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Molecular and Morphological Phylogenies of Spirorbinae (Serpulidae, Polychaeta, Annelida) and the Evolution of Brooding Modes

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Abstract: Spirorbinae, a ubiquitous group of marine calcareous tubeworms with a small body size as adults, have a fascinating diversity of brooding modes that form the basis for their taxonomic division into six tribes (traditionally subfamilies): in-tube incubation, with varying degrees of attachment to adult structures (four tribes), and external incubation in a modified radiole (opercular brood chambers; two tribes). We investigated the evolutionary transitions among these brooding modes. Phylogenetic reconstruction with molecular (28s and 18s rDNA) and morphological data (83 characters) among 36 taxa (32 ingroup spirorbins; 4 filogranin outgroups) of the combined data set, using maximum parsimony, maximum likelihood, and Bayesian analyses, inferred Spirorbinae to be monophyletic, with strong support for the monophyly for five tribes (Circeini, Januini, Romanchellini, Paralaeospirini and Spirorbini), but non-monophyly for Pileolariini. However, deeper relationships among some tribes remain unresolved. *Neomicrorbis* was found to be the sistergroup to all other Spirorbinae. Alternative coding strategies for assessing the ancestral state reconstruction for the reproductive mode allowed for a range of conclusions as to the evolution of tube and opercular brooding in Spirorbinae. Two of the transformations suggest that opercular brooding may be ancestral for Spirorbinae, and the tube-incubating tribes may have been derived independently from opercular-brooding ancestors.

Keywords: polychaete; Spirorbidae; Serpulinae; Filograninae; Sabellida; Annelida; reproductive mode

Citation: Rouse, G.W.; Macdonald, T.A.; Kupriyanova, E.K. Molecular and Morphological Phylogenies of Spirorbinae (Serpulidae, Polychaeta, Annelida) and the Evolution of Brooding Modes. *Diversity* **2024**, *16*, 237. https://doi.org/10.3390/d16040237

Academic Editors: Michael Wink, Michel Baguette and Luc Legal

Received: 31 December 2023
Revised: 10 April 2024
Accepted: 11 April 2024
Published: 17 April 2024

1. Introduction

The diversity of reproductive modes in the calcareous tube-dwelling serpulid polychaetes (Serpulinae Rafinesque, 1815, Filograninae Rioja, 1923, and Spirorbinae Chamberlin, 1919) is intriguing, with Spirorbinae showing a variety of brooding modes (Figure 1, [1–3]). Spirorbins have distinctive spirally coiled tubes and a consistently small size (2–5 mm body length) with approximately 130 described species and clearly form a monophyletic group [4–9], and, therefore, they present an opportunity to assess the evolution of reproductive traits.

The morphological distinctiveness of Spirorbinae among serpulids prompted Pillai [4] to elevate them from a subfamily within Serpulidae to family rank, Spirorbidae. This spurred the development of the taxonomic structure within Spirorbidae into six subfamilies (see [10,11]). These subfamilies were defined by brooding modes elucidated by Bailey [1]. However, the recognition of Spirorbidae renders Serpulidae paraphyletic [12] and, therefore, recent authors [3,9,13–15] have retained the name Spirorbinae and referred to the subfamilies erected by Knight-Jones [10] as tribes. Some spirorbins have never been placed in

any of the tribes, including *Neomicrorbis* Zibrowius, 1972 (Figure 1A), because its brooding mode remains unknown. In this study, we sampled a wide range of Spirorbinae (Table 1), though some genera were not available for study (see [5,14]).

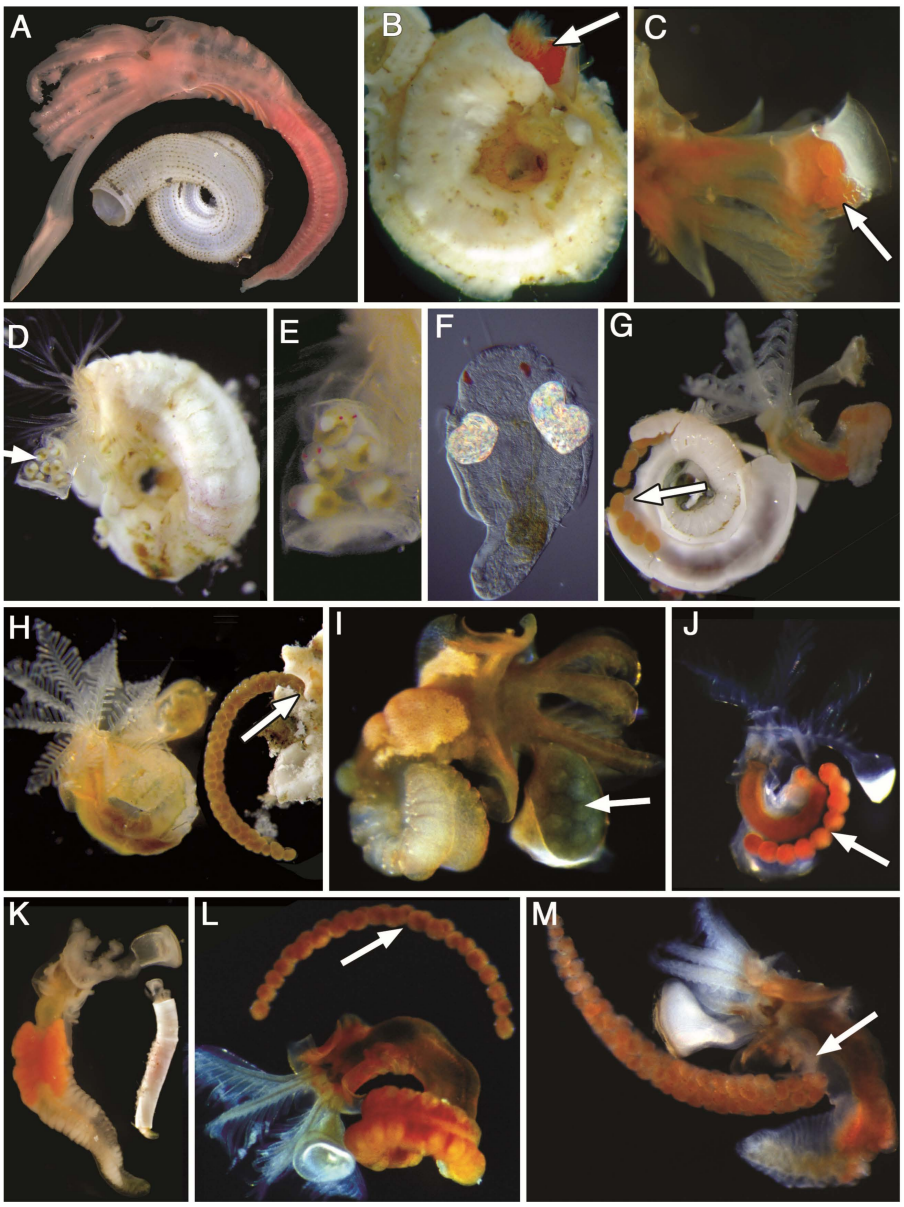

Figure 1. Representatives of Spirorbinae. (**A**) *Neomicrorbis* cf. *azoricus*: preserved specimen removed from the tube with a whole tube at a smaller scale. (**B**) *Amplicaria spiculosa*: live specimen with a distinctive hooked operculum and brood in the operculum (arrow). (**C**) Live *A. spiculosa* anterior showing brood chamber (arrow). (**D**) *Janua heterostropha* in the tube; arrow indicates opercular brood chamber, used for only one brood. (**E**) Closeup of operculum of *J. heterostropha* showing well-developed larvae. (**F**) Larva of *J. heterostropha* showing paired larval attachment glands. (**G**) An opened tube of *Circeis spirillum*, showing embryos adhering to each other and the tube wall (arrow). (**H**) *Spirorbis* cf. *tridentatus* removed from the tube, showing an embryo string that is attached to the posterior inner surface of the tube. (**I**) *Pileolaria* sp. (Pileolariini) (from Bondi, Australia and not included in this study) with an epithelial opercular brood chamber, used for more than one brood (arrow). (**J**) *Metalaeospira tenuis* with an unattached brood string (arrow). (**K**) *Helicosiphon biscoeensis* removed from the tube and in the tube at a smaller scale. (**L**) *Paralaeospira* sp. from Tasmania (and not included in this study) removed from the tube with its loose embryo string (arrow). (**M**) *Protolaeospira* sp.: arrow indicates thoracic brood stalk, attaching brood mass to the adult body.

Table 1. List of species analyzed. Voucher numbers are references for South Australia Museum (SAM E), Australian Museum (AM W), and Scripps Institution of Oceanography (SIO-BIC) collections. **Bolded** sequences were generated for this study.

Taxa	Source	Voucher Number	18S	28S
Filograninae outgroup				
Chitinopoma serrula	Norway	E3524	DQ317112	EU195350
Pomatostegus actinoceras	Mexico	W.42378	OQ379437	OQ389670
Protula bispiralis	Japan	E3657	OQ379439	OQ389672
Salmacina sp.	Edithburgh, SA, Australia	E3499	DQ317126	EU256545
Spirorbinae				
Neomicrorbis cf. *azoricus*	Cocos (Keeling) Isl. Australia	W.54342	**PP002544**	**PP002543**
Pileolariini				
Amplicaria spiculosa	Whyalla, SA, Australia	E3490	**DQ242560**	**DQ242579**
Bushiella abnormis	Barkley Sound, BC, Canada	E3488	**DQ242563**	**DQ242598**
Jugaria cf. *quadrangularis*	Barkley Sound, BC, Canada	E3479	**DQ242564**	**DQ242599**
Pileolaria cf. *marginata*	Barkley Sound, BC, Canada	E3478	**DQ242565**	**DQ242594**
Pileolaria cf. *militaris*	Pt. Cartwright, QLD, Australia	E3492	**DQ242567**	**DQ242593**
Pileolaria sp. 1 (orange eggs)	Whyalla, SA, Australia	E3493	**DQ242562**	**DQ242596**
Pileolaria sp. 3 (gold eggs)	Rapid Bay, SA, Australia	E3494	**DQ242568**	**DQ242597**
Simplaria cf. *potswaldi*	Barkley Sound, Canada	E3504	**DQ242566**	**DQ242595**
Vinearia cf. *koehleri*	Pt. Cartwright, QLD, Australia	E3475	**DQ242561**	**DQ242592**
Januini				
Janua heterostropha	Sangerdi, Iceland	E3506	**DQ242548**	**DQ242585**
Neodexiospira cf. *brasiliensis*	Barkley Sound, BC, Canada	E3498	**DQ242550**	**DQ242586**
Neodexiospira cf. *nipponica*	Barkley Sound, BC, Canada	E3486	**DQ242549**	**DQ242587**
Neodexiospira cf. *steueri*	Encounter Bay, SA, Australia	E3523	**DQ242551**	**DQ242588**
Paralaeospirini				
Paralaeospira sp.	Encounter Bay, SA, Australia	E3485	**DQ242555**	**DQ242580**
Eulaeospira convexis	North Bondi, NSW, Australia	E3496	**DQ242552**	**DQ242582**
Eulaeospira cf. *orientalis*	Encounter Bay, SA, Australia	E3495	**DQ242553**	**DQ242581**
Romanchellini				
Helicosiphon biscoensis	Antarctica	BIC A4000	OQ379432	OQ392408
Protolaeospira cf. *eximia*	Barkley Sound, BC, Canada	E3482	**DQ242556**	**DQ242584**
Protolaeospira cf. *tricostalis*	Bondi, NSW, Australia	E3487	**DQ242557**	**DQ242606**
Protolaeospira cf. *capensis*	Bondi, NSW, Australia	E3484	**DQ242558**	**DQ242607**
Romanchella quadricostalis	Kangaroo Isl., SA, Australia	E3491	**DQ242559**	**DQ242608**
Metalaeospira tenuis	Port Lincoln, SA, Australia	E3480	**DQ242554**	**DQ242583**
Circeini				
Circeis cf. *armoricana*	Barkley Sound, BC, Canada	E3476	**DQ242545**	**DQ242589**
Circeis spirillum	Stykkishlómør, Iceland	E3507	**DQ242546**	**DQ242590**
Paradexiospira cf. *vitrea*	Barkley Sound, BC, Canada	E3483	**DQ242547**	**DQ242591**
Spirorbini				
Spirorbis cf. *bifurcatus*	Barkley Sound, BC, Canada	E3489	**DQ242569**	**DQ242600**
Spirorbis cf. *marioni*	PJs Pets, Edmonton, Canada	E3481	**DQ242570**	**DQ242605**
Spirorbis corallinae	Finnøy, Norway	E3497	**DQ242572**	**DQ242603**
Spirorbis rupestris	Finnøy, Norway	E3500	**DQ242571**	**DQ242601**
Spirorbis spirorbis	Sangerdi, Iceland	E3357	AY577887	**DQ242604**
Spirorbis cf. *tridentatus*	Finnøy, Norway	E3477	**DQ242573**	**DQ242602**

The two opercular-brooding forms (Figure 1B–E,I) are morphologically distinct and arguably not homologous [16]. The cylindrical and cuticular brood chamber of Januini Knight-Jones, 1978 (Figure 1D,E), is formed by the swelling and subsequent degeneration of the opercular ampulla and can be used for only one brood, with larvae (Figure 1F) released by the dehiscence of the brood chamber (referred to hereon as OBC-SHED). Members of Januini Knight-Jones, 1978, included here are *Janua* Saint-Joseph, 1894, and *Neodexiospira* Pillai, 1970. The brood chamber of Pileolariini Knight-Jones, 1978 (Figure 1B,C,I), is formed by invagination of the opercular ampulla, resulting in walls consisting of a double epithelium and a pore for larval release (and possibly entrance [17,18]). These can be used for more than one brood (referred to hereon as OBC-REUSE). Members of Pileolariini included

here are *Amplicaria* Knight-Jones, 1984, *Bushiella* Knight-Jones, 1973, *Jugaria* Knight-Jones, 1978, *Pileolaria* Claparède, 1868, *Simplaria* Knight-Jones, 1984, and *Vinearia* Knight-Jones, 1984. Opercular brooders comprise more than 70% of described Spirorbinae species ([19]; one-fifth of which belong to the Januini [2]), which has prompted speculation of the possible advantages of opercular brooding (e.g., [5,14,20,21]). Four of the six tribes brood embryos and larvae inside their tubes: Paralaeospirini Knight-Jones, 1978 (*Paralaeospira* Caullery and Mesnil, 1897, and *Eulaeospira* Pillai, 1970), brood an embryo/larval string loose within the tube (referred to hereon as LOOSE STRING; Figure 1G); Circeini Knight-Jones, 1978 (*Circeis* Saint-Joseph, 1894, and *Paradexiospira* Caullery and Mesnil, 1897), embryo/larval strings adhere to the tubes walls in a gelatinous matrix (referred to hereon as MATRIX; Figure 1D); in Spirorbini Chamberlin, 1919 (*Spirorbis* Daudin, 1800), the embryo/larval string is attached to the tube posteriorly by an epithelial string (referred to hereon as ATTACHED STRING; Figure 1E); and Romanchellini Knight-Jones, 1978 (*Helicosiphon* Gravier, 1907, *Romanchella* Caullery and Mesnil, 1897, and *Protolaeospira* Pixell, 1912), where most taxa have a thoracic attachment stalk connecting the brood mass to the adult body (referred to hereon as STALK; Figure 1H). This stalk has been suggested to be homologous with a recessed radiole [16]. In some species of *Metalaeospira* Pillai, 1970, such as *M. tenuis* Knight-Jones, 1973, included here (Figure 1J), there is no embryo attachment stalk, and *Metalaeospira* was thought to be part of Paralaeospirini by Knight-Jones [10]. *Metalaeospira* was subsequently moved to Romanchellini upon the observation of a reduced stalk in a new species described by Knight-Jones and Knight-Jones [22], and this is accepted here.

There have been a variety of hypotheses about the evolution of brooding mode in Spirorbinae, though most predate explicit repeatable phylogenetic methods. Caullery and Mesnil [23] focused on tube coiling direction, chaetal and opercular form, and the number of thoracic chaetigers as key characters, and so their scheme of relationships shows multiple occurrences of tube and opercular incubation. Some authors [24,25] proposed that tube brooding represents the ancestral state while Nishi [26] suggested a polyphyletic origin of Spirorbinae from different serpulid ancestors. Two previous morphological phylogenetic studies resulted in conflicting conclusions as to the relationships among spirorbin tribes and, hence, brooding mode evolution [5,14]. Macdonald's [5] genus-level, equally weighted maximum parsimony analysis suggests that opercular brooders form a clade, with Januini recovered as a derived clade within a paraphyletic Pileolariini. A Romanchellini + Paralaeospirini clade was recovered to be the sistergroup to opercular brooders. This lent some support to the idea that the romanchellin thoracic brood stalk is a 'step' in evolution towards opercular brooding in Pileolariini at least (suggested by Knight-Jones and Thorp [16]). The remaining tube-brooding tribes were found to form a grade with respect to the remaining Spirorbinae [5]. A similarly genus-level morphology-based analysis by Rzhavsky and Kupriyanova [14] suggested that two independent transitions from a tube-brooding ancestral state to opercular brooding occurred in the history of the group, with a subsequent reversal from operculum brooding to the tube brooding form seen in Spirorbini (ATTACHED STRING).

Opercular brooders tend to be dominant in the tropics [27], with Januini largely confined to warm latitudes [28]. Various hypotheses have been presented to explain this based on physiological constraints [14,20]. Some representatives of Pileolariini extend to higher latitudes, and some are boreal or boreal-arctic (especially *Jugaria*, *Bushiella*, and *Protolaeodora* Pillai, 1970) [29]. Paralaeospirini and Romanchellini are almost all found in the southern hemisphere, with few exceptions [27]. Thus, almost all tube-brooders of the northern hemisphere are members of Spirorbini or the circumboreal Circeini [29]. These geographic patterns and physiological constraints are of interest in our understanding of the evolutionary history of Spirorbinae. However, we first need a strong phylogenetic basis to assess these hypotheses.

The purpose of this study is to incorporate 18S and 28S rDNA nuclear sequences and, combined with morphological data, assess the phylogenetic hypotheses generated by two recent studies based on morphology [5,14]. These data should improve the resolution

of the existing morphology-based phylogenies of Spirorbinae and provide a framework to interpret and reassess our ideas of the evolution of their morphology. We addressed the following questions: (1) Did opercular brooding evolve more than once? and (2) What is the ancestral brooding mode of Spirorbinae?

2. Materials and Methods

2.1. Taxon Sampling

The data sets consisted of 36 taxa; 32 ingroup spirorbin species and 4 outgroup taxa, where 31 spirorbins were newly sequenced for this study; 4 outgroup sequences and 1 ingroup (*Helicosiphon*) were used by us in a previous study and mined from GenBank (Table 1).

Outgroup taxa included representatives of Filograninae according to Kupriyanova et al. [9]. Ingroup terminals encompassed the diversity of brooding modes observed in the Spirorbinae to date [11]. The missing genera were either rare, with unknown brooding modes (*Crozetospira* Rzhavsky, 1997, *Anomalorbis* Vine, 1972), or had clear morphological affinity with other genera represented here (*Nidificaria* Knight-Jones, 1984, *Protolaeodora*, *Pillaiospira* Knight-Jones, 1973, and *Leodora* Saint-Joseph, 1894). Outgroup taxa included representatives of Filograninae based on the most recent phylogenetic analyses of Serpulidae in Kupriyanova et al. [9]. Ingroup terminals encompassed the diversity of brooding modes observed in Spirorbinae to date [11].

2.2. Collection and Preservation

Specimens were predominantly collected in British Columbia, Canada, as well as in Scandinavia (Iceland and Norway) and Australia (Table 1). Collections were mostly performed between 2001 and 2004, although *Helicosiphon* was collected in 2011 and *Neomicrorbis* in 2022. Specimens were preserved in 95% ethanol for DNA extraction, as well as in formalin for identification purposes and morphological study.

2.3. DNA Extraction, Amplification, and Sequencing

Worms were removed from their tubes and sliced longitudinally. Visible traces of the digestive tract of the non-operculum-bearing half of the worm were removed, and this tissue was used in subsequent DNA extraction. The remaining half, including the diagnostic operculum, was saved as a voucher and deposited at the South Australia Museum (SAM), Adelaide, SA, Australian Museum (AM), Sydney, NSW, and Scripps Institution of Oceanography Benthic Invertebrate Collection (SIO-BIC) (Table 1). One specimen was sequenced for each terminal.

To remove traces of ethanol, the tissue was rinsed in 1× Phosphate-Buffered Saline (PBS) three times and left to soak for approximately 1 h at the last rinsing step. Genomic DNA was extracted using a Qiagen DNA Mini Kit (Qiagen Inc., Venlo, The Netherlands) and eluted in 50–100 µL of sterile distilled water. Two nuclear genes 18S and 28S ribosomal DNA were amplified. Outgroup sequences were taken from previous studies using methods described therein [6,9], and ingroup sequences for *Neomicrorbis* cf. *azoricus* and *Helicosiphon biscoeensis* were generated in the same way, and we recommend these as the most efficient (see Table S1). For most ingroup specimens, 18S rDNA was amplified in two overlapping fragments of approximately 1100 bp each using the primers 18s1F & 1R and 18s2F & 2R [30,31]. In some cases, reamplification using nested PCR (see Table S1 for list of internal primers) was necessary. For 28S, rDNA was amplified in either a 1000 bp fragment (D1 plus subsequent region with primers 28sF [32] and Po28R4 [33]; most taxa), or when this amplification failed, a 400 bp region (D1 only with primers 28sF and 28sR: *Protolaeospira tricostalis*, *Protolaeospira capensis*, and *Romanchella quadricostalis*) (see Table S1 for primer sequences).

For the newly generated sequences apart from *Neomicrorbis* cf. *azoricus* (where methods follow [9]), PCR reactions were 25 µL and contained the following: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2–4 mM $MgCl_2$, 0.5 mM of each primer, 100 mM of each dNTP, 1.5 Units Taq

(M. Pickard, University of Alberta), and 20–100 ng template DNA (usually 1–2 µL). For taxa that did not amplify well, 2% DMSO was added to the reaction mix, which often resulted in a successful amplification. The following PCR temperature profiles were used: 18S–95 °C for 3 min, 40 cycles of 94 °C for 30 s, a °C for 1 min, 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min; where a = 47–49 °C. For 28S, the cycling protocol was the same, except that the first extension step was reduced to 1 min at 72 °C, and a = 46–48 °C.

Amplification products were separated via electrophoresis on a 1.1% agarose gel in TAE buffer, stained with ethidium bromide. PCR products were either purified directly with a PCR Purification Kit (Qiagen Inc.), or bands were excised from the gel and purified with a QIAQuick™ Gel Extraction Kit (Qiagen Inc.). Elution was performed in sterile distilled water in both cases.

Sequences were obtained directly with the BigDye v 3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA). Full reactions were 20 µL: 2 µL Big Dye, 6 µL buffer (200 mM Tris-HCl pH 9.0, 5 mM $MgCl_2$), µL 1 µM primer, and 1–6 µL PCR product. Cycling sequencing was performed according to the manufacturer's instructions and separated on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Basecaller v.3.4.1 was used to read the chromatograms and GeneTool 2.0 to assemble gene fragments.

2.4. Morphological Data

The morphological matrix used in this study is based on the genus-level matrix of Macdonald [5] but edited in several ways. The C-coding method of Pleijel [34] was used to score for inapplicable characters. Also, as taxa included in this study did not encompass all genera represented in the morphological studies, the matrix of morphological characters used here was condensed to include only taxa that were sequenced. The number of outgroup taxa was reduced to just some members of Serpulidae, making many of the characters used in [5] unnecessary. A total of 83 morphological characters were used in this study, including gross morphological characters of adults and larvae and chaetal characteristics. Some significant changes to character construction and coding from [5] were also made. The most notable were those pertaining to brooding mode. Instead of having separate characters for tube brooding and opercular brooding, these were combined into a single character; 13. 'Location of embryo incubation': 0: tube, 1: operculum. Subsidiary characters based on the kind of brooding were also modified. A single character for the kind of tube brooding was used with multiple states: 14. 'Tube incubation': 0: loose in tube, 1: loose string, 2: gelatinous matrix, 3: attached string, 4: thoracic stalk. Similarly, the kind of opercular brooding was coded as a single character but with three states as outlined in [16]: 15. 'Opercular brood chamber': 0. distal cuticular plate (for Januni); 1. Epithelial cup (for most Pileolariini); 2. Paired plates. The state 'paired plates' was used to accommodate the different forms of brood chamber for two members of Pileolariini, *Amplicaria* and *Vinearia*. This allowed for the different forms of opercular brooding to be scored. Other changes from Macdonald [5] concern *Metalaeospira* and *Eulaeospira*, which were both coded as having a romanchellin-like reproduction based on the observations of an epithelial funnel arising from the posterior thorax in *Metalaeospira* species [11], and the assumption that *Metalaeospira* and *Eulaeospira* were closely related due to the similar brush-like abdominal chaetae, a character thought to be unique to Romanchellini [22]. However, further investigation revealed that the 'oviducal funnel' only appears in *Metalaeospira* (e.g., [35]) and *Eulaeospira* brood embryos and larvae either as a string loose in the tube as in Paralaeospirini, or with some unknown posterior attachment in the fecal groove [22]. *Metalaeospira tenuis* and the two *Eulaeospira* terminals used here were coded as having the same state as *Paralaeospira* (LOOSE STRING). Some character states were also altered for *Amplicaria*. Both formalin-preserved and fresh specimens were studied when available, as well as the available literature. See Appendix A and Supplemental File S1 (Spirorbinae Morphology Matrix) for the list of morphological characters and the character matrix, respectively.

2.5. Phylogenetic Analyses

Analyses were performed on three datasets: morphology data only, molecular data only, and a combined morphology and molecular dataset. The 83-character morphology dataset was analyzed under the maximum parsimony criterion (MP) using PAUP* v.4.0a166 [36] following export from Mesquite 3.81 [37]. Characters were treated as unordered. One hundred random addition searches were executed on the data using the tree–bisection–reconnection algorithm, and the results were summarized with a strict consensus tree.

2.5.1. Molecular and Molecular + Morphology Analyses

The gene partitions were aligned using MAFFT [38] and concatenated using RAxML-NG GUI 2.0.10 [39], resulting in an alignment of 3423 base pairs of molecular data that was saved in phylip format. This data set was partitioned, and the appropriate model for each of the two DNA partitions selected by ModelTest-NG [40] was TIM3 + I + Γ. A maximum likelihood analysis with RaxML-NG [41] was then executed with 50 replicates followed by 1000 bootstrap pseudoreplicates to assess node support. For the combined molecular + morphology analyses, the morphology dataset was manually appended to the phylip file of DNA data, formatted with numerical character states, giving a total dataset of 3506 characters. The data were partitioned with two DNA partitions, each using TIM3 + I + Γ and the morphology partition set to the MULTIx_MK model (with x as 5 for the maximum five-character states). A maximum likelihood analysis with RaxML-NG was then run as previously outlined. A Bayesian inference (BI) analysis of the partitioned concatenated molecular + morphology data was conducted using Mr. Bayes v.3.2.7a [42]. Both DNA partitions were run using GTR + I + Γ (the closest model to TIM3 + I + Γ), with the morphology partition analyzed under the Mkv model [43]. Two iterations of four Markov Chain Monte Carlo (MCMC) chains were run for 50 million generations, sampling every thousand generations. A majority rule consensus tree was made from the trees remaining after burn-in (discarding 10% of trees) following assessment with Tracer 1.7.1 [44]. An MP analysis of the concatenated molecular + morphology dataset was also executed in PAUP* with 100 random addition searches followed by 1000 bootstrap pseudoreplicates that were summarized using a majority-rule consensus tree.

2.5.2. Transformations

The best tree based on the ML analysis of the combined molecular and morphology dataset was used to assess transformations for several morphological characters of main interest. These characters were recoded from that seen in Appendix A (and Supplemental File S1; Spirorbinae Morphology Matrix) in different ways owing to the influence of inapplicable states (coded as —) and to explore the various possibilities for the evolution of brooding modes in Spirorbinae. Character 13. 'Location of embryo/larvae incubation' was recoded from 0. Tube; 1. Operculum. 2. On tube to also add state 3. Absent, and it is referred to in the Results as 'Brooding General'. Character 15. 'Opercular brood chamber' was recoded from 0. Distal cuticular plate; 1. Epithelial cup; 2. Paired plates to also add state 3. Absent, and it is referred to in the Results as 'Opercular Brooding'. Finally, Character 14. 'Tube incubation' was recoded from 0. Loose in tube; 1. Loose string; 2. Gelatinous matrix; 3. Attached string; 4. Thoracic stalk to also add states 5. Operculum, 6. On tube and 7. Absent, and it is referred to in the Results as 'Brooding Multistate Tube'. Transformations were visualized in Mesquite 3.81 using likelihood ancestral state reconstruction on ball and sticks tree form, with the Mk1 probability model.

3. Results

3.1. Morphology Data Analysis

The parsimony analysis of the morphology data set resulted in 28 most parsimonious trees of length 297. The strict consensus tree (Figure 2) recovered Circeini, Januini, and Spirorbini as clades but Paralaeospirini, Pileolariini, and Romanchellini were non-

monophyletic. *Neomicrorbis* was recovered as the sistergroup to all other Spirorbinae. *Pileolaria* was paraphyletic owing to the placement of *Simplaria potswaldi*, and *Eulaeospira* was paraphyletic with respect to Januini.

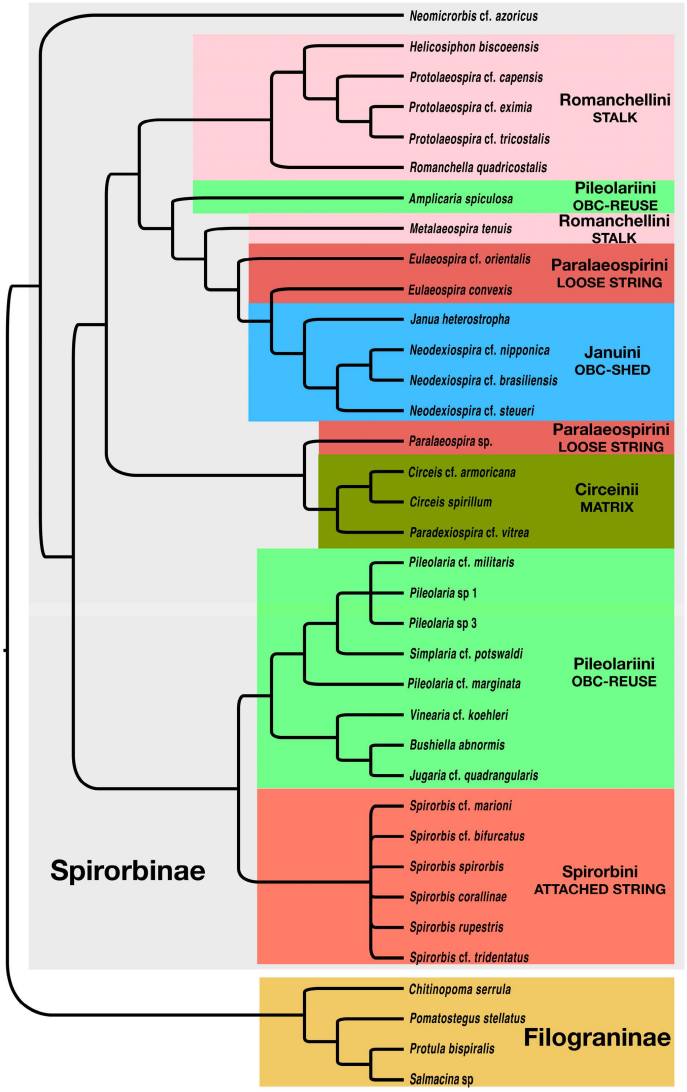

Figure 2. Strict consensus tree of 28 most parsimonious trees (297 steps) derived from the 83-character morphology matrix using PAUP*.

3.2. Molecular-Only and Combined DNA + Morphology Data Analyses

The analyses of the morphology-only dataset and the combined *DNA + morphology* dataset, as well as the ML analysis of the molecular-only data, gave similar tree topologies. The ML analysis of the *DNA + morphology* dataset with branch lengths is shown in Figure 3. Supplemental Figure S1 shows the molecular-only data ML analysis. As with the morphology-only analysis, *Neomicrorbis* was recovered as the sistergroup to all other Spirorbinae. Circeini, Januini, Romanchellini, Paralaeospirini, and Spirorbini were all recovered as clades with only Pileolariini appearing as non-monophyletic. *Amplicaria* was recovered as the poorly supported sistergroup to the Romanchellini and Paralaeospirini clade, and *Bushiella* and *Jugaria* formed a clade that was a sistergroup to Spirorbini. The clade of most terminals of Pileolariini plus Spirorbini was well supported as were the nodes for most of the other tribes. Most genera that had multiple representatives were recovered as clades. The exceptions were as follows: 1. *Pileolaria*, which was paraphyletic owing to the placement of *Simplaria potswaldi*, and 2. *Protolaeospira*, which was rendered paraphyletic by the placement of *Romanchella* and *Helicosiphon* (Figure 3).

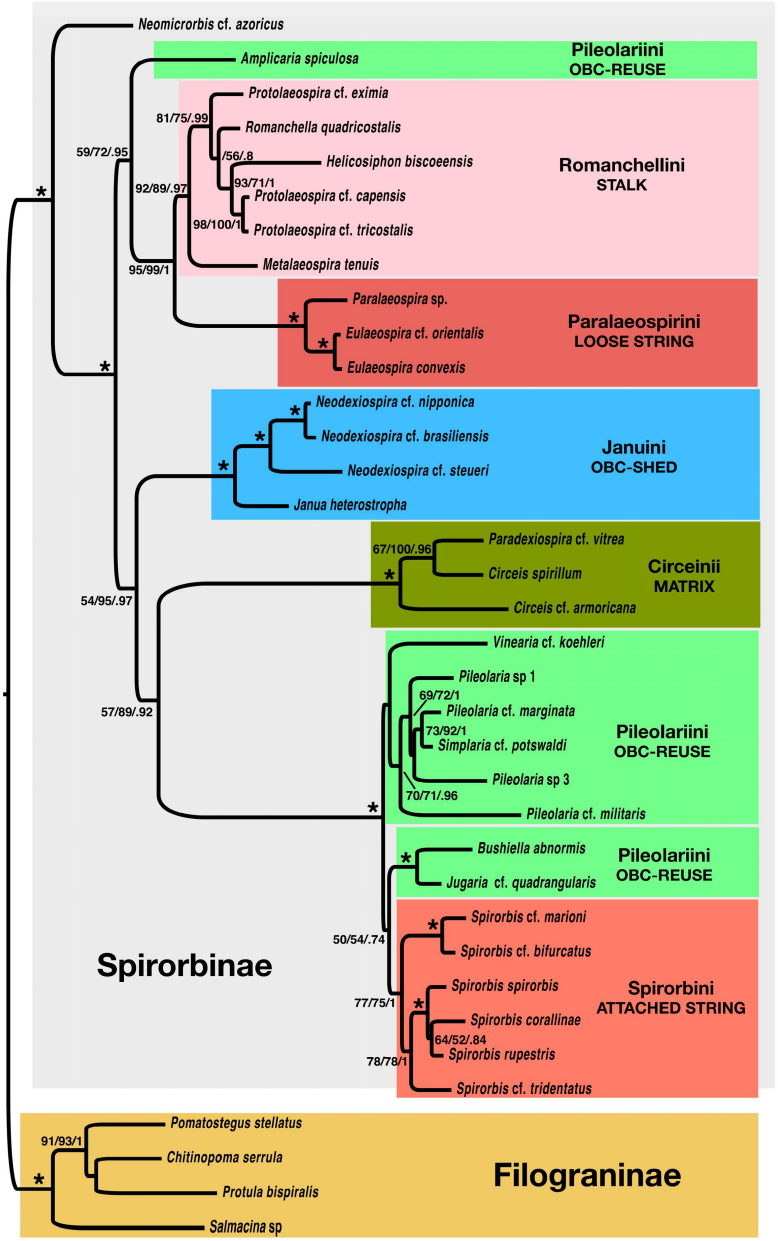

Figure 3. Maximum likelihood tree with branch lengths derived from the morphology plus molecular (18S rDNA, 28S rDNA) concatenated datasets. The MP and Bayesian analyses gave the same tree topology. Numbers at nodes are ML and MP bootstrap (BS) values followed by Bayesian posterior probability (PP). BS values below 50 and posterior probabilities below 0.7 are not shown. A * indicates that both BS scores were 95 or greater, and the PP was 1.

3.3. Transformations

The transformations for three characters mapped onto the ML analysis of the combined dataset are shown in Figure 4. Unfortunately, the brooding mode for *Neomicrorbis* was coded as unknown.

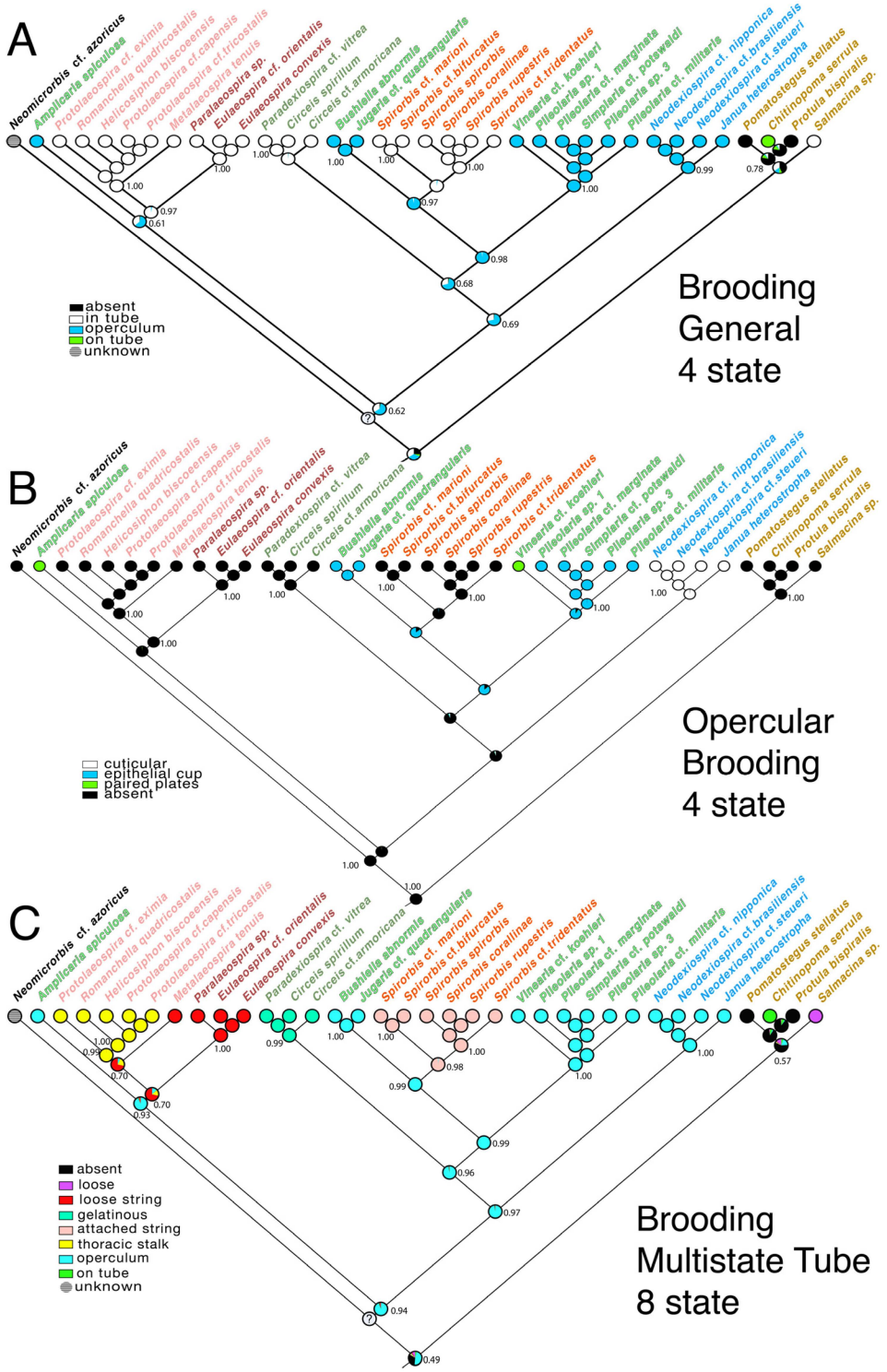

Figure 4. Transformation for three versions of characters based on brooding mode mapped onto the ML tree topology, visualized in Mesquite 3.81 using likelihood ancestral state reconstruction on ball and sticks tree form, with the Mk1 probability model. (**A**) Brooding general character with four states, only two of which occur in Spirorbinae, in the tube or in the operculum. (**B**) Opercular brooding only character with three states, two for kinds of opercular brooding. (**C**) Brooding character with eight states to cover the various forms of tube brooding and with only one state for opercular brooding. Numerical values at the nodes show the maximum likelihood score, as a proportion of 1, for the most likely state. The ancestral state for Spirorbinae for Brooding (**C**) is unknown owing to the lack of knowledge for *Neomicrorbis* cf. *azoricus*.

The 'Brooding General' transformation (Figure 4A) suggests that opercular brooding may have been the plesiomorphic state for Spirorbinae, but the likelihood score was less than 65 for the more inclusive nodes. It does appear that Spirorbinae show a loss of opercular brooding to brooding in the tube. When the opercular brooding form was recoded to accommodate three states, the transformation suggested that opercular brooding evolved three times, once in *Amplicaria*, once for Januini, and once for the Pileolariini plus Spirorbini clade (Figure 4B). The 'Brooding Multistate Tube' character, however, was coded with a single opercular brooding state, and the form of tube brooding was broken into a series of states (Figure 4C). This transformation recovered opercular brooding as a plesiomorphic state for Spirorbinae with only a single origin and then three multiple transformations to tube brooding, separately in Circeini, Spirorbinae, and the Romanchellini + Paralaeospirini clade. The latter clade LOOSE STRING brooding was transformed into THORACIC STALK brooding.

4. Discussion

4.1. Phylogeny of the Spirorbinae

The sister group to Spirorbinae is not Serpulinae, as hypothesized by Macdonald [5] and Kupriyanova [45] based mostly on morphology, but Filograninae, as revealed by molecular data by Kupriyanova et al. [6] and Lehrke et al. [46], and most recently, Kupriyanova et al. [9]. This taxon was used to root our phylogenetic analyses which did support a monophyletic Spirorbinae that also includes *Neomicrorbis*, a taxon that has had unclear affinities [47]. Zibrowius [48] regarded *Neomicrorbis* as an 'intermediate between the subfamilies Spirorbinae and "Serpulinae"'. He drew on similarities of *Neomicrorbis* with both Spirorbinae and the filogranin *Vermiliopsis*, which, as of the time he was writing, was part of Serpulinae. If *Neomicrorbis* has a closer relationship to Filograninae than to Spirorbinae, our sampling across Filograninae (Figures 2 and 3) would likely have shown this, but a more comprehensive analysis of Serpulidae including *Neomicrorbis* may be warranted. It is unfortunate that the reproductive mode for *Neomicrorbis* remains unknown. It has never been found with broods of any kind. This means the inference of the ancestral reproductive mode for Spirorbinae also remains unknown (Figure 4). We can explore the current taxonomic arrangements for the remaining Spirorbinae and their reproductive modes.

The analyses of morphology and molecular datasets all inferred strong support for a clade comprising all the spirorbin terminals except for *Neomicrorbis* (Figure 4). The tribes Circeini, Januini, Paralaeospirini, Romanchellini, and Spirorbini were all recovered as clades, though Pileolariini was paraphyletic with Spirorbini nested inside. These results match in many details the scenarios proposed by Ippolitov and Rzhavsky [49,50] based on tube ultrastructure studies.

The pileolariin *Amplicaria* was recovered as the sistergroup to the Paralaeospirini, Romanchellini clade (Figure 4), though with weak to moderate support. *Amplicaria* (Figure 1B,C) also did not group with other Pileolariini in the morphology-only analysis (Figure 3) and was nested in a Romanchellini grade. This differed from its placement in the morphological cladistic analysis by Macdonald [5] and Rzhavsky and Kupriyanova [14], where *Amplicaria* grouped with other Pileolariini. This difference may be related to the coding of the characters concerning opercular brooding where *Amplicaria* was given a different coding than other Pileolariini (see Materials and Methods 2.4, Appendix A, and Supplemental File S1; Spirorbinae Morphology Matrix). The placement of *Amplicaria* with the Romanchellini + Paralaeospirini is not surprising given its morphological similarity to members of these taxa such as the large number (3–4) of thoracic uncinal tori, which are asymmetrically distributed (unlike in Pileolariini) [11,35]. Additionally, both Romanchellini and Paralaeospirini release sperm clusters in eights or tetrads, as does *A. spiculosa* (G. Rouse pers. obs.). Most other tribes release clusters of >100 spermatids. Romanchellini and Paralaeospirini also lack larval attachment glands (Pileolariini have one), but it remains unknown how many (if any) larvae of *A. spiculosa* possess. Thus, an examination of brooding specimens is required, as is an investigation into the anatomy and structure of the opercular brood chamber, but based on the present evidence, it should not be regarded as part of

Pileolariini. Interestingly, *Vinearia* has an operculum like that of *Amplicaria spiculosa*. It is an open 'nest' instead of the typical enclosed brood chamber of the other members of the Pileolariini [16]. This similarity appears to be convergent, with *Vinearia* clearly falling with Pileolariini (Figures 2 and 3).

Metalaeospira, represented here by *M. tenuis*, was recovered as part of Romanchellini (STALK) in the morphology and molecular analyses (Figure 3); even though it has the loose string brooding mode found in Paralaeospirini, the tribe it was originally placed in by Knight-Jones [10]. The morphology-only analysis, however, showed that *M. tenuis* did not group with the Romanchellini (Figure 2), likely owing to its coding for the brooding characters. The morphology and molecular analyses support the move from Paralaeospirini to Romanchellini by Knight-Jones and Knight-Jones [22] with the discovery of a thoracic stalk in other *Metalaeospira*. The placement of *M. tenuis* as the sister to the remaining Romanchellini (Figure 3) and the transformation for the form of tube brooding (Figure 4C) suggest that loose string brooding is the plesiomorphic state for Romanchellini and that thoracic stalk brooding is an apomorphy within the tribe. Further sampling of other *Metalaeospira*, especially *M. armiger* Vine, 1977, which has thoracic stalk brooding, will be important to assess this further.

Januini (OBC-SHED) was recovered in the morphology analysis as a clade nested within a Romanchellini + *Amplicaria* + Paralaeospirini (partial) grade (Figure 2), while it was sistergroup to a clade comprised of most Pileolariini + Spirorbini in the DNA plus morphology analysis (Figure 3). In Macdonald [5], Januini was nested inside Pileolariini, suggesting a single origin of opercular brooding. In analyses of Rzhavsky and Kupriyanova [14], Januini was sistergroup to most Spirorbinae, including *Neomicrorbis*, suggesting opercular brooding evolved twice. These various placements are due to variations in the morphological character coding in the matrices used. The morphology and molecular analyses placed Circeini as nested well within Spirorbinae but among various clades of tube brooders and not particularly close to Pileolariini. This has various implications for the evolution of brooding, as discussed below. Circeini (MATRIX) also had a varying position in our morphological-only analysis and the morphology and molecular analyses and when compared with previous phylogenetic hypotheses [5,14].

A close relationship between Pileolariini (OBC-REUSE) (exclusive of *A. spiculosa*) + Spirorbini (STRING) was found in both the morphology-only and the morphology and molecular analyses (Figures 2 and 3). In the former, the tribes were reciprocally monophyletic sister taxa (Figure 3), while in the latter, Spirorbini was nested inside Pileolariini (Figure 4) as the sister taxon to a *Bushiella* + *Jugaria* clade of pileolariins, though this had poor support. The Pileolariini + Spirorbini clade was well-supported by morphological apomorphies such as the presence of a single-larval attachment gland (Character 18), abdominal uncinal tooth transverse rows diagonal (Character 71), and the absence of multiple abdominal uncini tooth rows (character 72). The paraphyly of Pileolariini with Spirorbini closest to taxa such as *Bushiella* was also shown in the morphology analysis of Rzhavsky and Kupriyanova [14]. Spirorbini should arguably be synonymized with Pileolariini, and as Spirorbini is the senior name, the clade would be referred to as Spirorbini. Further sampling of Pileolariini is warranted.

4.2. Evolution of Brooding Modes

The various coding strategies for a brooding mode allow for a range of conclusions as to the evolution of tube and opercular brooding in Spirorbinae. Accepting the morphology and molecular analyses as the correct placement for Januini leads to the implications for the evolution of opercular brooding that varied depending on the transformation applied (Figure 4A–C). Under a general homology coding for opercular brooding, the mode in Januini (OBC-SHED) and most Pileolariini (OBC-REUSE) may be homologous (Figure 4A), but the likelihood was low at key nodes owing to the placement of the tube-brooding Circeini (MATRIX) and Spirorbini (ATTACHED STRING). When opercular brooding was made its own character with different states for Januini and Pileolariini, the two forms of

opercular brooding were recovered as independently derived (Figure 4B). When opercular brooding was coded as a single state in a multistate character with various states for tube brooding (Figure 4C), then the forms of opercular brooding appeared to be homologous and would appear to be the ancestral state for Spirorbinae (not including *Neomicrorbis*). This would then suggest that the tube brooding modes of Circeini (MATRIX), Paralaeospirini (LOOSE STRING), Romanchellini (STALK), and Spirorbini (ATTACHED STRING) are derived from opercular brooding (Figure 4A,C). Considering that the Romanchellini (STALK) condition appears derived from the Paralaeospirini (LOOSE STRING) state (Figure 4C) means there were three transformations from opercular brooding (also seen in Figure 4A). This hypothesis of Romanchellini (STALK) condition transforming from a Paralaeospirini state is supported by Pillai's [51] assertion that the brood-stalk of Romanchellini is not a likely precursor to the opercular brood chamber because it occupies a different position from the radioles of the opercular crown. The hypothesis that the thoracic brood-stalk of Romanchellini is derived from a recessed radiole [16] and is a precursor to opercular brooding (e.g., [5,19]) is not supported by the analyses here. Under the coding shown in Figure 4B, only Spirorbini (ATTACHED STRING) appears to have been derived from opercular brooding. Given that it is also shown to have transformed from opercular brooding in the other coding examples (Figure 4A,C) and its nested position within Pileolariini, this switch would appear to be the most well supported.

Given the poor bootstrap support for several key nodes, further molecular data would be ideal to establish the position of Januini in relation to Pileolariini, and the placement of *Amplicaria* (Figures 3 and 4) is currently strongly influencing the transformations (Figure 4). Further investigation into the development of the *Amplicaria* operculum is needed to determine which coding scheme is most justified.

4.3. Future Studies

The brooding mode for *Neomicrorbis* remains a mystery, and given its phylogenetic position, it is important to establish the ancestral state of brooding for Spirorbinae. The placement of *Amplicaria spiculosa* remains unclear. Given that opercular brooding may be the ancestral state for Spirorbinae (or most of the clade allowing for the uncertainly of *Neomicrorbis*), an understanding of the morphology and development of the brood chamber and larvae of *A. spiculosa* would be valuable. Further investigation into phylogenetic relationships among the Spirorbinae with more molecular data such as transcriptomes or mitogenomes would be welcome. Also, the inclusion of genera not represented here, such as *Leodora* (Januini; OBC-SHED), *Nidificaria* (Pileolariini; OBC-REUSE), *Pillaiospira* (Januini; OBC-SHED), *Velorbis* (Spirorbini; STRING), and more representatives of *Metalaeospira* (Romanchellini STALK) and *Paralaeospira* (Paralaeospirini; LOOSE), is needed. Improving on the existing analyses will not only clarify the evolution of spirorbin brooding modes, but also questions of broader evolutionary significance, such as the evolution of tube coiling, its directional asymmetry, and its relationship to miniaturization.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16040237/s1, File S1: Matrix of morphological characters used in this study in nexus format, SpirorbinaeMorphology.nex; Table S1: List of primers used for amplification and sequencing; Figure S1: Maximum likelihood tree with branch lengths derived from the molecular-only concatenated datasets (18S rDNA, 28S rDNA). References [52,53] are cited in the Supplementary Materials).

Author Contributions: Conceptualization, G.W.R. and T.A.M.; Data curation, G.W.R., T.A.M. and E.K.K.; Formal analysis, G.W.R.; Funding acquisition, G.W.R.; Investigation, E.K.K.; Methodology, G.W.R. and T.A.M.; Resources, G.W.R.; Visualization, G.W.R.; Writing—original draft, G.W.R., T.A.M. and E.K.K.; Writing—review and editing, G.W.R. and E.K.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant to A. Richard Palmer (Department of Biological Sciences, University of Alberta, Canada) and the Australian Research Council (ARC) grant DP0558736 to G.W.R.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Data are contained within the article, on GenBank, or in the Supplementary material.

Acknowledgments: Lauren Helgen (formerly South Australia Museum), staff of the University of Alberta's Molecular Biology Services Unit, and Avery Hiley (SIO) provided laboratory assistance; Ken H. Halanych provided advice and unpublished primer sequences. Nerida Wilson, Daniel Jackson, Valerie Macdonald, and Heidi Krajewsky helped with collections. Felix Sperling and A. Richard Palmer provided comments on early versions of the manuscript. Thanks also to the director and staff of the Bamfield Marine Sciences Centre for their support. We thank the CSIRO Marine National Facility (MNF) for its support in the form of sea time on RV 'Investigator', support personnel, scientific equipment, and data management. All data and samples acquired on the voyage are made publicly available in accordance with MNF Policy. We also thank all the scientific staff and crew who participated in voyage IN2022_V08 where specimens of the elusive *Neomicrorbis* were collected.

Conflicts of Interest: Author Tara A. Macdonald, was employed by the Biologica Environmental Services Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A

List of characters used in morphology data set

(1) Tube coiling: 0. absent; 1. flat spiral; 2. flat spiral posteriorly, uncoiled anteriorly;
(2) Coiling direction: 0. dextral; 1. sinistral; 2. both;
(3) Calcareous tube type: 0. opaque; 1. porcellanous: 2. vitreous;
(4) Longitudinal ridges: 0. absent; 1. present;
(5) Growth rings: 0. absent; 1. present;
(6) Peripheral flange: 0. absent; 1. present;
(7) Crystalline patch: 0. absent; 1. present;
(8) Form of crystalline patch: 2. paired; 3. single; 4. diffuse;
(9) Sexuality pattern: 0. simultaneous hermaphrodite; 1. gonochoric or sequential hermaphrodites;
(10) Sperm head: 0. spherical; 1. elongate;
(11) Larval feeding: 0. lecithotrophic; 1. planktotrophic;
(12) Embryo/larvae incubation: 0. absent; 1. present;
(13) Location of embryo/larvae incubation: 0. tube; 1. operculum. 2. on tube;
(14) Tube incubation: 0. loose in tube; 1. unattached string; 2. gelatinous matrix; 3. posterior filament; 4. thoracic stalk;
(15) Opercular brood chamber; 0. distal cuticular plate; 1. epithelial cup; 2. paired plates;
(16) Dorsal convex collar flap: 0. absent; 1. present;
(17) Number of radioles: 0. <10; 1. >10;
(18) Larval attachment gland: 0. absent; 1. single; 2. paired;
(19) Position of larval attachment gland: 0. anterior; 1. posterior;
(20) Operculum: 0. absent; 1. present;
(21) Opercular calcification: 0. absent; 1. present;
(22) Opercular peduncle: 0. smooth; 1. with pinnules;
(23) Opercular plate: 0. absent; 1. present;
(24) Distal opercular plate calcified: 0. absent; 1. present;
(25) Orientation of opercular plate relative to tube mouth: 0. perpendicular; 1. oblique;
(26) Opercular plate spines: 0. absent; 1. present;
(27) Secondary opercular plate below embryos: 0. absent: 1. present;
(28) Primary operculum becomes brood chamber: 0. absent; 1. present;
(29) Opercular plates retained after molting: 0. absent; 1. present;

(30) Brood chamber-talon fusion: 0. absent: 1. present;
(31) Primary opercular plate rim: 0. absent; 1. present;
(32) Secondary opercular plate rim: 0. absent; 1. present;
(33) Fiber connecting talon and tori: 0. absent; 1. present;
(34) Primary talon: 0. absent; 1. present;
(35) Secondary talon: 0. absent; 1. present;
(36) Primary talon type: 0. spatulate; 1. vestigial; 2. tooth;
(37) Terminal talon bifurcation: 0. absent; 1. present;
(38) Talon projection: 0. absent; 1. present;
(39) Primary talon location: 0. eccentric; 1. peripheral;
(40) Primary talon external: 0. absent; 1. present;
(41) Collar margin fused: 0. absent; 1. present;
(42) Number of segments; concave side: 0. three; 1. four; 2. five; 3. >five;
(43) Number of thoracic tori, concave side: 0. two; 1. three; 2. four; 3. >five;
(44) Number of segments, convex side: 0. Three; 1. four; 2. five; 3. >five;
(45) Number of thoracic tori, convex side: 0. two; 1. three; 2. >five;
(46) Thoracic tori symmetry: 0. symmetric; 1. asymmetric;
(47) Collar chaetae type: 0. fin-and-blade; 1. capillary and limbate;
(48) Collar chaetae distribution: 0. symmetric; 1. more on convex; 2. more on concave;
(49) Same collar chaetae form convex and concave side: 0. present; 1. absent;
(50) Capillary collar chaetae: 0. absent; 1. present;
(51) Capillary collar chaetae distribution: 1. both sides; 2. convex only;
(52) Gap between fin and blade: 0. absent; 1. present;
(53) Collar chaetae blade teeth: 0. fine; 1. coarse;
(54) Form of collar chaetae fin teeth: 0. fine; 1. coarse;
(55) Collar chaetae cross-striations: 0. absent; 1. present;
(56) Collar chaetae cross striation distribution: 0. present both sides; 1. absent concave side;
(57) Capillary chaetae in second thoracic fascicle: 0. absent; 1. present;
(58) Capillary chaetae in third thoracic fascicle: 0. absent; 1. present;
(59) Sickle (*Apomatus*) chaetae in third thoracic fascicles: 0. absent; 1. present;
(60) Shape of sickle (*Apomatus*) chaetae: 0. parallel-sided; 1. pennant-shaped;
(61) Thoracic uncini distribution: 0. more on concave; 1. more on convex; 2. same number on both;
(62) Multiple rows of thoracic uncinal teeth (rasp-shaped): 0. absent; 1. present;
(63) Transverse uncini rows: 0. straight;1. diagonal;
(64) Thoracic uncini peg: 0. blunt; 1. pointed;
(65) Thoracic uncinal peg lateral teeth: 0. absent; 1. present;
(66) Chaetiger with smallest number of thoracic uncini: 0. last thoracic chaetiger; 1. 2nd convex; 2. 3rd convex: 3. first thoracic chaetiger;
(67) Number of abdominal chaetigers: 0. 0–10; 1. 11–20; 2. 21–30; 3. 30+;
(68) Abdominal uncini on convex side: 0. absent; 1. present;
(69) Location of largest abdominal tori: 0. anterior; 1. posterior; 2. even distribution; 3. middle;
(70) Abdominal uncini symmetry: 0. symmetric; 1. asymmetric;
(71) Abdominal uncinal tooth transverse rows: 0. straight; 1. diagonal;
(72) Multiple abdominal uncini tooth rows: 0. absent; 1. present;
(73) Number of multiple abdominal uncini rows: 0. <ten; 1. >ten;
(74) Abdominal uncinal peg: 0. flat; 1. gouge-shaped: 2. pointed;
(75) Flat geniculate abdominal chaetae type: 0. pennant-shaped; 1. parallel-sided; 2. brush-like;
(76) Paired abdominal chaetae: 0. absent; 1. present;
(77) Distribution of abdominal chaetae: 0. entire abdomen; 1. posterior; 2. anterior;
(78) Capillary abdominal chaetae: 0. absent; 1. present;
(79) Capillary abdominal chaetae left/right distribution: 0. concave; 1. both sides;

(80) Abdominal chaetal teeth: 0. fine; 1. coarse;
(81) First abdominal chaetal tooth size: 0. same as other teeth; 1. first two small; 2. larger than other teeth; 3. first tooth small;
(82) Abdominal chaetae heel projection: 0. absent; 1. present;
(83) Size of abdominal chaetae vs. collar chaetae: 0. same; 1. larger; 2. Smaller.

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