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

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Animals vary widely in their ability to regenerate, suggesting that regenerative ability has a rich evolutionary history. However, our understanding of this history remains limited because regenerative ability has only been evaluated in a tiny fraction of species. Available comparative regeneration studies have identified losses of regenerative ability, yet clear documentation of gains is lacking. We assessed ability to regenerate heads and tails either through our own experiments or from literature reports for 35 species of Nemertea spanning the diversity of the phylum, including representatives of 10 families and all three orders. We generated a phylogenetic framework using sequence data to reconstruct the evolutionary history of head and tail regenerative ability across the phylum and found that all evaluated species can remake a posterior end but surprisingly few could regenerate a complete head. Our analysis reconstructs a nemertean ancestor unable to regenerate a head and indicates independent gains of head regenerative ability in at least four separate lineages, with one of these gains taking place as recently as the last 10–15 Myr. Our study highlights nemerteans as a valuable group for studying evolution of regeneration and identifying mechanisms associated with repeated gains of regenerative ability.

1. Introduction

Regeneration, the ability to regrow a body part following traumatic loss, is a fascinating phenomenon that occurs in many animal groups. Regeneration of specific body structures (e.g. heads, tails, appendages) and regeneration from a tiny fragment (whole body regeneration) are both found scattered across metazoans [1,2]. Lineages that are sister to Bilateria (i.e. Porifera, Ctenophora and Cnidaria) generally possess high regenerative ability, suggesting that early animals had high regenerative ability [1,3,4]. Within Bilateria, however, regenerative ability is extremely variable, indicating a complex pattern of regeneration evolution. Within phyla of Ecdysozoa, regenerative abilities are generally very restricted, with limb regeneration in Arthropoda being the main exception [5]. Both across and within most other bilaterian phyla, however, regenerative ability ranges widely. Species with extensive regenerative ability are common in Xenacoelomorpha [6,7], in deuterostome phyla such as Echinodermata [8], Hemichordata [8], and Chordata [9,10], and in spiralian phyla such as Platyhelminthes, Mollusca, Annelida and Nemertea [11]. However, most of these same phyla also include representatives with modest or even extremely limited regenerative ability, indicating that the pattern of regeneration evolution is complicated.

Estimating where and when changes in regenerative abilities have occurred across animal phylogeny is a fundamental step towards understanding how regeneration evolves. However, the vast majority of relevant basic and applied research on regeneration has focused on a small set of species that are deeply diverged from one another [2,4,12–15], while knowledge about regenerative ability remains extremely sparse for most animal phyla, limiting our ability to infer patterns and mechanisms of regeneration evolution. The few studies comparing closely-related species that differ naturally in their ability to regenerate homologous body parts have proved extremely informative, demonstrating for example that variation in regenerative ability can result from just a few changes in key molecular and developmental processes [16–18]. Expanding the number of groups in which regeneration increases (i.e. gains) and regeneration decreases (i.e. losses) are well documented is likely to provide new insights into regeneration evolution.

Losses of regeneration have been inferred in several animal groups [19]. Early animals probably could regenerate well, with restrictions in regenerative ability evolving later, especially within Bilateria. Comparisons of regenerative ability between phyla are difficult to interpret, however, owing to issues regarding homology (e.g. how can regenerative ability across species be compared if the structures being regenerated have unclear homologies?) and because there may be considerable variation within each of the phyla being compared (such that the ancestral states for the phyla being compared are unclear). To date, only one study has analysed regenerative ability across an entire phylum to reconstruct ancestral states and formally identify putative gains and losses [20]. This recent study inferred the last common ancestor of extant Annelida as having both anterior and posterior regenerative ability. The study also identified many losses, of both anterior and posterior regeneration, but despite the large dataset of several hundred annelid species, it identified no gains of regeneration.

Evidence for clear increases of regenerative abilities in animals is thus far very limited. Limb regeneration in arthropods probably represents a gain of regenerative ability, given the extremely limited regenerative abilities of most ecdysozoans [5]. Limb regeneration in salamanders and tail regeneration in lizards also may represent gains, given the weak or absent regeneration of these same structures in the closest relatives of these groups [21]. Although these putative gains are interesting, they would have happened at phylogenetic nodes so deep that comparative approaches have little chance to uncover meaningful mechanistic insights into their underlying causes. By contrast, identifying more recent gains of regenerative ability would potentiate studies of the proximate (developmental) and ultimate (evolutionary) causes behind regeneration enhancements. To date, no comparative studies have yet uncovered clear gains of regeneration across relatively close groups.

Ribbon worms (phylum Nemertea) are a promising group for investigating the evolution of regeneration. Nemerteans are elongated, primarily marine predatory worms. The phylum has a reputation for possessing high regenerative abilities, but this reputation is based almost entirely on the remarkable ability of one species: *Lineus sanguineus* Rathke. This species unquestionably is one of the champions of regeneration, possessing some of the highest regenerative abilities known among animals [11]. Individuals of this species can be repeatedly amputated until the resulting worms that regenerate are just 1/200 000th of the volume of the original individual. Furthermore, a complete animal can regenerate not only from a thin transverse slice of the body, but even from just one *quadrant* of a thin slice (with a large majority of the fragment's surface area being wound surface) [22]. Yet such regenerative ability does not appear to be typical for this phylum. Nemertea comprises approximately 1200 known species and regenerative abilities have been described from a few of those, yet none comes close to the remarkable ability of *L. sanguineus*. Furthermore, there probably has been a publication bias against reporting findings from poor regenerators, as is suggested for other groups [19,20]. Thus, regenerative ability appears to be variable among nemerteans, but the phylogenetic pattern within this phylum remains very poorly understood.

In the context of current understanding of nemertean phylogeny, the limited regeneration data available yield at best a blurry picture of regeneration evolution in this phylum [11]. Nemerteans comprise three groups: Palaeonemertea, Hoplonemertea, and Pilidiophora [23–26]. Palaeonemertea are probably a paraphyletic assemblage of basal lineages, and no regeneration data is available in the literature for any species in this order. Hoplonemertea is a well-supported clade, with most species reported in the literature to have quite limited regenerative ability; unfortunately, most reports of regeneration are presented as blanket statements, without specifying the species examined [27]. Pilidiophora (Heteronemertea) is a large and well-supported clade, and many species are frequently cited as examples of nemerteans with outstanding regenerative ability [28,29]. However, all of these 'many species' [22,27,30–33] now have been synonymized to *L. sanguineus* [34–36]. Thus, regeneration data remain very cursory across Nemertea but do suggest that high regenerative ability—in particular, the ability to regenerate a head—may be uncommon in the phylum. Systematic testing of regenerative ability of well identified species is clearly needed to resolve the pattern of regeneration evolution in this phylum. We addressed this knowledge gap by surveying regenerative abilities across the phylum, performing new regeneration experiments and compiling data from existing reports, and by analysing the resulting data in a phylogenetic context to reconstruct the pattern of gains and losses of regenerative ability across the phylum.

2. Material and methods

(a) Regeneration survey

Nemerteans were collected worldwide between 2012 and 2014, on coasts along the United States, Argentina, Spain, and New Zealand. Tables S1 and S2 in the electronic supplementary material provide a full list of locations, collectors and taxonomic nomenclature. Owing to the patchy distribution and low abundance of many species, sampling was opportunistic and sample size per species was often low, but we aimed to collect specimens from all major lineages within the phylum.

For regeneration experiments, we bisected worms by cutting transversely, generating an anterior and a posterior fragment. We cut at approximately one-third the total body length (see the electronic supplementary material), so that the amputation plane was posterior to the mouth and the cephalic nervous system (brain and cerebral organs). Sample sizes ranged from 1 to greater than 40 animals cut per species (resulting in twice

this many fragments). We maintained amputated specimens in sea or fresh water, without food, and scored survival and externally visible post-amputation phenotypes for days to weeks (depending on the species). We used standardized morphological and behavioural criteria (detailed in the electronic supplementary material) to determine whether amputated specimens showed evidence of posterior and/or anterior regeneration of the missing end, as well as the time to complete regeneration (when present). Regeneration of each type (anterior, posterior) was scored as present if at least one individual regenerated fully, even if not all experimental individuals completed all landmarks. When multiple individuals were scored, approximate times for each landmark were summarized and reported as a range. For completion of regeneration, the fastest cases were reported; for survival without regeneration, the longest survival times were reported. Experimental specimens showing clear signs of poor health or abnormal development were excluded from timing estimations.

We expanded our dataset with previously published data, using literature searches as described in [20]. 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.

(b) Molecular marker sequencing

DNA was extracted using a DNeasy 96 Blood & Tissue Kit (69581, Qiagen) from at least one individual of each species used in regeneration experiments. Whenever possible, the extraction was made from an individual included in our amputation experiments; else we used a conspecific individual from the same field collection. We amplified fragments of cytochrome oxidase subunit I (COI), 16S ribosomal RNA (16S), small subunit ribosomal RNA (18S) and large subunit ribosomal RNA (28S). Primer sequences and polymerase chain reaction (PCR) parameters are detailed in the electronic supplementary material. PCR products were purified using ExoSAP-IT (Thermo-Fisher) and sequenced in paired reactions using respective forward and reverse primers with the BigDye™ Terminator v. 3.0 Cycle Sequencing Kit v. 3.0 (Applied Biosystems). Sequencing products were analysed using an ABI Prism 3730xl Genetic Analyzer capillary sequencer (Applied Biosystems). For several species of *Lineus*, sequences were obtained from published transcriptomes [36]. In the few cases in which we had regeneration data (either from our experiments or the literature) but no associated sequence data, we retrieved relevant sequence data available from NCBI.

(c) Sequence alignment and phylogenetic reconstruction

Sequence quality assessment, assembly, alignment and phylogenetic reconstruction were performed using Geneious 8.1.9 [37]. Markers were aligned into multiple sequence alignments (MSA) using MAFFT [38], curated by eye and concatenated. Concatenated MSAs were inputted to RAxML v. 8.2.11 [39], 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 on six partitions. The inference was run first without topological constraints and then re-run with alternative topological constraints reflecting different hypotheses about deep phylogenetic relationship within the Nemertea (see the electronic supplementary material for details). We also performed Bayesian inference using MrBayes 3.2.6 [40], specifying a GTR model with four categories of gamma distributed rate heterogeneity and a proportion of invariant sites, and no topological constraints. 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.

(d) Ancestral trait estimation by maximum likelihood

Best scoring trees from each analysis were used as phylogenetic frameworks for character mapping and ancestral trait estimation. We coded regenerative ability as two binary variables, presence/absence of anterior regeneration and presence/absence of posterior regeneration. Then, we generated a matrix that included these two variables for each taxon (species or population) in our MSA. We used the *ace* function from the *ape* package [41], which models discrete trait state evolution as a Markovian process and incorporates phylogenetic tree branch length information to estimate the rates of change of the trait and the likelihood of each character state at every node of the tree, including the basal node. A two-parameter model was specified allowing for separate estimation of the rate of gain $(0 \rightarrow 1)$ and rate of loss $(1 \rightarrow 0)$. We repeated this procedure for all the trees inferred using the different constraint sets. All analyses were run within the R computing environment [42].

3. Results

(a) Regeneration survey

We collected and performed regeneration experiments on 22 nemertean species: four species of Palaeonemertea, six species of Hoplonemertea and 12 species of Pilidiophora. We also obtained data from the literature for 13 additional species, producing a final regeneration dataset of 35 species (see the electronic supplementary material, table S4). Although the number of species in our dataset is a small fraction of the known nemertean diversity, it nonetheless represents 10 of the most diverse families, and spans all three orders (two out of three palaeonemertean families, four out of 20 hoplonemertean families and all four pilidiophoran families [43]).

Outcomes of regeneration experiments for each species are described in the electronic supplementary material. Overall, we found that in all species, most individuals (greater than 90%) survived the initial amputation, and fragments usually healed the wounds within 5 days post-amputation (dpa; electronic supplementary material, table S4). All species were able to complete posterior regeneration (figure 1). However, most species (27 out of 35) were not capable of regenerating a complete head (including a brain), despite many species being able to survive without the missing structures for several weeks or months (figure 2; electronic supplementary material, table S4).

Successful head regeneration was documented in four species where it was previously unreported: *Tubulanus ruber* and *Tubulanus sexlineatus* (Palaeonemertea), and *Baseodiscus delineatus* and *Cerebratulus lineolatus* (Pilidiophora). We also observed head regeneration in *L. sanguineus* (Pilidiophora) (previously known to regenerate anteriorly) from several collection locations. In addition, our literature review identified three more species able to regenerate a head: *Lineus pseudolacteus* [44] and *Lineus pictifrons* [30] (Pilidiophora); and *Prostoma graecense* [45] (Hoplonemertea).

(b) Sequencing and phylogenetic framework inference

We collected 114 new Sanger sequences, 55 RNAseq-based sequences, and 35 sequences retrieved from NCBI, for four phylogenetic markers (COI, 16S, 18S and 28S). New sequences have been deposited at NCBI (see the electronic supplementary material, table S5 for accessions). Sequences were aligned into an MSA 8123 bp long that was used to
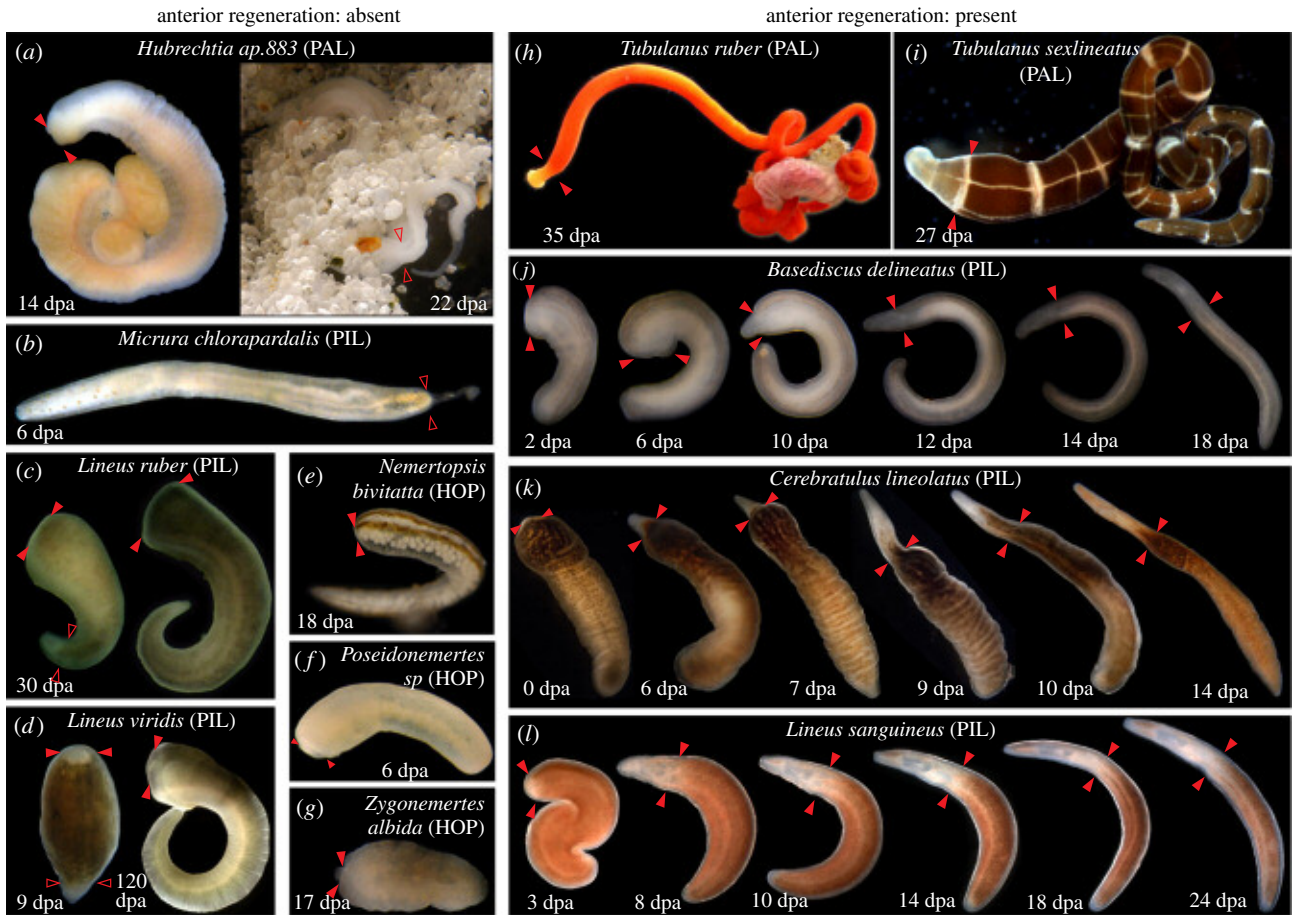
Figure 1. Regenerative ability in representatives of Nemertea. Photos of representative individuals undergoing successful posterior regeneration (*a*–*d*), failed anterior regeneration (*a*–*g*) and successful anterior regeneration (*h*–*l*). Individuals shown were amputated posteriorly and/or anteriorly (time since amputation is shown at bottom). Species in which anterior regeneration was scored as absent are on the left; species in which anterior regeneration was scored as present are on the right. Plane of posterior amputation is indicated by paired, empty arrowheads (*a*–*d*); plane of anterior amputation is indicated with paired, filled arrowheads (*a*,*c*–*l*). Panels *j*–*l* show regeneration time series of the same experimental individual over time. All individuals within the same panel are at the same scale. Anterior is left, upper left, or up. PAL: Palaeonemertea; HOP: Hoplonemertea; PIL: Pilidiophora; dpa: days post-amputation.

infer phylogenetic trees using maximum-likelihood searches (RAxML trees) and Bayesian inference (MrBayes tree). When no topology constraint was enforced, both methods found mostly congruent trees, with monophyletic Palaeonemertea, Hoplonemertea and Heteronemertea (figure 2 and electronic supplementary material, figures S1–S6). The only difference between the inferences was that the RAxML tree grouped Palaeonemertea and Heteronemertea into a sister group to Hoplonemertea, while in the MrBayes tree the branching order of the three clades was not resolved. When topology constraints were enforced (see Methods), the resulting inferences differed only in the enforced bipartitions, but the internal topology of the remaining clades did not differ from the unconstrained trees. Our results are similar overall to those of previous studies [23–26,46], and are further described in the electronic supplementary material.

(c) Ancestral character estimation analysis

Given that posterior regenerative ability was invariant (present throughout our dataset), no further formal analyses were performed for this trait. As for anterior regenerative ability, we found that analyses based on any of the inferred phylogenetic trees gave the same qualitative results. Specifically, all analyses strongly support the absence of anterior regenerative ability at the root node of Nemertea (figure 2).

Runs on alternative topologies yielded only minor differences in the resulting log-likelihoods (ranging from −12.60 to −12.54) and transition rate parameters (ranging from 0.9 to 1.4 for gains, and 0 for losses).

Based on the likelihood of anterior regeneration being present or absent at each node of the trees, our analyses suggest at least four independent gains of anterior regeneration across the phylum (figure 2): one in the *Tubulanus* lineage, one in the *B. delineatus* lineage, one in the *C. lineolatus* lineage and one in the *L. sanguineus* lineage.

4. Discussion

We found that all nemertean species investigated were able to reform a posterior end but that most (27 out of 35) species were not able to regenerate a complete head (including a brain), even if individuals survived several weeks or months after amputation. This general pattern was previously suggested by several nemertean researchers based on more limited and largely unpublished observations [27,43,47,48]. Our study, which includes far more species than previously considered and broader coverage across the phylum, supports these early inferences and provides strong evidence that anterior regenerative ability is neither common nor ancestral in Nemertea. The gains of anterior
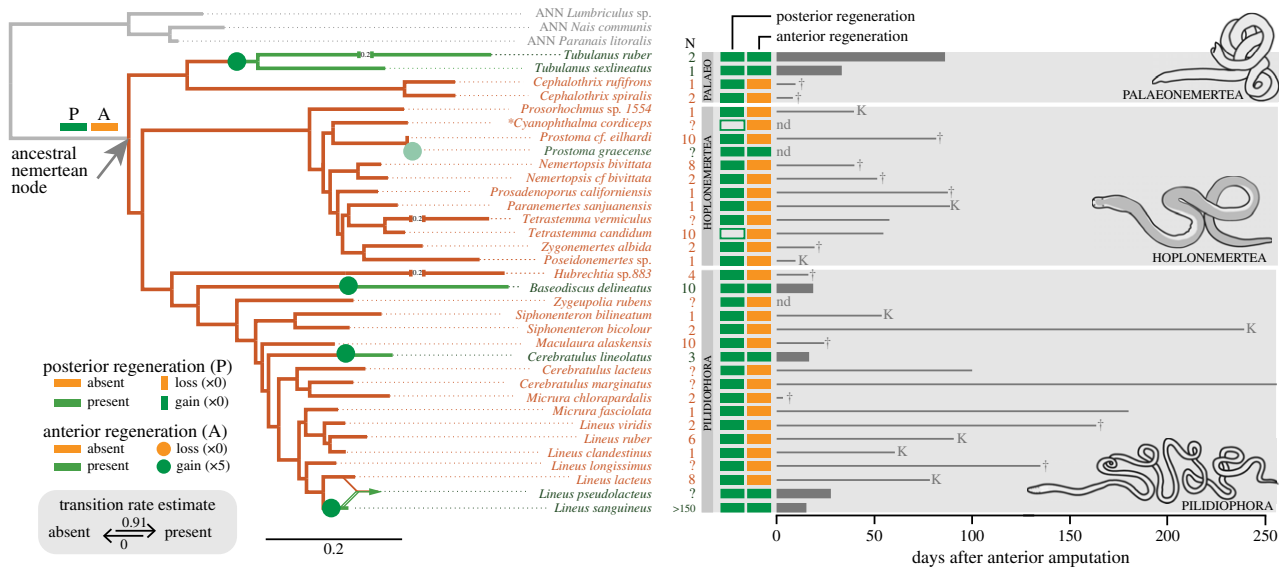
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Figure 2. Phylogenetic distribution of regenerative abilities in the phylum Nemertea. Maximum-likelihood tree from aligned and concatenated sequences of two mitochondrial (COI, 16S) and two nuclear (18S, 28S) markers, inferred by RAxML with a GTRGAMMA partitioned model constrained to the Pilidiophora hypothesis (see Methods). Branch colours represent the estimated state for anterior regenerative ability (orange: absent, green: present); grey branches lead to three outgroup species (ANN: annelids). Numbers on broken branches indicate abridged distances. Green circles represent evolutionary transitions; the fifth, lighter green circle indicating a gain in *Prostoma graecense* is placed based on the position of *P. eilhardii* in our analyses. Location of a transition along a given branch is arbitrary. Converging lines leading to *Lineus pseudolacteus* represent the suggested hybrid origin of this species. Column N provides sample sizes of regeneration experiments for each species; if sample size was not indicated in a report, sample size is shown as '?'. Orange and green boxes to the right of species names indicate regenerative abilities (orange: absent, green: present; outlined boxes represents putative results) scored experimentally or obtained from the literature. For anteriorly regenerating species, bars to the right indicate days to complete anterior regeneration. For non-anteriorly regenerating species, lines to the right indicate longest survival time in days without a head. For species experimentally assessed, dagger symbol indicates natural death while K indicates sacrifice or accidental death of the longest surviving individual. Data from literature reports with no survival time are marked 'ND'. *Lineus pictifrons* is not included in this diagram because there are no molecular data available to infer the phylogenetic position of this species.

regeneration ability we identify represent some of the most clearly documented increases of regenerative ability, and, to our knowledge, the first well-documented gains of head regeneration ability among animals.

(a) All nemertean species investigated can reform a posterior end, but most cannot regenerate a complete anterior end

The ability to reform the posterior end appears to be widespread, and far more common than anterior regeneration in many animal groups, including annelids, platyhelminths, molluscs, and vertebrates [1,11]. Thus, finding that posterior regeneration is also prevalent among nemerteans is not unexpected. However, it should be noted that scoring for the reformation of the posterior end is challenging in nemerteans, especially when assessments are limited to external observation (as was the case in our study). Many species lack any morphologically distinctive posterior structures, and, in the absence of these, the only externally scorable posterior traits are the anus and a diffuse posterior growth zone [49]. Observing defecation and/or elongation of the newly formed posterior end is the only definitive way to determine that posterior regeneration is indeed complete, but this is challenging to do as most species will not feed in laboratory settings (precluding defecation and growth). We scored for the reformation of the posterior end based on the reappearance of any distinctive posterior structures (if these were present in the species), of the anus, and of the diffuse posterior growth zone. The possibility remains that posterior

regenerative abilities have been overestimated in our survey and thus future studies involving feeding (so that defecation and posterior elongation can be scored) and histological analysis (to definitively score for anus formation) should be performed to confirm our results.

The ability to regenerate anteriorly was found to be far more limited across Nemertea than the ability to regenerate posteriorly. Of the eight species found capable of anterior regeneration, four were previously known; this study represents, to our knowledge, the first report of anterior regenerative ability in four other species. The anteriorly-regenerating species *L. sanguineus* (including forms described as *Lineus nigricans*, *Lineus socialis*, *Lineus vegetus* and *Lineus bonaerensis*) plus the hybrid species *Lineus pseudolacteus* [36] were previously described as possessing outstanding regenerative abilities. We also found reports in the literature of complete head regeneration after amputation, at a narrow range of positions, for the hoplonemertean *P. graecense* [45] and the pilidiophoran *L. pictifrons* [30]. Our work is, to our knowledge, the first to report the presence of anterior regenerative ability in the palaeonemerteans *T. ruber* and *T. sexlineatus*, and the pilidiophorans *B. delineatus* and *C. lineolatus*.

Our confidence in accurately scoring species for anterior regenerative ability is high for several reasons. First, unlike posterior regeneration, anterior regeneration in nemerteans involves clearly recognizable intermediate stages, including the formation of a blastema that is evident morphologically (being composed of a tightly packed mass of cells with low pigmentation). Second, amputation of the head removes the mouth, and thus halts the ability to feed, such that food availability cannot influence regeneration output. Therefore, we

expect high accuracy in detecting both the presence and the absence of anterior regenerative ability, despite low sample sizes. Nonetheless, evidence for the absence of anterior regeneration is necessarily weaker than evidence for the presence of anterior regeneration, especially in those species for which only a few specimens were available for experimental assessment. Thus, we hope that future regeneration studies will be performed on a broader range of nemerteans to corroborate and expand our findings.

(b) The nemertean last common ancestor probably lacked anterior regenerative ability

Reconstructing the ancestral regenerative abilities of individual animal phyla is a key step towards understanding the broad pattern of regeneration evolution in animals. Knowing the ancestral regenerative condition is necessary to polarize changes in regenerative ability within a phylum (e.g. to determine whether regeneration gains or losses have occurred) and is critical for making meaningful comparisons of regeneration between phyla. We thus used our data to investigate whether the ancestral nemertean could regenerate a complete head.

Our ancestral character estimation analyses consistently yielded a zero likelihood for anterior regeneration being present in the last common ancestor of Nemertea. This outcome was found even when considering alternative topologies (including one where tubulanids—anteriorly-regenerating palaeonemerteans—represent the most basally branching lineage). This result stands in sharp contrast to the widespread regenerative capabilities of basal bilaterians [1,4] and also to results from a similar analysis made on the phylum Annelida that found strong support for anterior regeneration being present at its basal node [20]. The contrast between Nemertea and Annelida is all the more striking as both phyla are within the same bilaterian subclade, Spiralia, and both are soft-bodied elongated animals ('worms') with a similar level of body complexity.

The closest relatives of Nemertea have been relatively poorly sampled for regenerative ability, but regeneration of particular body regions is known from three phyla closely related to Nemertea: Phoronida, some of which can regenerate the primary body axis [50], Brachiopoda, some of which can regenerate the shell, lophophore and pedicle [51], and Mollusca [52], some of which can regenerate the foot, tentacles, mantle, and eyestalks but which, as a group, does not appear to have widespread, extensive regenerative abilities [11]. Also within Spiralia are the Platyhelminthes, with both highly and weakly regenerating representatives [11]. Even though more extensive regeneration surveys and formal ancestral state estimation are needed for these other spiralian phyla, placing our results for nemerteans in the broader context of our current knowledge suggests that the Spiralia subclade of bilaterian animals has had a rich evolutionary history with respect to regeneration and that regenerative ability was highly variable even at deep nodes within this clade.

(c) Head regenerative ability evolved independently at least four times within Nemertea

The most unexpected finding of our study is that anterior regenerative ability evolved several times among Nemertea.

Mapping our regeneration dataset to nemertean phylogeny indicates four separate gains of anterior regeneration: one among Palaeonemertea and three among Pilidiophora. The origin within Palaeonemertea involves two species of the same genus (T. sexlineatus and T. ruber) that represent two fairly diverged subclades within the genus [53], indicating a gain of anterior regeneration that could be quite old. By contrast, within Pilidiophora, two gains involve a single species each and the third involves a pair of very closely related species, indicating that some origins of anterior regeneration within Nemertea could be relatively recent.

The number of origins of anterior regeneration in Nemertea is likely to be greater than the four formally identified in our analysis. In particular, two additional species are also reported in the literature as being capable of regenerating a full head, albeit under a narrow range of conditions: the pilidiophoran L. pictifrons [30] and the hoplonemertean P. graecense [45]. Lineus pictifrons was described by Coe [30] as being able to regenerate an anterior end including the brain when amputated behind the mouth (which is posterior to the brain in this species), an observation we consider reliable given that Coe did extensive work on nemertean regeneration (including groundbreaking work on regeneration in L. sanguineus). Unfortunately, absence of sequence information precluded us from including this species in our analysis. Determining whether L. pictifrons represents yet another origin of anterior regeneration must await further studies that can place this species within the nemertean phylogeny. As for P. graecense, this species was reported by Kipke to regenerate a complete head [45], although only when the amputation plane is immediately behind the brain. We were unable to procure specimens of this species, precluding us from confirming this report. However, we did have regeneration and sequence information for another species of Prostoma, Prostoma eilhardii. Although P. eilhardii is thought to be either very closely related to P. graecense or even its junior synonym [54], it showed no evidence of anterior regeneration in our experiments. Thus, if Kipke's report is confirmed, P. graecense would represent another very recent gain of anterior regeneration and would also indicate that gains have also occurred within the third major nemertean clade, the Hoplonemertea. If future studies corroborate these preliminary conclusions, then six gains of anterior regeneration would be inferred within Nemertea, including gains within all three major clades of the phylum.

Sampling additional nemertean species will be critical for strengthening or revising our understanding of the evolution of anterior regeneration in this phylum. Assessing additional basal pilidiophorans is important to better evaluate the ancestral condition of Pilidiophora (which in our dataset is strongly influenced by the lack of anterior regeneration in the pilidiophoran Hubrechtia). More extensive sampling of Palaeonemertea is also needed, as only four species were included in our dataset, yet sampling within this group is critical for confidently reconstructing the nemertean ancestral regeneration ability.

Finding evidence of several independent gains of head regeneration within nemerteans suggests the possibility that certain nemertean traits might facilitate evolving this developmental capability. One such trait could be the ability to survive without a head for an extended period. We documented survival of cut fragments for up to many months without regeneration (figure 2; electronic supplementary material,

table S4), consistent with anecdotal observations made by other researchers. This finding is important for several reasons. First, the confidence in determining that a species fails to regenerate increases with survival time of the amputee. Second, long-term observations of amputees are crucial to assess regenerative abilities, as regeneration rates can vary widely, both among and within species. And third, the ability to survive without a lost structure long enough to allow for regeneration is considered a fundamental requirement for regenerative ability to be acted upon by selection [55]. Thus, the ability of nemerteans to survive without a head may be a key pre-adaptation enabling evolutionary gains of anterior regeneration.

But how can regeneration be gained? A potential mechanism for evolving head regeneration in adults is redeployment of embryonic or larval regeneration. In several animal groups, embryos and larvae have higher regenerative abilities than adults [29]. Relevant data for nemerteans are limited, but it is known that a bisected embryo of the pilidiophoran *Cerebratulus lacteus* can regulate development to form two complete larvae, and that a bisected embryo of the hoplonemertean *Nemertopsis bivittata* develops instead as two half-larvae [56] while neither of these species can regenerate their heads as adults. High larval regenerative ability has also been documented for some pilidiophorans [57]. Comparative developmental studies across life stages are needed to test whether such early life-stage regenerative capabilities underlie gains of adult regenerative ability.

(d) *Lineus sanguineus* and its close relatives can be studied as a model system of regeneration gain

Phylogenetic distribution of regenerative abilities across Metazoa suggests that early animals, including the bilaterian stem group, were likely to have high regenerative ability [1] and that evolutionary loss of regenerative abilities appears to be far more common than gains [19]. As a consequence, our understanding of evolutionary change in regenerative ability is based almost exclusively on studies of the loss of regeneration [16,17]. Studying gains of regeneration would not only greatly improve our understanding of the developmental strategies that enable and enhance regenerative processes, but also offer insights into the organismal traits that can facilitate or constrain such gains. Unfortunately, the few cases of evolutionary gains of regeneration previously described map to deep branches of the metazoan tree, and thus are too ancient to provide strong insight into the proximate causes of regeneration gains.

The trio of *Lineus* species including *L. sanguineus*, *L. pseudolacteus* and *L. lacteus* constitutes a powerful group in which to study the gain of regenerative ability. Of the gains identified in our study, the one represented by *L. sanguineus* and *L. pseudolacteus* stands out: our analysis demonstrates that the spectacular and well-documented regenerative ability of *L. sanguineus* emerged recently from a clade in which anterior regeneration is ancestrally absent. The closest relative of *L. sanguineus*, *L. pseudolacteus*, has recently been identified by transcriptome sequencing as a hybrid species descended by exclusive asexual reproduction from a triploid founding individual, probably resulting from the fertilization of an unreduced *L. sanguineus* egg by a *L. lacteus* sperm [36]. This hybrid origin could explain why *L. pseudolacteus* individuals possess regenerative ability intermediate between that of

L. sanguineus and *L. lacteus* (which cannot regenerate anteriorly) [44]. Thus, this species group provides an unparalleled set of advantages for future study of the evolution of regeneration: the two non-hybrid species, *L. sanguineus* and *L. lacteus*, straddle a clear gain of regeneration; the age of the regeneration gain is recent (estimated at less than 10 Ma [36,58]); three degrees of regenerative ability are represented by the group, from non-anteriorly regenerating (in *L. lacteus*), to anteriorly regenerating in limited contexts (in *L. pseudolacteus*), to extremely robust anterior regeneration (in *L. sanguineus*); the three species are accessible, being found in similar inter- and subtidal substrates along the European coasts in reasonably large numbers to make their study convenient; and many aspects of their biology have been well described [22,31–33,48,49,59–64], providing a solid foundation on which to base new studies, including ones using the newest molecular tools.

5. Conclusion

Although Nemertea includes a few species with outstanding regeneration capabilities, and although posterior regeneration was found to be widespread, our study indicates that the ability to regenerate a head is a derived feature within the phylum. Specifically, anterior regenerative ability is uncommon, is inferred to have been absent in the last common ancestor of the phylum and is inferred to have evolved independently at least four times. One of these gains, involving *L. sanguineus*, appears to be evolutionarily very recent [36], making this species and its close relatives an excellent system in which to further investigate regeneration evolution. Our findings contrast strongly with the pattern of regeneration evolution inferred in Annelida, a group of worms relatively closely related to Nemertea, and the one other group in which evolution of regeneration has been inferred at a phylum-wide scale [20]. Thus, our study shows that evolutionary histories of regeneration may differ markedly across phyla, and demonstrates the high evolutionary lability of regenerative abilities in metazoans. Available data thus highlight the need to perform such studies in additional groups and provide strong justification for future comparative studies of the developmental mechanisms underlying the evolution of regeneration.

8

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