturias, Spain	FFA	April 2013
	Siphonenteron bicolour	whma	Woods Hole, MA, USA	Susan Hill	June 2012
	Maculaura alaskensis	coos12	Charleston, OR, USA	S. Maslakova	September 2012
	Maculaura alaskensis	port12	Charleston, OR, USA	S. Maslakova	June 2012
	Cerebratulus lineolatus	vkfl	Virginia Keys, FL, USA	EEZ, JLN	February 2013
	Micrura chlorapardalis	fpfl	Fort Pierce, FL, USA	EEZ, JLN	February 2013
	Lineus viridis	nama	Nahant, MA, USA	JLN	June 2012
	Lineus ruber	nama	Nahant, MA, USA	JLN	June 2012
	Lineus clandestinus	mnes	Muros de Nalón, Asturias, Spain	FFA	April 2013
	Lineus lacteus	mnes	Muros de Nalón, Asturias, Spain	FFA	April 2013
	Lineus sanguineus "sanguineus"	swed	Kristineberg, Sweden	JLN	May 2014
	Lineus cf sanguineus	nzsi2	Kaikoura, South Island, New Zealand	JLN	March 2013
	Lineus sanguineus " sanguineus"	mnes	Muros de Nalón, Asturias, Spain	FFA	April 2013
	Lineus sanguineus " sanguineus"	mnes	Muros de Nalón, Asturias, Spain	FFA	April 2013
	Lineus sanguineus " sanguineus"	tces	Tapia de Casariego, Asturias, Spain	FFA	April 2013
	Lineus sanguineus "vegetus"	boat12	Charleston, OR, USA	S.Maslakova	September 2012
	Lineus sanguineus "vegetus"	coos13	Charleston, OR, USA	S. Maslakova	July 2013
	Lineus sanguineus "vegetus"	port13	Charleston, OR, USA	S. Maslakova	July 2013
	Lineus sanguineus "socialis"	whma	Woods Hole, MA, USA	A.Matthewson	July 2012
	Lineus sanguineus "socialis"	nama	Nahant, MA, USA	JLN	June 2012
	Lineus sanguineus "bonaerensis"	mcba	Mar Chiquita, BA, Argentina	EEZ, Pia Floria	December 2013
	Lineus sanguineus "bonaerensis"	scba	Santa Clara, BA, Argentina	EEZ, Pia Floria	December 2013

Table S2: Nomenclature and taxonomic references

Higher Order Ranks		***Family***	***Species***	***Taxonomic reference***
"Palaeonemertea"		*Tubulanidae*	*Tubulanus ruber* Griffin 1898	[1]
			Tubulanus sexlineatus Griffin 1898	[2]
		Cephalothricidae	*Cephalothrix rufifrons* (Johnston, 1837)	[2]
			Cephalothrix spiralis Coe, 1930	[2]
Neonemertea *Thollesson Norenburg, 200* [3]	Hoplonemertea	*Prosorhochmidae*	*Prosorhochmus sp.* JLN1554	
			Prosadenoporus (=Pantinonemertes) californiensis (Gibson, Moore Crandall, 1982)	[4]
		Emplectonematidae	*Nemertopsis bivittata* (Delle Chiaje, 1841)	[5]
			Paranemertes sanjuanensis Stricker, 1982	[6]
		Tetrastemmidae	*Cyanophthalma cordiceps* (Friedrich, 1933)	[2]
			Prostoma graecense (Böhmig, 1892)	[7,8]
			Prostoma cf *eilhardi* (Montgomery, 1894)	[9,10]
			Tetrastemma vermiculus (Quatrefages, 1846)	[2]
			Tetrastemma candidum (Müller, 1774)	[2]
		Amphiporidae	*Zygonemertes albida* Coe, 1901	[11]
			Poseidonemertes sp. Kirsteuer, 1967	[12]
	Pilidiophora Thollesson Norenburg, 2003 [	*Hubrechtidae*	*Hubrechtia* sp. JLN883	
		Baseodiscidae	*Baseodiscus delineatus* (delle Chiaje, 1825)	[13,14]
		Valenciniidae	*Zygeupolia rubens* (Coe, 1895)	[14]
		Lineidae	*Siphonenteron bilineatum* Meneghini in Renier, 1847	[15]
			Siphonenteron bicolour (Verrill, 1892)	[15,16]
			Euborlasia nigrocincta Coe, 1940	[17]
			Maculaura alaskensis Hiebert & Maslakova, 2015	[18]
			Cerebratulus lineolatus Coe, 1905	[19]
			Cerebratulus lacteus (Leidy, 1851)	[2]
			Cerebratulus marginatus Renier, 1804	[5]
			Micrura chlorapardalis Schwartz & Norenburg, 2005	[20]
			Micrura fasciolata Ehrenberg, 1828	[2]
			Lineus pictifrons Coe, 1904	[21]
			Lineus viridis (Müller, 1774)	[22]
			Lineus ruber (Müller, 1774)	[22,23]
			Lineus clandestinus Krämer, Schmidt, Podsiadlowski, Beckers, Ho & Von Döhren, 2016	[22]
			Lineus longissimus (Gunnerus, 1770)	[5]
			Lineus lacteus (Rathke, 1843)	[23]
			Lineus pseudolacteus (Gontcharoff, 1951) *(=Line sanguineus×lacteus)*	[23,24]
			Lineus sanguineus (Rathke, 1799)	[23,25,26]

Table S3: Primer sequences

Locus	Primer	Sequence, 5' – 3'	Source
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	[27]
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	[27]
16S	16Sar-L	CGCCTGTTTATCAAAAACAT	[28]
	16Sbr-H	CCGGTCTGAACTCAGATCACGT	[28]
18S	1F	TACCTGGTTGATCCTGCCAGTAG	[29]
	5R	CTTGGCAAATGCTTTCGC	[29]
	3F	GTTCGATTCCGGAGAGGGA	[29]
	9R	GATCCTTCCGCAGGTTCACCTAC	[29]
	a2.0	ATGGTTGCAAAGCTGAAAC	[30]
28S	LSU5	ACCCGCTGAAYTTAAGCA	[31]
	R2762	CCGCCCCAGCCAAACTCCCC	[32]
	rd5B	CCACAGCGCCAGTTCTGCTTAC	[33]
	28Sa	GACCCGTCTTGAAACACGGA	[30]
	rd7b1	GACTTCCCTTACCTACAT	[33]
	28Z	CTTGGTCCGTGTTTCAAGAC	[34]

Table S4: Regeneration survey results

			Posterior wound surfaces				Anterior wound surfaces						
Grp	*Species*	N	wound healing	growth/ elongation	cirrus regrown	Days to regenen	wound healing	growth/ elongation	mouth/ groove	brain	ocelli	Days to regener.	Days to death
PAL	*Tubulanus ruber*	2	2-4 d	4-20 d	NA	~20	2-4 d	~35-90 d	Yes	Yes	NA	*88*	—
PAL	*Tubulanus sexlineatus*	1	3 d	3-20 d	NA	~20	3 d	4-35 d	Yes	Yes	Yes	*35*	—
PAL	*Cephalothrix rufifrons*	1	2-3 d	3-10 d	NA	~10	2-3 d	No	No	No	NA	—	10
PAL	*Cephalothrix spiralis*	2	2-3 d	3-9 d	NA	~9	2-3 d	No	No	No	NA	—	9
HOP	*Prosorhochmus sp.*	1	1 d	2-9 d	NA	~9	1 d	No	No	No	No	—	40[K]
HOP	*†Cyanophthalma cordiceps*	?	?	?	?	?	Yes	No	No	No	No	?	?
HOP	*†Prostoma graecense*	?	1-3 d	4-18 d	NA	~18	3 d	4-8 d	Yes	Yes	Yes	*~60*	—
HOP	*Prostoma* cf *eilhardi*	10	1-3 d	4-18 d	NA	~18	3 d	No	No	No	No	—	83
HOP	*Nemertopsis bivittata*	10	1-2 d	3-7 d	NA	~7	2-5 d	No	No	No	No	—	41
HOP	*Prosadenoporus californiensis*	1	1 d	2-9 d	NA	~9	1 d	No	No	No	No	—	90
HOP	*Paranemertes sanjuanensis*	1	1 d	2-9 d	NA	~9	1 d	No	No	No	No	—	90
HOP	*†Tetrastemma vermiculus*	?	?	?	?	?	2-4d	No	No	No	No	—	58
HOP	*†Tetrastemma candidum*	10	?	?	?	?	2-4d	No	No	No	No	—	58
HOP	*Zygonemertes albida*	2	1 d	1-11 d	NA	~11	1 d	No	No	No	No	—	20
HOP	*Poseidonemertes sp.*	1	4 d	4-6 d	NA	~6	4 d	No	No	No	No	—	10[K]
PIL	*Hubrechtella* sp.883	4	2-4 d	12-14 d	NA	~15	2-4 d	No	No	No	No	—	18
PIL	*Baseodiscus delineatus*	10	2-4 d	5-16 d	NA	~16	1-5 d	6-12 d	Yes	Yes	Yes	*19*	—
PIL	*†Zygeupolia rubens*	?	Yes	Yes	Yes	?	Yes	No	No	No	No	?	?
PIL	*Siphonenteron bilineatum*	1	1 d	2-12+ d	NA	~27	1 d	No	No	No	No	—	53[K]
PIL	*Siphonenteron bicolour*	2	1-2 d	3-7 d	NA	~7	1-2 d	No	No	No	No	—	240
PIL	*†Euborlasia nigrocincta*	?	Yes	Yes	NA	?	?	No	No	No	NA	**?**	?
PIL	*Maculaura alaskensis*	10	2 d	3-9 d	7-9 d	~10	2 d	No	No	No	NA	—	25
PIL	*Cerebratulus lineolatus*	3	1 d	2-7 d	NA	~7	1 d	2-7 d	Yes	Yes	Yes	*17*	—
PIL	*†Cerebratulus lacteus*	?	Yes	Yes	?	?	Yes	No	No	No	NA	—	90
PIL	*†Cerebratulus marginatus*	?	Yes	Yes	?	?	Yes	No	No	No	NA	—	256
PIL	*Micrura chlorapardalis*	2	1-2 d	3-6 d	3 d	~6	No	No	No	No	NA	3	—
PIL	*†Micrura fasciolata*	1	Yes	Yes	Yes	~270	20 d	Some?	No	No	NA	—	180[K]
PIL	*†Lineus pictifrons*	?	Yes	Yes	NA	~25	Yes	Yes	Yes	Yes	NA	*25*	—
PIL	*Lineus viridis*	2	1-2 d	3-9 d	NA	~9	2 d	No	No	No	No	—	164
PIL	*Lineus ruber*	6	1-2 d	3-9 d	NA	~9	2 d	No	No	No	No	—	91
PIL	*Lineus clandestinus*	1	2 d	3-8 d	NA	~8	2 d	No	No	No	No	—	61[K]
PIL	*†Lineus longissimus*	?	7-28 d	?	?	?	7-28 d	No	No	No	No	—	135
PIL	*Lineus lacteus*	8	1-5 d	3-7 d	NA	~16	1-5 d	No	No	No	No	—	79
PIL	*†Lineus pseudolacteus*	?	Yes	Yes	NA	Yes	Yes	Yes(~11d)	Yes	Yes	?	*>28*	—
PIL	*Lineus sanguineus*	>150	1-2 d	3-10 d	NA	~6-10	1-2 d	3-7 d	Yes	Yes	Yes	*15*	—

Group (PAL: Palaeonemertea, HOP: Hoplonemertea, PIL: Pilidiophora), species (*†* indicates species with data from literature), experimental sample size (N), landmarks of anterior and posterior regeneration ("No" indicates absence, while presence is indicated by time in days or "Yes" when timing data are lacking; [NA] indicates a structure not present in the species), average time to completion of anterior regeneration (if present) or longest survival time an amputated individual not showing an anterior regenerative response ([K] indicates specimen was sacrificed for DNA extraction). Cells with "?" indicate data were not available.

Table S5: Sample OTUs, OUT id codes and NCBI nucleotide sequence accessions

Samples			**NCBI Accesion**			
Grp	**OTU**	**OTU ID**	**COI**	**16S**	**18S**	**28S**
ANN	*Lumbriculus sp.*	ANN-LUMva	**MK047673**	**MK067298**	**MK076297**	**MK076419**
ANN	*Nais communis*	ANN-NAICO	**MK047674**	**MK067299**	**MK076298**	**MK076420**
ANN	*Paranais litoralis*	ANN-PARli	KP204261	**MK067300**	**MK076299**	**MK076421**
PAL	*Tubulanus ruber*	TUBpo	HQ848623	JF277598	JF293061	HQ856899
PAL	*Tubulanus sexlineatus*	TUBse	HQ848621	JF277596	JF293063	HQ856895
PAL	*Cephalothrix rufifrons* nzsi2	CEPsi- nzsi2	**MK047675**	**MK067301**	**MK076300**	**MK076422**
PAL	*Cephalothrix spiralis* port	CEPsp-port	**MK047676**	**MK067302**	**MK076301**	**MK076423**
HOP	*Prosorhochmus sp.* nz13	PROne-nz13	**MK047678**	**MK067303**	**MK076302**	**MK076424**
HOP	*Cyanophthalma cordiceps**	CYAob	EF208980		AY039667	
HOP	*Prostoma* cf *eilhardi* blin	PROei-blin	**MK047681**		**MK076303**	
HOP	*Prostoma* cf *eilhardi* bost	PROei-bost	**MK047682**	JF277620	**MK076304**	**MK076425**
HOP	*Prostoma gracense*	PROgr	EU489490		AY928356	
HOP	*Nemertopsis bivittata* NA vkfl	NEMbi-vkfl	**MK047680**	**MK067304**	**MK076305**	**MK076426**
HOP	*Nemertopsis cf. bivittata* SA mcba	NEMbi-mcba	**MK047679**	**MK067305**	**MK076306**	**MK076427**
HOP	*Prosadenoporus californiensis* coos13	PROca-coos	**MK047685**	**MK067306**	**MK076307**	**MK076428**
HOP	*Paranemertes sanjuanensis* coos13	PARsa-coos	**MK047686**	**MK067307**	**MK076308**	**MK076429**
HOP	*Tetrastemma vermiculus*	TETve	AY791996		AY928378	
HOP	*Tetrastemma candidum*	TETca	KP697777		AY928357	AB505827
HOP	*Zygonemertes albida* fpfl	ZYGal- fpfl	**MK047684**	**MK067308**	**MK076309**	**MK076430**
HOP	*Poseidonemertes sp.* fpfl	POSsp- fpfl	**MK047683**	**MK067309**	**MK076310**	**MK076431**
PIL	*Hubrechtella* sp.883 fpfl	HUB883-fpfl	**MK047677**		**MK076311**	**MK076432**
PIL	*Baseodiscus delineatus* vkfl	BASde-vkfl	**MK047687**	**MK067310**	**MK076312**	
PIL	*Zygeupolia rubens*	ZYGru	HQ997773	HQ997773	JF293045	EF124961 +HQ856861
PIL	*Siphonenteron bilineatum* ases	SIPbi-ases	GU392015	**MK067311**	**MK076313**	**HQ856844**
PIL	*Siphonenteron bicolour* whma	TENbi-whma	**MK047688**	**MK067312**	**MK076314**	**MK076433**
PIL	*Maculaura alaskensis* coos12	MACal-coos	**MK047691**	**MK067313**	**MK076315**	**MK076434**
PIL	*Maculaura alaskensis* port12	MACal-port	**MK047692**	**MK067314**	**MK076316**	**MK076435**
PIL	*Cerebratulus lineolatus* vkfl	CERli-vkfl	**MK047689**	**MK067315**	**MK076317**	**MK076436**
PIL	*Cerebratulus lacteus*	CERla	KC424754	EF124868	AY145368	AY145396
PIL	*Cerebratulus marginatus*	CERma	HQ848575	JF277576	JF293042	HQ856858
PIL	*Micrura chlorapardalis* fpfl	MICch- fpfl	**MK047690**	**MK067316**	**MK076318**	KF935348
PIL	*Micrura fasciolata*	MICfa	HQ848577	JF277585	JF293038	HQ856846
PIL	*Lineus viridis* nama	LINvi-nama	**MK047696**	**MK067317**	**MK076319**	**MK076437**
PIL	*Lineus ruber* nama	LINru-nama	**MK047693**	**MK067318**	**MK076320**	**MK076438**
PIL	*Lineus clandestinus* wifr	LINru2-wifr	**MK047694**	**MK067319**	**MK076321**	**MK076439**
PIL	*Lineus clandestinus* mnes	LINvi-mnes	**MK047695**	**MK067320**	**MK076322**	
PIL	*Lineus longissimus*	LINlo-galt	**MK047697**	**MK067321**	**MK076323**	**MK076440**

PIL	*Lineus lacteus* frfr	LINla-frfr	**MK047698**	**MK067322**	**MK076324**	**MK076441**
PIL	*Lineus lacteus* mnes	LINla-mnes	**MK047699**	**MK067323**	**MK076325**	**MK076442**
PIL	*Lineus pseudolacteus* rofr	LINps-rofr	**MK047700**	**MK067324**	**MK076326**	**MK076443**
PIL	*Lineus sanguineus* "sanguineus" rofr	LINsa-san-rofr	**MK047701**	**MK067325**	**MK076327**	**MK076444**
PIL	*Lineus sanguineus* "sanguineus" swed	LINsa-san-swe	**MK047702**	**MK067326**	**MK076328**	**MK076445**
PIL	*Lineus* cf *sanguineus* nzsi2	LINsa-san- nzsi2	**MK047703**	**MK067327**	**MK076329**	**MK076446**
PIL	*Lineus sanguineus* "sangra" mnes	LINsa-sag-mnes	**MK047705**	**MK067329**	**MK076330**	**MK076447**
PIL	*Lineus sanguineus* "sangra" mnes	LINsa-sag-nmes13	**MK047704**	**MK067328**	**MK076331**	
PIL	*Lineus sanguineus* "sanpe" mnes	LINsa-sap-mnes	**MK047706**	**MK067330**	**MK076332**	**MK076448**
PIL	*Lineus sanguineus* "sanpe" tces	LINsa-sap-tces	**MK047707**	**MK067331**	**MK076333**	
PIL	*Lineus sanguineus* "vegetus" boat12	LINsa-veg-boat	**MK047708**	**MK067332**	**MK076334**	**MK076449**
PIL	*Lineus sanguineus* "vegetus" coos13	LINsa-veg-coos	**MK047709**	**MK067333**		
PIL	*Lineus sanguineus* "vegetus" port13	LINsa-veg-port	**MK047710**	**MK067334**	**MK076335**	
PIL	*Lineus sanguineus* "socialis" whma	LINsa-soc-whma	**MK047711**	**MK067335**	**MK076336**	**MK076450**
PIL	*Lineus sanguineus* "socialis" nama	LINsa-soc-nama	**MK047712**	**MK067336**	**MK076337**	
PIL	*Lineus bonaerensis* mcba	LINbo-mcba	**MK047713**	**MK067337**	**MK076338**	**MK076451**
PIL	*Lineus bonaerensis* mcba-arg10	LINbo-mcba-arg10	**MK047714**	**MK067338**	**MK076339**	**MK076452**
PIL	*Lineus bonaerensis* scba	LINbo-scba	**MK047715**	**MK067339**	**MK076340**	
PIL	*Lineus bonaerensis* scba-arg5	LINbo-scba-arg5	**MK047716**	**MK067340**	**MK076341**	**MK076453**

Operating taxonomic units (OTUs), codes (used in trees shown in Figures S1-S6) and NCBI accessions for sequences of cytochrome oxidase subunit I (COI), and ribosomal RNAs 16S, 18S and 28S. New accesions generated for this work are in **bold.** Codes after certain species names indicate site codes (see Table S1). *Sequences from *Cyanophthalma obscura* were used as a species proxy for *C. cordiceps*, since no sequence was available for this species.

Extended methods

Regeneration survey

Nemerteans were collected worldwide (see main text for regeneration survey overview, Table S1 for a full list of locations and collectors, and Table S2 for nomenclature and taxonomic references).

For our regeneration experiments, we performed transverse body amputations at two specific body locations (see main text for cutting scheme). The number of individuals per species that was tested for regeneration ability varied from a single individual (for rarer species) to 10-40 individuals (for more abundant or more easily accessed species) (Table S4). When enough individuals had been collected, one or more uncut controls were kept in the same conditions as the regenerated individuals. When numbers were too low (1 or 2 individuals), then specimens were kept under daily observation for 5-10 days before amputating, to ensure their condition was stable and not deteriorating over time. Species in which all specimens showed high mortality in the days after collection were not furthered considered (this only happened in the case of *Carinoma* sp.).

For each species tested, amputated specimens were scored for wound healing, formation of a blastema (the mass of undifferentiated cells that accumulates at the wound site during some forms of regeneration and is typically noticeably less pigmented), and re-formation of anterior or posterior end structures.

Posterior regeneration was scored as present if the posterior cut surface of an anterior fragment healed the wound (forming an intact outer epithelium) and reformed a posterior end with a shape not distinguishable from the original end. In those species with a distinctive posterior structure (like a caudal cirrus), we also scored reconstitution of this structure. Time to completion of posterior regeneration was scored as the time until there was no obvious size mismatch between the stump and the newly formed end. Restoration of function could potentially be assessed by observation of defecation; however, specimens from most species did not feed in captivity, and thus would not be expected to defecate. Posterior regeneration in nemerteans appears to occur primarily by tissue remodeling (morphallaxis), with no intermediate blastema [17]; thus, presence/absence of a blastema could not be used to score posterior regeneration. For species where multiple individuals were scored, approximate times for each landmark are reported as a range (for example, “2-9 d” means that specific landmark was attained between 2 and 9 days post-amputation), except for completion of regeneration, where the fastest cases were reported, and survival without regeneration, where the longest survival times were reported. Experimental specimens showing clear signs of poor health or abnormal development were excluded from timing estimations.

Anterior regeneration was scored as present if the anterior cut surface of a posterior fragment healed the wound (forming an intact outer epithelium), if the cut end formed a regeneration blastema, and if the blastema eventually developed into an anterior end complete with all cephalic structures visible in intact living specimens (i.e., brain, cerebral organs, mouth, and ocelli and lateral grooves if these were present in that species). The presence of a blastema was evaluated based on mismatches in body width and pigmentation between the stump (wider, more pigmented) and the regenerated tissue (less wide, less pigmented). Time to completion of anterior regeneration was scored as the time until the normal size proportions were restored between the regenerated head and the rest of the body (either by regenerate growth or reshaping of the stump). In addition to reformation of morphological structures, movement behaviors of whole individuals and fragments were noted and considered when evaluating whether regeneration was complete.

For specimens that regenerated, we scored the approximate time (in days post-amputation, or dpa) it took to complete regeneration, and for specimens that did not regenerate, we scored the time to death (i.e., days survived without evidence of regeneration). For species for which sample size was very low (1-2 individuals), we had to eventually sacrifice the experimental specimens for DNA extraction; in these cases, specimens were fixed only after they started to show clear signs of deteriorating health, and

always after the outcome of the amputation experiment had been scored. Species in which survival time was truncated for DNA extraction are marked with a "K" in Figure 2 and Table S4.

To expand the number of nemertean species for which regeneration data could be included in our dataset, we also searched the literature for previous reports of anterior and posterior regeneration, as previously described [35]. Data were included in our dataset only if regeneration results were unambiguous, based on amputations similar to those from our own experiments, and involved identifiable, valid species. The World Marine Species database [36] was used as a baseline taxonomic reference for nomenclature and synonymies; species specific taxonomic references are shown in Table S2.

Molecular marker sequencing

DNA was extracted from at least one individual of each species used in regeneration experiments. Whenever possible, the extraction was made from individuals that had undergone the amputation experiments; when that was not possible, we used conspecific individuals from the same field collection. DNA was extracted at the Laboratories of Analytical Biology (Smithsonian Institution, National Museum of Natural History) using a DNeasy 96 Blood & Tissue Kit (69581, Qiagen).

Fragments of four genes were amplified by PCR (polymerase chain reaction) and sequenced using standard primers (Table S3): two mitochondrial genes, cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S), and two nuclear genes, small subunit ribosomal RNA (18S) and large subunit ribosomal RNA (28S). COI was amplified using the primer pair LCO1490/HCO2198 [27]; 16S was amplified using the primer pair 16Sar-L⁄16Sbr-H[28]; 18S was amplified using primer pairs 1F⁄5R and 3F⁄9R [29]; 28S was amplified using primer pairs LSU5/R2762, LSU5/rd5B, LSU5/rd7b, 28Sa/rd7b1, rd4.8a/R2762 [30–33,37]; the 3' end of 18S and 5' end of 28S were amplified using the overlapping primer pair 18S-a2.0/28S-28Z [30,34].

PCR was performed using ~5-50ng template in a 25 µL volume of 1x Biolase buffer, 5 mM $MgCl_2$, 20mM of each dNTP, 2µL of each primer (10µM stock) and 5U of Biolase DNA polymerase (Bioline BIO-21042). PCR was carried out using an initial denaturation step of 5 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 180 s at 72°C; and a final 7-min sequence extension at 72°C. Reaction products were run on a 1.5% agarose gel to confirm the presence of amplicons of the right size. Amplicon purification was performed using an enzymatic reaction with EcoSAP-IT (USB Corp., Cleveland, OH). Each purified PCR product was sequenced in both directions (in paired reactions using respective forward and reverse primers) in 10 µL reactions containing 1 µL PCR product, 0.5 µL ABI BigDye Terminator ver. 3.0 (Applied Biosystems, Foster City, CA), 1.75 µL ABI BigDye buffer, and 0.5 µL of primer (10µM stock). Sequencing reaction products were analyzed using an ABI Prism 3730xl Genetic Analyzer capillary sequencer (Applied Biosystems).

For several species of *Lineus*, gene marker sequences were obtained from published transcriptomes [23], which were searched with megablast [38] using sequences from related species as queries. For a few other species for which we had regeneration data (either from our experiments or the literature) but for which no specimens were available, we retrieved sequences from Genbank (Table S5).

Sequence alignment and phylogenetic reconstruction

Sequence quality assessment, assembly and alignment were performed using the Geneious 8.1.9 platform [39]. Chromatograms for each sequencing reaction were trimmed and quality assessed and, whenever possible, reaction pairs (forward and reverse sequencing reactions) were assembled. 28S sequences from overlapping primer pairs were further assembled using the "De Novo Assembly" tool in Geneious. A multiple sequence alignment (MSA) for each marker was initially estimated automatically using the MAFFT algorithm [40]; each MSA was inspected and curated by eye, and then the MSAs from the different markers were concatenated. For species where we had collected specimens

from different populations or locations, each sampled population was represented by a separate sequence (Tables S1 and S5).

The concatenated MSA was used to infer fully resolved phylogenetic trees, as required for the maximum likelihood ancestral character estimation analyses (see below). The MSA was analyzed with RAxML v8.2.11 [41], set up to perform 100 rapid bootstrap inferences followed by a thorough maximum likelihood search, using a General Time Reversible (GTR) model with gamma-distributed rate heterogeneity. The MSA was divided into six partitions, each run with different models: three partitions were used for the protein coding marker COI (one partition for each codon position) and one partition was used for each of the rRNA markers (16S, 18S and 28S). The inference algorithm was run without topological constraints ("unconstrained", Figure S1), and then repeated with each of the following constraints:

a) traditional Nemertea orders enforced: [((Palaeonemertea), (Hoplonemertea), (Heteronemertea))] (Figure S2);

b) monophyletic Palaeonemertea basal to reciprocally monophyletic Hoplonemertea and Heteronemertea: [((Palaeonemertea), (Hoplonemertea, Heteronemertea))] (Figure S3);

c) non- monophyletic Palaeonemerteans but monophyletic Hoplonemertea and Heteronemertea [(Hoplonemertea, Heteronemertea)] (Figure S4);

d) Pilidiophora hypothesis [42], with non-monophyletic Palaeonemerteans, monophyletic Hoplonemertea and Hubrechtidae as sister to monophyletic Heteronemertea: [(Hoplonemertea, (HUB883,(Heteronemertea)))] (Figure S5);

e) basal Cephalothricidae and Pilidiophora enforced: [((Cephalothricidae, (Tubulanidae, (Hoplonemertea, (HUB883, (Heteronemertea)))))];

f) basal Tubulanidae and Pilidiophora enforced: [((Tubulanidae, (Cephalothricidae, (Hoplonemertea, (HUB883, (Heteronemertea)))))]

To confirm that the choice of heuristic strategy was not introducing a method-specific bias in our follow up analyses, we also performed Bayesian inference from the MSA using MrBayes 3.2.6 [43], specifying a GTR model with 4 categories of gamma distributed rate heterogeneity and a proportion of invariant sites. Four heated chains were run for 1,100,000 steps and subsampled every 200 steps; the initial 100,000 steps were discarded as burn-in. We only used Bayesian inference on an unconstrained topology (Figure S6).

Ancestral trait estimation by maximum likelihood

The best scoring trees from each unconstrained and constrained run were used as fully resolved, rooted phylogenetic frameworks for character mapping and ancestral trait estimation. After removing the outgroup species, we generated a matrix matching each species/population in the MSA (rows) with the results of the survey (columns). The columns held two binary variables (absent or present): posterior regeneration and anterior regeneration. We used the *ace* function from the *ape* package[44], which models discrete trait state evolution as a Markovian process[45], and incorporates phylogenetic tree branch length information to estimate the rates of change of the trait, and the likelihood for each character state at every node of the tree, including the basal node [46]. A two-parameter model was specified allowing for separate calculation of the rate of gain (0→1) and rate of loss (1→0). We repeated this procedure for all the trees inferred using the different constraint sets (see above). All analyses were run within the R computing environment [47].

Data plotting and figure assembly

Tree diagrams, character mapping, and ancestral node likelihood were plotted in R, exported as vector files and assembled as figures using Adobe Illustrator CS6. For the summary tree figure, tips representing different sampled populations from the same species were collapsed into a single tip.

Experimental results by species

This section describes observation and results of amputation experiments for each species. "*" indicates data derived from reports in existing literature. "†" indicates species for which no DNA sequence data is available. Species are presented in the same order than Figure 2 and Table S4.

Palaeonemertea

Tubulanus ruber sensu [1]: Two individuals collected at Oregon, USA, were bisected at one-third of their body length.

- *Posterior cut:* The anterior fragments closed and healed wounds within a few days. By 20 dpa, posterior regeneration of a distinct tail had occurred in both fragments.
- *Anterior cut:* The posterior fragments closed its wound by folding over, laterally, and healed wounds within a few days. By 20 dpa, the anterior ends were small but rounded; crawling in the dish was distinctly lead by this regenerated anterior portion. By 35 dpa, the regenerated anterior ends were still small relative to the rest of the body. After ~90 dpa, regenerated ends were indistinguishable in size from the rest of the body but remained paler in color.

Tubulanus sexlineatus: One individual collected at Oregon, USA, was bisected at one-third of its body length.

- *Posterior cut:* The anterior fragment closed and healed its wound within 3 dpa. By 20 dpa, regeneration of the posterior ends was apparent, with a distinct tail.
- *Anterior cut:* The posterior fragment closed and healed its wound within 3 dpa. By 20 dpa, it had formed a small head with conspicuous ocelli. By 35 dpa, the regenerated head of the posterior fragment was fully formed and similar in size (width) to the rest of the body. *Notes:* Pigment of regenerated regions remained pale for several months, but distinct white transverse lines were apparent after 28 dpa.

Cephalothrix rufifrons: One individual collected at Ruahine Corner, Invercargill, South Island, New Zealand, was bisected at one-third of its body length.

- *Posterior cut:* The anterior fragment closed the wound about 2-3 dpa. By 10 dpa, a normal-looking posterior end had formed.
- *Anterior cut:* The posterior fragment closed the wound at ~2-3 dpa. It showed no sign of blastema formation or regeneration and died at 10 dpa.

Cephalothrix spiralis: Two individuals collected at Oregon, USA, were bisected at one- and two-thirds of their body length respectively.

- *Posterior cut:* The anterior fragments closed their wounds about 2-3 dpa. By 9 dpa, they had re-formed a normal-looking posterior end.
- *Anterior cut:* The posterior fragments closed the wound at ~2-3 dpa. After that, they showed no sign of blastema formation or regeneration, started swelling and died at 9 dpa.

Hoplonemertea

Prosorhochmus sp. JLN1554: One individual collected at New Zealand was bisected at one-third of its body length.

- *Posterior cut:* The anterior fragment closed the wound within 1 dpa. By 9 dpa, it had re-formed a posterior end.

- *Anterior cut:* The posterior fragment closed the wounds but failed to form any blastema-like structure in the following days. The fragment survived for 40 days before it was sacrificed for DNA extraction but did not show any signs of regeneration.

**Cyanophthalma cordiceps:* Henri Sandoz [48] reported results from experiments on individuals collected at Marseille, France, and referred to as *Tetrastemma vittatum*. He stated that anterior regeneration was not observed unless the amputation plane was anterior to the brain, and that ablations at the posterior extremity do not even seem to provoke a morphallactic remodeling. No details on timing of regeneration or posterior regenerative ability are given in his report.

**Prostoma graecense:* Senta Kipke [7] reported results from experiments on individuals collected at Graz, Austria. She amputated several individuals at four different planes along the body axis: 1) immediately anterior to the brain; 2) immediately posterior to the brain; 3) halfway along the body axis; and 4) ¾ of the total body length.

- *Posterior cuts:* anterior fragments cut at 1 or 2 died soon after the operation (1) or after 2 days (2). Anterior fragments cut at 3 or 4 closed their wounds at 1 dpa and wound-healed after 3 dpa. Despite no blastema being observed, fragments had a normal-looking posterior end by 18 dpa. Defecation was observed in a specimen at 54 dpa.
- *Anterior cuts*: Posterior fragments cut at 1 (in front of the brain) wound-healed by 1-2 dpa, regenerated their missing eyes after 14 dpa, and had regrown eyes, cerebral organs, frontal organs and a new mouth opening by 18-19 dpa. Posterior fragments cut at 2 (behind the brain) wound-healed by 3 dpa, developed a swelling at the front end by 8 dpa, and after 21 dpa had regenerated a head with brain, cerebral organs and one pair of eyes, with a second pair formed between 31 and 69 dpa; however, amputees that had ejected their proboscis after the amputation failed to regenerate cerebral organs. Posterior fragments cut at 3 wound-healed by 3-4dpa, and some formed blisters or rudimentary blastemata, but failed to regenerate any anterior structure; most of these fragments survived for 75-85 dpa, with some reaching up to 97 to 111 dpa. Posterior fragments cut at 4 died within a few hours.
- *Notes:* Encystment of fragments was commonly observed. One sexually mature posterior fragment laid eggs 1 day after amputation. Note that *no amputation plane resulted in regeneration of two viable individuals*.

Prostoma cf. eilhardii: Individuals collected at Bloomington, IN (N=4) and Boston, MA (N=6), USA, were amputated at one third or two thirds of their body length.

- *Posterior cuts:* The anterior fragments closed their wounds within 1 dpa and had and wound-healed after 3 dpa. By 18 dpa, the posterior end was not distinguishable from the original.
- *Anterior cut:* The posterior fragments closed their wounds within 1 dpa and had and wound-healed after 3 dpa. Most fragments started forming a blister at the anterior cut surface around 4 dpa; while the degree of blistering varied depending on the individuals and the position of the cut (amputation at a more posterior position resulted in larger blisters at the anterior cut surface), in no case was a blastema or any kind of anterior structure formed. Posterior fragments survived up to over 80 dpa.
- *Notes:* Encystment of fragments was observed in few cases.

Nemertopsis bivittata and *N. cf bivittata:* Individuals collected at Virginia Keys, FL, USA (N=8) and Mar Chiquita, Buenos Aires, Argentina (N=2), were amputated at one third or two thirds of their body length.

- *Posterior cuts:* The anterior fragments closed their wounds and healed within 1-2 dpa, regained normal-looking posterior ends by about 7dpa.
- *Anterior cuts*: Posterior fragments closed by folding over, taking up to 5 days to complete healing. Anterior cut surfaces did not develop any blastema-like structure, at most some

developed hollow blister-like epidermal edemas. Some fragments lacking an anterior end survived over 40 dpa.
- *Notes:* Several individuals lacking an anterior end underwent accelerated sexual maturation relative to non-amputated individuals, becoming loaded with eggs starting at 4dpa; several individual laid eggs between 18 and 24 dpa.

Prosadenoporus (=Pantinonemertes) californiensis: One individual collected at Charleston, OR, USA, was bisected at one-third of its body length.
- *Posterior cut:* The anterior fragment closed the wound within 1 dpa. By 9 dpa, it had re-formed a posterior end.
- *Anterior cut:* The posterior fragment closed the wounds within the first day but failed to form any blastema-like structure in the following days. The fragment survived for 90 days but did not show any signs of regeneration.

Paranemertes sanjuanensis: One individual collected at Charleston, OR, USA, was bisected at one-third of its body length.
- *Posterior cut:* The anterior fragment closed the wound within 1 dpa. By 9 dpa, it had re-formed a posterior end.
- *Anterior cut:* The posterior fragment closed the wounds within the first day but failed to form any blastema-like structure in the following days. The fragment survived for 90 days but did not show any signs of regeneration.

**Tetrastemma candidus* and *T. vermiculus*: Oskar Carlgren [49] reported results from experiments on individuals collected at Kristineberg, Sweden. He amputated individuals of both species (mainly *T. candidus*) at two types of positions near the brain: 1) locations anterior to the brain and 2) locations posterior to the brain.

- *Posterior cuts:* the fate of anterior fragments is not reported, but they likely did not survive long.
- *Anterior cuts*: Posterior fragments cut at 1 had regenerated the frontal organ, proboscis, some eyes and the cerebral organs by 8 dpa or less and formed the remaining eyes by 11dpa. Posterior fragments cut at 2 healed the wound within a few days, and survived for up to 58 days, with a few individuals showing a small pointed blister; regeneration of the head with its characteristic organs was not observed.
- *Notes:* Carlgren observed that oftentimes the cut ends of the lateral nerve cords would form a large commissure below the wound.

Zygonemertes albida: Two individuals collected at Fort Pierce, FL, USA, were amputated at one third or two thirds of their body length.
- *Posterior cuts:* the anterior fragments wound-healed by 1 dpa and re-formed a normal-looking posterior end by 11 dpa.
- *Anterior cuts:* Wounds had healed by 1 dpa. The posterior fragments started swelling by 7 dpa. A hollow blister-like epidermal edema formed at the anterior cut surface by 11 dpa, after which the fragment shriveled and eventually died around 20 dpa. No blastema-like structure was observed.

Poseidonemertes sp.*:* A single individual collected at Fort Pierce, FL, USA, was amputated at one third of its body length.
- *Posterior cut:* the anterior fragment had wound-healed by 4 dpa and re-formed a normal-looking posterior end by 6 dpa.

- *Anterior cut:* The posterior fragment had wound-healed by 4 dpa and started swelling by 6 dpa. No blastema-like structure was observed up to 10 dpa, after which the fragment was sacrificed for DNA extraction.

Pilidiophora

Hubrechtella sp.883: Four individuals collected at Fort Pierce, FL, USA, were at one third or two thirds of their body length.

- *Posterior cuts:* Anterior fragments closed their wounds and had wound-healed by 2 to 4 dpa. A thin regenerated tail could be seen by 12-14 dpa, which kept growing until being undistinguishable from the original.
- *Anterior cuts:* posterior fragments closed their wounds and wound healed by 2 to 4 dpa, but no blastema was observed until they had all died by 18 dpa.

Baseodiscus delineatus: Eight individuals collected at Virginia Keys, FL, USA were amputated at one third or two thirds of their body length.

- *Posterior cuts:* the anterior fragments had closed their wounds after 1 dpa and wound-healed around 2 to 4 dpa. They had re-formed normal-looking posterior ends by 16 dpa.
- *Anterior cuts:* posterior fragments closed their wounds after 1 dpa and all had wound-healed by 5 dpa. Between 6 and 12 dpa, fragments developed a very small blastema at the anterior cut surface, initially looking like a small fluid-filled blister, but later becoming solid. Blastema grew and extended forward, and by 12 dpa it nearly matched the stump width and had developed an anterior pair of ocelli. Cerebral organs were evident by 14 dpa, and by 16-18 dpa the regenerated head already has three pairs of ocelli and looks like a lighter pigmented version of the amputated head.
- *Notes:* After the initial round of experiments, two additional individuals were cut in five fragments (A1 to A5) of about equal length plus a posterior fragment (P) comprising a third of the total original length. The timing of wound closing and healing by both anterior and posterior surfaces, and of posterior regeneration, was similar to the previously described experiments. However, timing of anterior regeneration varied: by 6 dpa, only P had developed a very small blastema at the anterior cut surface; A2 to A5 developed blastemata by 10 dpa. An antero-posterior gradient in blastemal growth rates was evident by 12 dpa, as blastemata of A2 and A3 were much larger than those of A4, A5 and P. Regeneration rate of P had caught up with A2 and A3 by 14 dpa, and all fragments had fully formed heads with 4 ocelli by 20 dpa.

**Zygeupolia rubens:* Wesley Coe [17] reported a "*remarkable capacity for posterior regeneration*" in this species, but no anterior regeneration in cuts removing the brain. "*Wound healing takes place rapidly at both ends of cut pieces and posterior regeneration restores a new midgut in pieces taken from the foregut region or leads to the formation of a slender posterior end piece in fragments from the midgut portion of the body […]. But no true blastema appears at the anterior end of the fragment and the brain was not restored in any of the many fragments observed. Regulation also proceeds slowly in fragments from the midgut region and disintegration eventually occurred in every case*." Minimal challenge experiments are successful: "*Anterior regeneration occurs rapidly in front of the brain if a part of the foregut region remains attached to the head, but small fragments consisting of the brain region only appear to lack the necessary amount of tissues, particularly mesenchyme, for regeneration in either direction*.".

Siphonenteron bilineatum: A single individual collected at Villaviciosa Estuary, Asturias, Spain, was amputated at one third of its body length.

- *Posterior cut:* the anterior fragment closed the wound after 1 dpa and formed a small posterior swelling of lighter color by 12 dpa. It re-formed a normal-looking posterior end by 27 dpa.

- *Anterior cut:* The posterior fragment closed the wound after 1 dpa but did not form any blastema-like structure until 53 dpa, when it was sacrificed for DNA extraction.

Siphonenteron bicolour: Two individuals collected near Woods Hole, MA, USA were amputated at one third of their body length.

- *Posterior cuts:* the anterior fragments closed their wounds within 1 dpa and had wound-healed by 2 dpa. The fragments elongated over the following days and had re-formed a posterior end within 7 dpa.
- *Anterior cuts*: the posterior fragments closed their wounds within 1 dpa and had wound-healed by 2 dpa, but no blastema-like structure developed, even though they survived for several months; one posterior fragment survived over 8 months, slowly shrinking in size and becoming rounder over time.

**†Euborlasia nigrocincta:* Wesley Coe [17] reported presence of posterior but not anterior regenerative ability. "*Only those fragments which remained connected with the brain regenerated completely. When the body was cut into several pieces there was a slow restoration and regulation of the posterior ends, but in no case was a new head formed*." Minimal challenge experiments were successful: "*restoration of the parts anterior to the brain is rapid but cuts made through the brain always proved fatal to both pieces*."

Maculaura alaskensis: Ten individuals collected at two sites near Charleston, OR, USA, were bisected at one-third or two-thirds of their body length.

- *Posterior cuts:* the anterior fragments closed their wounds after 2 dpa. Anterior fragments had regenerated a small posterior cirrus by 7 to 9 dpa.
- *Anterior cuts:* posterior fragments closed their wounds after 2 dpa. Some developed blisters at their cut surfaces and/or swelling of the anterior end but showed no evidence of a blastema in the following days. All posterior fragments were dead by 25 dpa.
- *Notes:* By 14 dpa, one of the posterior fragments became heavily loaded with oocytes.

Cerebratulus lineolatus: Three individuals collected at Virginia Keys, FL, USA, were amputated at one third or two thirds of their body length.

- *Posterior cuts:* the anterior fragments closed their wounds within 1 dpa and had re-formed normal-looking posterior ends by 7 dpa.
- *Anterior cuts:* Posterior fragments closed their wounds within 1 dpa. By 4 dpa, their anterior cut surface had formed a small blastema, which grew forward and developed into a cephalic structure. By 7 dpa, the structure was spearhead-shaped and lateral cephalic grooves were evident. By 14 dpa, they had developed ocelli and begun to adjust its size to match the rest of the body.
- *Notes:* Two of the three field collected individuals showed distinctly smaller heads of a lighter color; the third instead had larger head with dark pigmentation similar to that of the rest of the body, except at the posterior end, where it was noticeably lighter. This pattern is consistent with the expected sampling from an asexually reproducing population.

**Cerebratulus lacteus:* Wesley Coe [17] reported high capacity for posterior regeneration "if cut through any part of the midgut region but restitution is usually incomplete, except in very young individuals, if the cut passes through the foregut". In contrast, he observed that in "this and in other species of the genus anterior regeneration appears to be limited to the tissues in front of the brain [a minimal challenge]. Headless fragments may live for several months, with the cut end entirely healed, but in no case, has the formation of a new brain been observed."

**Cerebratulus marginatus*: John Dalyell [50] gave an extensive account of his observations on two individuals collected in Scotland by fishermen, which he referred to as *Gordius fragilis*. From his report on the fate of the individual fragments, it can be concluded that while posterior regeneration took place

often, in no case was restoration of the anterior head observed. In his notes, he remarks that some headless fragments did survive over 250 days.

Micrura chlorapardalis: Two individuals collected at Fort Pierce, FL, USA, were amputated at one third or two thirds of their body length.

- *Posterior cuts*: anterior fragments closed their wounds and healed by 1-2 dpa. By 3 dpa, a very small caudal cirrus was visible, which had grown to normal size by 6 dpa.
- *Anterior cuts:* posterior fragments extruded abundant internal body contents after amputation, and failed to heal the wound, dying within 1 dpa.

**Micrura fasciolata:* John Dalyell [50] noted that specimens from this species, which he referred to as *Gordius fasciatus spinifer*, were prone to fragmenting, and he followed several fragments. He remarks that in one case in which a worm broke in two fragments. In the anterior portion, "symptoms of regeneration were early manifested by the mutilated trunk, which, in nine months, was terminated by a white spinous regeneration of considerable extent, restoring the integrity of the specimen". In contrast, the posterior fragment "healed in twenty days by a prominence on the anterior; but although surviving six months, until lost accidentally, no specific indications of what would be considered a head were shewn".

**†Lineus pictifrons*: Wesley Coe [21] reported that amputations through a region between the brain and the middle of the foregut result in regeneration of two complete worms, but posterior pieces from cuts made further back failed to complete anterior regeneration. Regeneration of the anterior and posterior ends of a fragment cut between brain and foregut was complete by 25 dpa.

**†Lineus rubescens:* In one of his comparative analysis of nemertean regeneration, Wesley Coe [17] mentions that this species regenerates like *L. pictifrons* (see above), placing both species in an intermediate category between *L. sanguineus* and poor anterior regenerators like *L. ruber*, but gives no further description. In another comparative paper of the same year, *L. rubescens* is omitted from that same category and not mentioned [51]. Furthermore, no sequence data is available for this species. Thus, while mentioned here for completeness, we opted to omit this species in our analyses.

Lineus viridis: Two individuals collected at Nahant, MA, USA, were amputated at one third or two thirds of their body length.

- *Posterior cuts:* The anterior fragments closed their wounds and healed by 1-2 dpa. Posterior cut surfaces elongated over the next week to form a normal-looking posterior end by 9 dpa.
- *Anterior cuts:* posterior fragments wound-healed by 2 dpa, but anterior surfaces did not develop a blastema or show any signs of regeneration, despite surviving past 120 dpa.

Lineus ruber: Six individuals collected at Nahant, MA, USA, were amputated at one third or two thirds of their body length.

- *Posterior cuts:* The anterior fragments closed their wounds and healed by 1-2 dpa. Posterior cut surfaces usually elongate over the next week to form normal-looking posterior end by 9 dpa.
- *Anterior cuts:* posterior fragments wound-healed by 2 dpa, but anterior cut surfaces did not form any blastema-like structure, giving the fragment a comma-like shape. Over time, smaller fragments began to shrink and rounded into a ball, developing blister-like epidermal edemas, some surviving past 90 dpa.
- *Notes*: After the initial round of experiments, one additional individual was cut in one anterior fragment cut immediately posterior to the mouth (A), six middle fragments (T1 to T6) of about equal length and a short posterior fragment (P) comprising a third of the total original length. The A fragment failed to wound heal and died within two days. Fragments T1, T4 and T6 failed to wound heal completely and died after two weeks. T2, T3, T5 and P healed their wounds, but

T3 died about two weeks later. T2 formed an elongated posterior end similar to that seen for posterior regeneration of anterior fragments, but still had no sign of an anterior blastema by 40 dpa. T5 and P never developed any signs of regeneration at healed wound sites, and over time adopted a round shape, surviving well over a month after amputation.

Lineus clandestinus: A single individual collected at Muros de Nalón, Asturias, Spain, was amputated at one-third of the body length.

- *Posterior cut:* the anterior fragment had wound-healed by 2 dpa and regenerated a normal-looking posterior end by 8 dpa.
- *Anterior cut:* the posterior fragment had wound-healed by 2 dpa but did not form a blastema or show any other evidence of regeneration until 61 dpa, when it was sacrificed for DNA extraction.
- *Notes:* This morphospecies belongs to the *L. ruber/viridis* complex, and has recently been described [22]. Being abundant and sympatric to *L. ruber* and *L. viridis sensu sctricto* in the European Atlantic shores, it has most likely been one of the *L. ruber* form A *sensu* Mieczysław Oxner and Józef Nisbaum [52,53] described as achieving posterior regeneration but not anterior regeneration.

**Lineus longissimus:* Dalyell [50] made detailed observations on the regeneration of several individuals, some of them having fragmented by autotomy, and others experimentally amputated. In one of his experiments, he made cuts to obtain "*an inch and a half of the anterior extremity, the other about three inches of the body below it. The wound of the former seemed to heal in about a week, and both those of the latter appeared to be so in three or four weeks. But although the thicker end was always in advance while crawling, it had not acquired that peculiar configuration distinguishing the head of the genus during the course of seven months, when both sections perished accidentally*". Another more recent communication (B. Vellutini, pers. comm. 2012) confirmed lack of anterior regeneration in an individual captured near Norway that had been living over 5 months in such condition.

Lineus lacteus: Eight individuals collected at Muros de Nalón, Asturias, Spain, were amputated at about half the body length.

- *Posterior cuts*: anterior fragments wound-healed between 1 and 5 dpa. Six fragments formed a small protrusion at the posterior cut site between 3 and 7 dpa, and two had regained normal-looking posterior ends by 16 dpa.
- *Anterior cuts:* several posterior fragments healed their anterior cut surfaces between 1 and 5 dpa, but none developed a blastema-like structure, despite surviving up to 79 dpa.
- *Notes:* Posterior fragments often autotomized in 2-4 pieces, none of which showed any signs of anterior regeneration. Additional experiments were performed in which the amputation plane was located right behind the brain, and the anterior fragment was followed; in five cases, those fragments were able to posteriorly regenerate a trunk in 34 ± 16.3 dpa.

**Lineus pseudolacteus (=Lineus sanguineus×lacteus):* Marie Gontcharoff [24] described the results of transversal amputations on individuals of this species.

- *Posterior cuts*: Posterior cut surfaces from amputations made behind the brain formed normal posterior ends.
- *Anterior cuts*: In posterior fragments cut after those levels, anterior cut surfaces present by 11 dpa a thin, elongated blastema with little evidence of head organ morphogenesis, and a very early developing brain. By 28 dpa, the central nervous system and cerebral organs have differentiated, but not the proboscis apparatus nor eyes. Bierne et al. [26] mentions "very late, biconvex blastemata" in this species, agreeing with Gontcharoff's description of anterior regeneration as much delayed relative to that of *L. sanguineus* (see below).

Lineus sanguineus: Over 150 individuals collected at various worldwide locations, representing most of the morphs originally described as separate species (see Table 2), were amputated between one and two-thirds of their body length.

- *Posterior cuts:* anterior fragments close their wounds by 2 dpa. Posterior cut surfaces elongate and gradually reform into normal-looking posterior ends within 6-10 dpa.
- *Anterior cuts*: posterior fragments close and heal their wounds by 2 dpa. Anterior cut surfaces present a small but evident blastema at by 6-7 dpa. By 8-10 dpa, development of new brain and cerebral organs becomes externally evident. A small invagination at the anterior end by 10-11 dpa indicates morphogenesis of the proboscis pore. Lateral grooves and a first pair of ocelli appear between 13 and 18 dpa. About 24 dpa, the regenerated anterior end matches in size the rest of the fragment and the regenerate looks like a normal, small individual.
- *Notes:* Timing of regeneration is variable depending on the size of the fragment, size and condition of the original individual, and level of the amputation plane along the antero-posterior body axis. Several additional experiments involving cutting individuals in multiple pieces showed that even small fragments will wound heal and regenerate a small but complete worm at high success rates.

Extended description of phylogenetic inferences

The main aim of this work was to survey regenerative abilities in Nemertea and see how such abilities are distributed across the phylum. To formally analyze the observed patterns of distribution, an adequate phylogenetic framework was necessary. Several papers aimed to elucidate the phylogenetic relationships across the Nemertea have been recently published, using either denser taxon representation or deeper sequencing and larger number of markers [1,3,10,42] than the dataset we obtained. However, we chose to generate new sequencing data and a corresponding new phylogenetic inference for two reasons: 1) none of the published papers include all of the species present in our survey, thus a working framework could not be obtained by pruning existing trees; 2) given the ambiguity of external morphological characters as a method of species identification, and the often reported existence of cryptic species [16,18,22], we consider that DNA sampling and barcoding is a fundamental component of this type of experimental surveys; and 3) a fully resolved phylogram with branch lengths is required for the ancestral trait estimation method we used. Thus, we sequenced, assembled and aligned data for four widely used barcoding/phylogenetic markers (COI, 16S rRNA, 18S rRNA and 28S rRNA), and used the concatenated multiple sequence alignment (MSA) to infer fully resolved trees using either maximum likelihood (ML) searches or a Bayesian approach (see Extended Methods section).

Since previous work using wider or deeper sampling has highlighted a number of problematic nodes within nemertean relationships [1,3,10,42], we also re-run our ML inferences using different sets of constraints aimed to test alternatives hypothesis (see Extended Methods section), and see how much they influenced the results of our ancestral trait estimations.

Phylogenetic trees inferred from our MSA under each set of conditions are shown in Figures S1-S6 of these Supplementary Electronic Materials. We describe below the main findings of our phylogenetic analyses and contrast them to previous work.

Among Palaeonemertea, both *Cephalothrix* and *Tubulanus* species pairs cluster together as reported in previous published analyses[1,3,10,42]. When unconstrained, *Hubrechtia* sp883 is positioned as sister group to these genera by maximum likelihood inference but as sister group to the Hoplonemertea by the Bayesian inference. The later inference resembles the position found for *Hubrechtella dubia* under some (but not all) parameter sets in [10], and reflects the instability for the phylogenetic position of the family Hubrechtiidae [54].

Hoplonemerteans sampled in this study show inferred relationships that are congruent across all analyses: *Prosorochmus* is the first to branch off, followed by a clade conformed by the brackish water *Cyanophthalma* and the freshwater *Prostoma*. Then the North and South American *Nemertopsis bivittata* branch off, followed by *Prosadenoporus*. The remaining clade splits in two, a *Zygonemertes+Poseidonemertes* clade and a *Paranemertes+Tetrastemma* clade. Our sampling of hoplonemertean species is quite different from that of other studies, and thus comparing congruence of our hoplonemertean tree with previous studies is not feasible.

As for Heteronemertea, inferred relationships are congruent across all analyses, and are mostly similar to those of previous studies [3,10,16,54,55].. *Baseodiscus* branches off basally to the rest of the groups, followed by *Zygeupolia*. A clade formed by *Siphonenteron* branches off next, followed by *Maculaura* [18]. Next to branch off is *Cerebratulus lineolatus*, followed by a clade comprising *C. lacteus*, *C. marginatus* and *Micrura chlorapardalis*. *Micrura fasciolata* branches off as sister to a clade comprising all the *Lineus* species. This clade splits in two: the *L. ruber/viridis* complex and the *L. longissimus/lacteus/sanguineus* group. Within the former complex, the North American specimens branch off sequentially, while the two European specimens both present very high sequence similarity to the haplotypes of the species recently re-described as *Lineus clandestinus* [22]. Relationship among the second group are the same as previously reported [23]. Our results also show that sequences from specimens of *Lineus bonaerensis* [56] collected near the type locality for the species fall nested within the different populations of *Lineus sanguineus*, strongly supporting *Lineus bonaerensis* Moretto, 1971 is a junior synonym for *Lineus sanguineus* (Rathke, 1799).

Supporting Figures

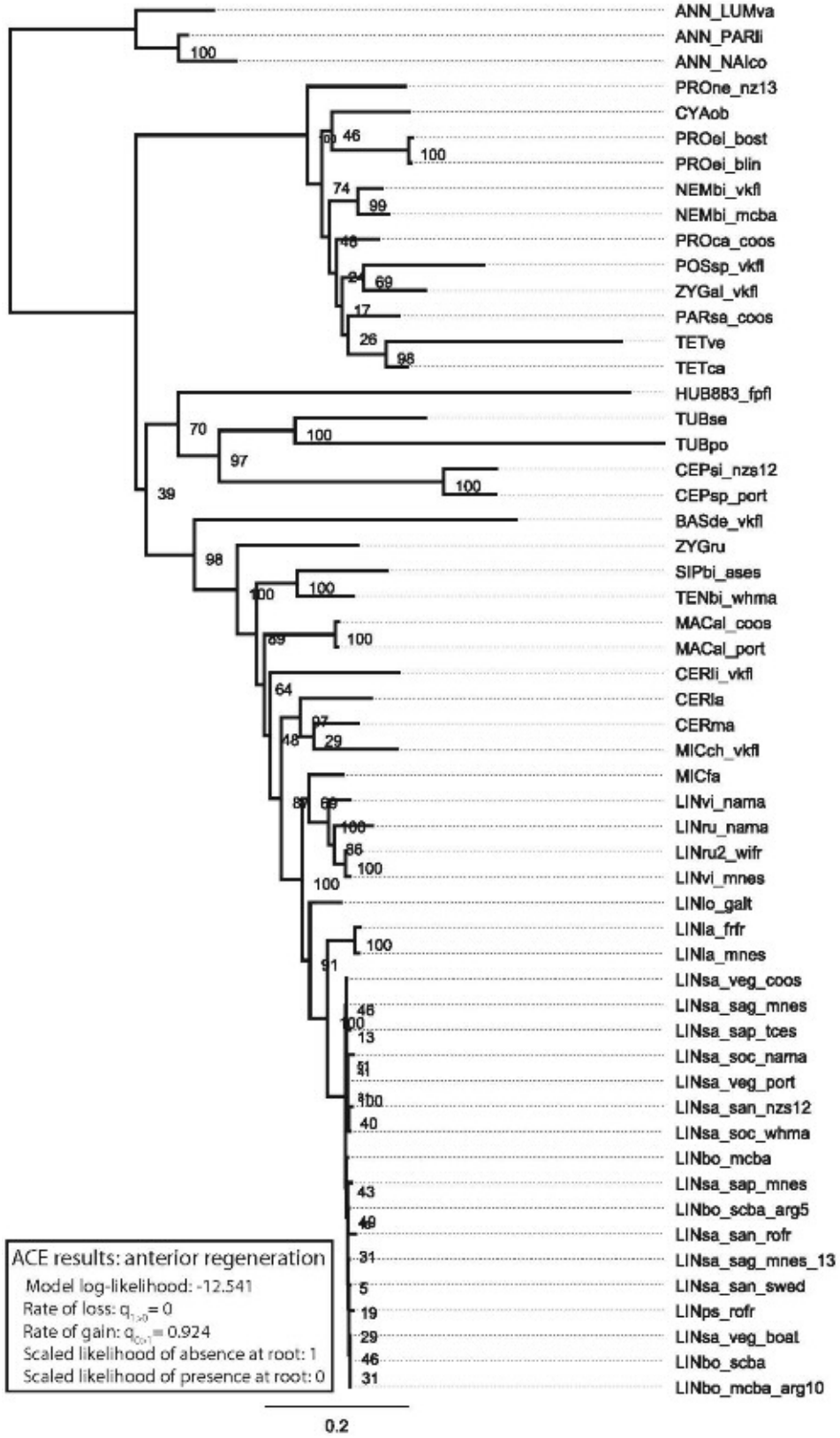

Figure S1: Best tree inferred using RaxML (rapid maximum likelihood) from the concatenated multiple sequence alignments of COI, 16S rRNA, 18S rRNA and 28S rRNA of OTUs analyzed in this study, using no constraint tree.

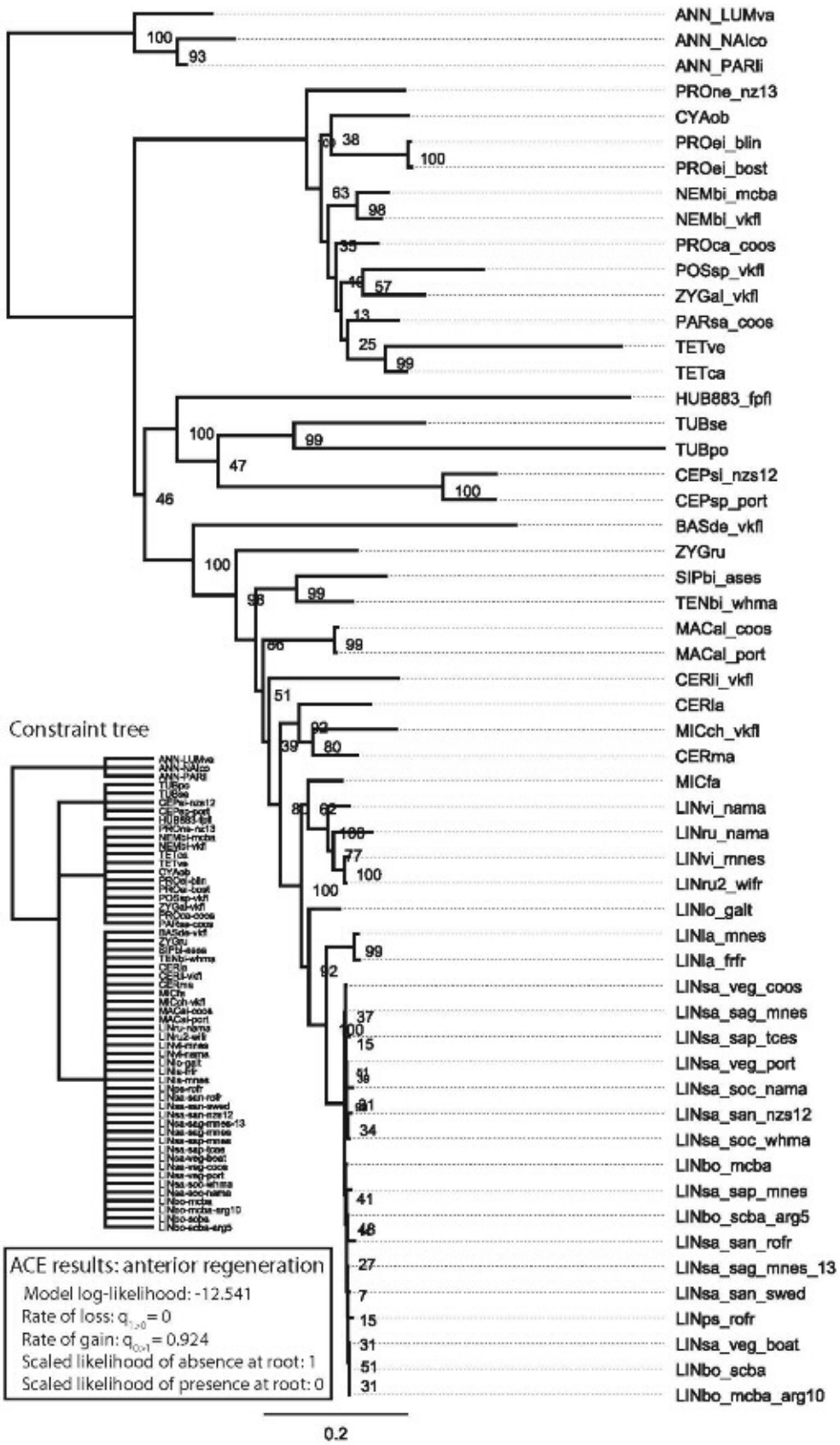

Figure S2: Best tree inferred using RaxML (rapid maximum likelihood) from the concatenated multiple sequence alignments of COI, 16S rRNA, 18S rRNA and 28S rRNA of OTUs analyzed in this study, using a constraint tree enforcing the traditional Nemertea orders.

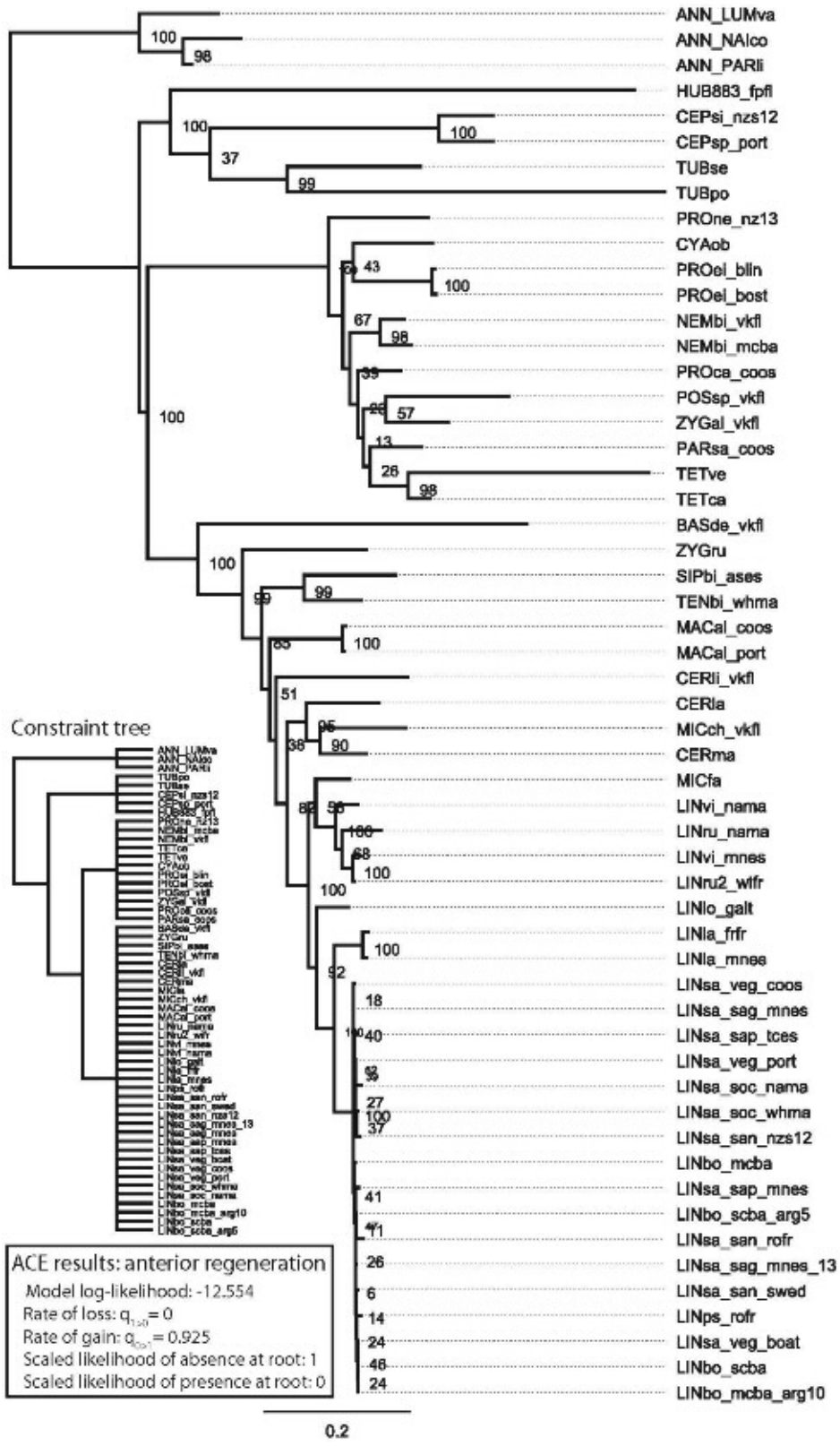

Figure S3: Best tree inferred using RaxML (rapid maximum likelihood) from the concatenated multiple sequence alignments of COI, 16S rRNA, 18S rRNA and 28S rRNA of OTUs analyzed in this study, using a constraint tree enforcing monophyletic Palaeonemertea basal to reciprocally monophyletic Hoplonemertea and Heteronemertea

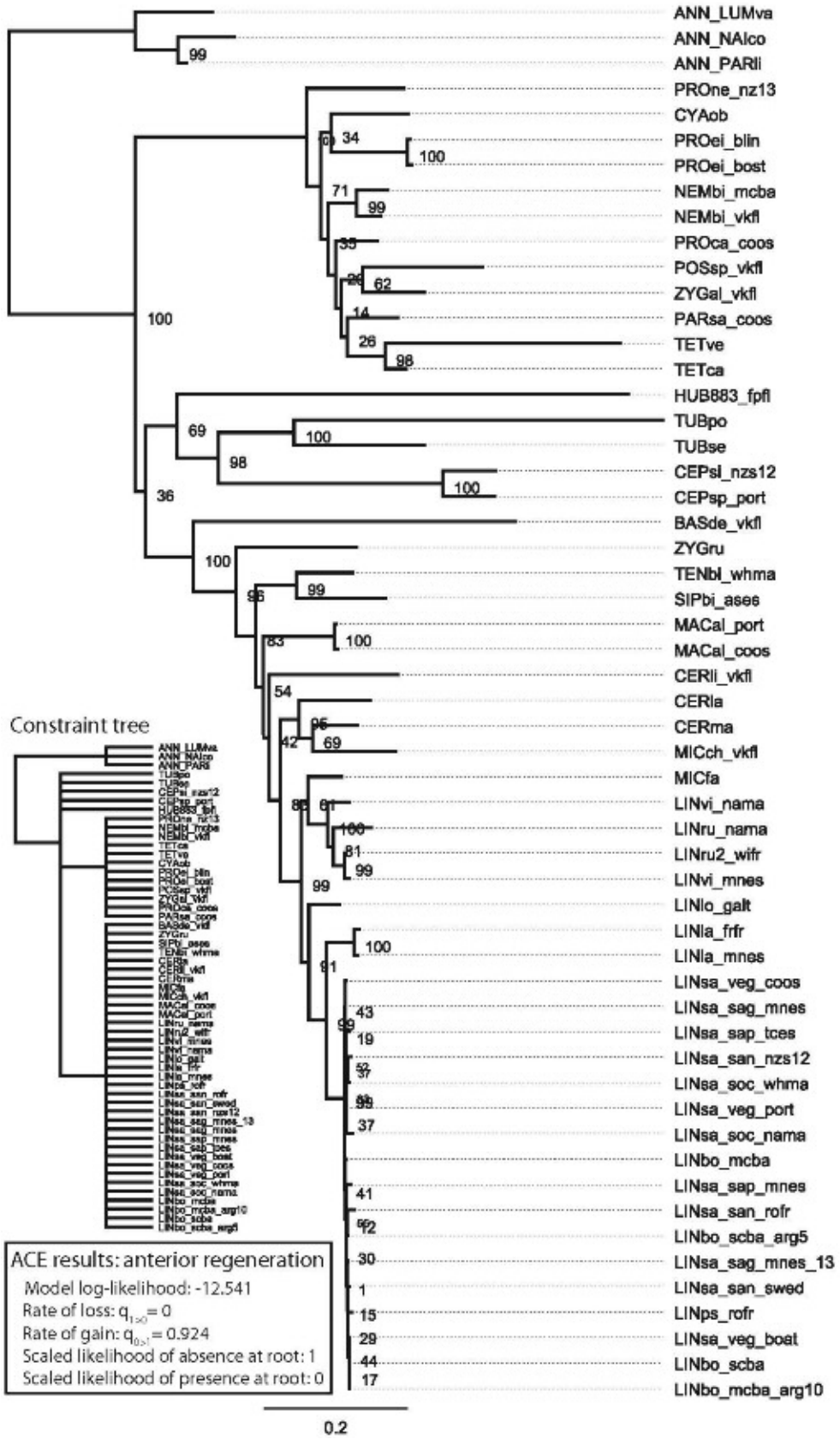

Figure S4: Best tree inferred using RaxML (rapid maximum likelihood) from the concatenated multiple sequence alignments of COI, 16S rRNA, 18S rRNA and 28S rRNA of OTUs analyzed in this study, using a constraint tree enforcing non-monophyletic palaeonemerteans but monophyletic Hoplonemertea and Heteronemertea.

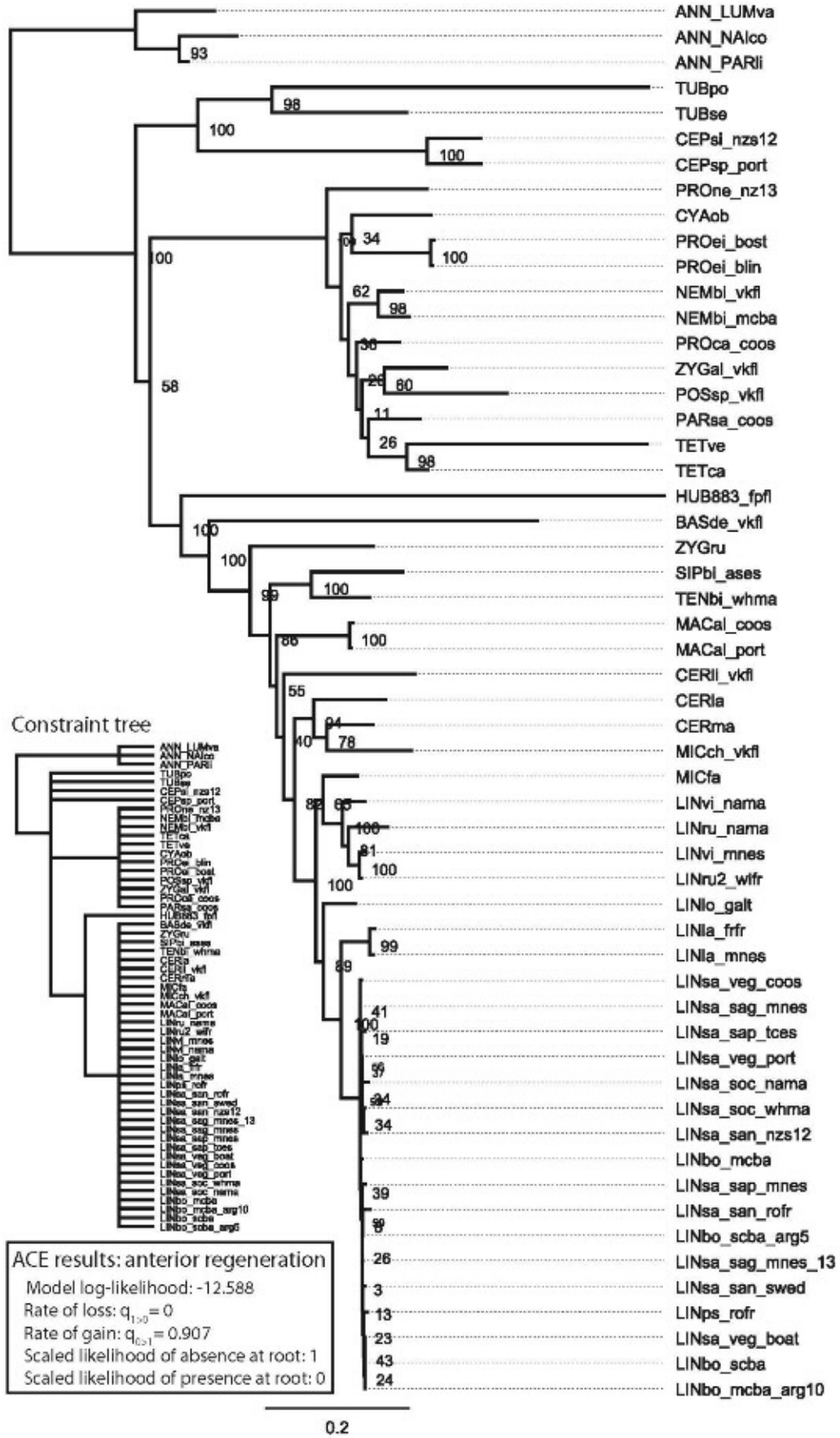

Figure S5: Best tree inferred using RaxML (rapid maximum likelihood) from the concatenated multiple sequence alignments of COI, 16S rRNA, 18S rRNA and 28S rRNA of OTUs analyzed in this study, using a constraint tree enforcing the Pilidiophora hypothesis.

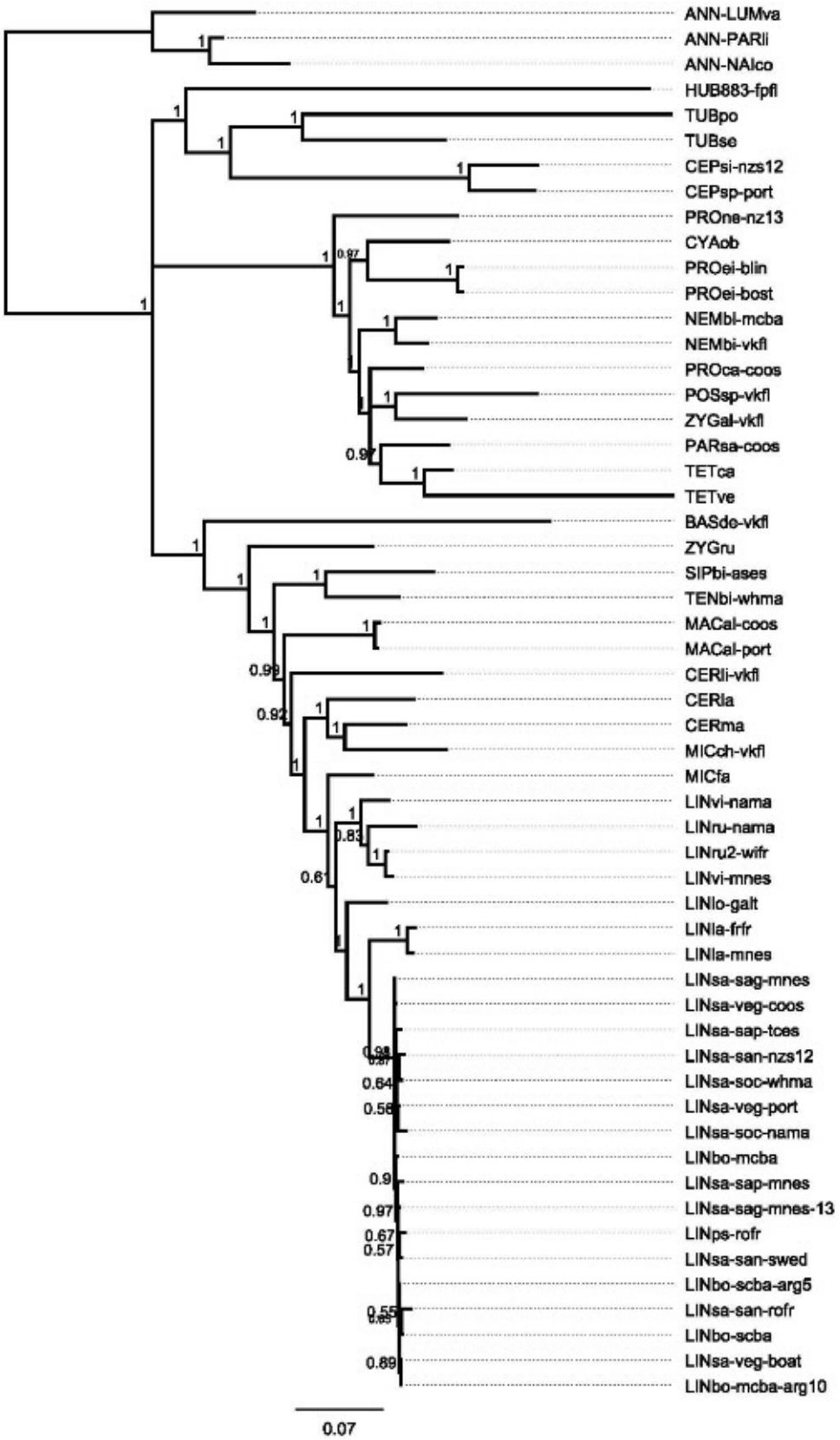

Figure S6: Best tree inferred using MrBayes (Bayesian inference) from the concatenated multiple sequence alignments of COI, 16S rRNA, 18S rRNA and 28S rRNA of OTUs analyzed in this study, using no constraint tree.

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