Branching out: a remarkable new branching syllid (Annelida) living in a *Petrosia* sponge (Porifera: Demospongiae)

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We describe the morphology and biology of a previously unknown form of branching annelid, ***Ramisyllis multicaudata* gen. et sp. nov.**, an endosymbiont of shallow-water marine sponges (*Petrosia* sp., Demospongiae) in northern Australia. It belongs to the polychaete family Syllidae, as does *Syllis ramosa* McIntosh, 1879, the only other named branching annelid, which was collected from deep-water hexactinellid sponges during the 1875 *Challenger* expedition. It differs from *S. ramosa* in parapodial and chaetal morphology. ***Ramisyllis multicaudata* gen. et sp. nov.** has segments of several types, including specialized posterior segments on the emergent portions of the worm, and simplified elongate segments that bridge larger cavities in the sponge interior. Aside from the obvious branching form, the new annelid is similar to *Parahaplosyllis*, differing from it in lacking pharyngeal armature and in the details of the parapodial chaetae and dorsal cirri. Molecular evidence from *16S* and *18S* rDNA supports a sister-group relationship with *Parahaplosyllis*, with both being sister to *Trypanosyllis* and *Eurysyllis*. The phylogenetic position of ***R. multicaudata* gen. et sp. nov.** indicates that branching has evolved independently in ***Ramisyllis* gen. nov.** and *Syllis*. This is supported by differences in the branching process between the two taxa: in *S. ramosa* branching is initiated by segment addition at the parapodium, whereas in ***R. multicaudata* gen. et sp. nov.** segments are added from a region between parapodia. A model for branching in ***R. multicaudata* gen. et sp. nov.** is proposed and possible developmental processes underlying branching in Annelida, and body symmetry comparisons with other invertebrates, are also discussed.

INTRODUCTION

Since McIntosh's (1879, 1885) first description of *Syllis ramosa*, collected during the *Challenger* expedition, which appeared to branch throughout the water passages of hexactinellid sponges, biologists have been fascinated by the existence of such annelids. McIntosh's specimens were collected in 140 fathoms, from off the Kai Islands, Indonesia, and in 95 fathoms near Cebu, Philippines. *Syllis ramosa* has since been reported several times from Japanese waters (Oka, 1895; Izuka, 1912; Okada, 1937), and from deep water off New Zealand (Read, 2001). Crossland (1933) very briefly reported a branching syllid from shallow water in the northern Red Sea, which he assigned to *S. ramosa*, although the sponge in which it was found is unlikely to have been a hexactinellid, as it was living in warm water at a depth of about 2 m. We believe that the presence of branching was accepted

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by most of these authors as diagnostic of *S. ramosa*, and that the possibility that more than one taxon of branching syllid might exist was not seriously entertained.

Here we report on the second ever case of a branching annelid species. The undescribed syllid, *Ramisyllis multicaudata* gen. et sp. nov., occurs in a shallow water tropical demosponge, *Petrosia* sp., from the vicinity of Darwin, Northern Territory, Australia. The worm was first observed in 2003 by divers collecting sponges for a different project; extra specimens of *Petrosia* sp., which occurs in both white and purple varieties, were preserved to permit study of the worm, and additional excursions have since been undertaken to permit observation of living material. Observations on adults and juveniles over the last 8 years have yielded sufficient specimens to describe the taxonomy and biology of this most unusual animal. Based on these observations, we discuss aspects of the morphological evolution of the species, particularly concerning branching, and compare it with other bilaterally symmetrical animals (Bilateria).

Among the Bilateria only the annelids, arthropods, and chordates possess segmental organization. The annelids, which include syllids, all have a head (prostomium), a tail (pygidium), and in between a series of segments arranged in a linear sequence along an anteroposterior (A–P) axis. They grow by the addition of new segments at the posterior end of the body in a region called the posterior growth zone (PGZ), immediately in front of the pygidium (Anderson, 1973). Although the precise genetic mechanism controlling post-embryonic segmentation and segmental organization in annelids has yet to be elucidated, it is thought to be similar to those of other bilaterians, viz. regulation by homeotic (= *Hox* family) genes, which in turn control the activity of hundreds of other target genes (Irvine & Martindale, 2001; Shimizu & Nakamoto, 2001).

Unlike most other bilaterians, some Syllidae can also add segments during reproduction in a process called gemmiparous schizogamy. In this process the mature individual (stock) proliferates new segments to produce a single, or multiple, gamete-filled stolon that resembles a miniature, simplified version of the parent stock. Segment proliferation in gemmiparous schizogamy can occur at the PGZ of the stock, near the tail of the stolon (producing chains of stolons), on the ventral surface of one or more posterior segments of the stock, or in the case of *S. ramosa*, from the parapodial dorsal dorsal cirrus (Garwood, 1991). We describe somatic and reproductive segment addition in *R. multicaudata* gen. et sp. nov. and compare it with that in *S. ramosa* and other syllidan annelids in order to explain the branching ability of this remarkable new form.

MATERIAL AND METHODS
SAMPLE COLLECTION, PREPARATION AND MORPHOLOGICAL ANALYSIS

Petrosia sponges and their annelid symbionts were collected by SCUBA and reef walking from Darwin Harbour, Bynoe Harbour, and the Wessell and English Company Islands, off the north coast of Australia. Some were fixed in 10% seawater formalin and preserved in 70% ethanol; others were preserved directly in 90% ethanol for DNA sequencing. The incidence of infestation and the number of worms per sponge was determined by fully dissecting each sponge collected. The worms could not be removed intact as they were very fragile; instead, fragments were removed by dissection, following the different branches through the internal cavities of the sponge. The most efficient method for locating the head of the worm was to select the smallest possible sponge for dissection and carefully divide it into cubes of about 1 cm^3, where possible using natural cleavage breaks. Each piece was then classified as 'surface' (purple coloured) or interior. The interior parts were searched first as being most likely to include the head. As the pigmented dorsal cirri of the posterior segments occur near the surface of the sponge, it was possible to establish roughly how close one was to the head.

Light microscope observations were made using a Nikon SMZ 1500 and a Nikon Eclipse 80i with Nomarski optics; photographs were taken on both microscopes using a Qimaging Micropublisher 5.0 RTV digital camera. The specimens examined for internal structures were first cleared in a 50 : 50 solution of lactic acid and glycerol. Scanning electron microscopy (SEM) observations were based on specimens fixed in 5–10% formalin, then transferred to 70% ethanol (including the holotype and the Japanese *S. ramosa*), or fixed with a solution of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. All samples were dehydrated through an ethanol series, air-dried or lyophilized, and coated with gold in a sputter coater. Scanning electron microscope images of the holotype were taken using a Jeol JSM 5610LV; all other SEM images were taken on a Hitachi S-570.

Type and voucher specimens have been deposited at the Los Angeles County Museum of Natural History, Los Angeles, formerly known as the Allan Hancock Foundation (LACM-AHF) and the Museum and Art Gallery of the Northern Territory, Darwin (NTM). Comparative material of *S. ramosa* was examined at the Natural History Museum, London (BMNH), and loaned from the National Science Museum, Tokyo, Japan (NSM).

MOLECULAR ANALYSIS

The nuclear gene *18S* rDNA and the mitochondrial gene *16S* rDNA were analysed. Most of the syllid sequences were used previously in the analysis performed by Aguado, Nygren & Siddal (2007) and Aguado *et al*. (in press) except for the sequences of *R. multicaudata* gen. et sp. nov. (Table 1). To get these new sequences, the same DNA extraction, purification, amplification, and sequencing processes were applied as described by Aguado *et al*. (2007). The sequences of the out-groups were obtained from GenBank (Table 1).

Gene sequences were aligned using MAFFT 6 (Katoh *et al*., 2002), using the iterative refinement method E-INS-i, with the default gap open and extension values. No regions were excluded from the alignments. The combined analyses of the two genes were performed though maximum parsimony (MP) and Bayesian inference (BI). The MP analysis was performed in TNT (Goloboff, Farris & Nixon, 2008) through a heuristic search using the tree–bisection reconnection (TBR) searching algorithm and 5000 replicates, with 100 trees saved per replication. All characters were left unweighted and non-additive, and gaps were treated as missing data. Strict consensus topologies and jackknife support values (Farris *et al*., 1996) for 1000 replicates were also generated with TNT. The combined data set was also analysed through BI in MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2001), in which four Markov chains were run for 10 million generations, trees were sampled every 1000 generations (sample freq = 1000), and 12 000 trees were excluded as burn-in. The model of evolution that best fits the data was chosen using Akaike's information criterion (AIC), available on MODELTEST 3.7 (Posada & Crandall, 1998). This test preferred the model GTR+I+Γ for *16S* and TrN+I+G for *18S*.

RESULTS

TAXONOMY

SYLLIDAE GRUBE, 1850

SYLLINAE GRUBE, 1850

RAMISYLLIS GEN. NOV.

Type species: Ramisyllis multicaudata sp. nov.

Description: Syllinae with dendriform body, more or less circular in section (flattened ventrally); three antennae; palps free to base; two pairs of tentacular cirri; pharynx slender, mid-dorsal tooth present (juveniles), lost in adults; dorsal cirri articulated, with alternating thick/slender pattern on mid-body and posterior segments; ventral cirri present, not articulated, inserted proximally; single type of simple chaeta present, tomahawk shaped; reproduction by schizogamy, stolons produced on short stalks arising from internal lengths of worm body within the sponge.

Etymology: Named derived from the Latin, *ramus*, masculine, meaning branch.

RAMISYLLIS MULTICAUDATA SP. NOV.
(FIGS 1–9, 11; TABLES 2, S1, S2)

Material examined: Holotype: Darwin Harbour, Channel Island (type locality), about 200 m north of bridge, 12°33.2′S, 130°52.4′E, coll. C. Glasby, 8 November 2006 (in two pieces, head end mounted for SEM and posterior end in ethanol; both NTM W23745). Paratypes: Darwin Harbour, Channel Island, same collection data as for holotype, one specimen (LACM-AHF on SEM stub; number unavailable), one specimen (NTM W23752), one specimen (NTM W23750); Darwin Harbour, same collection data except coll. C. & B. Glasby, 28 October 2007, one specimen (NTM W23753), one specimen (NTM W23749), one specimen (NTM W23747), one specimen (NTM W23746); Channel Island, 'Town Hall', 12°33.74′S, 130°51.67′E, 10–20 m depth, coll. C. & B. Glasby, 9 September 2004, one specimen (NTM W23748), one specimen (NTM W23751); Dudley Point, 12°24.87′S, 130°49.27′E, low water spring tide level, coll. B. Glasby, 30 September 2011, one specimen (NTM W23790). Non-types (all lacking heads): Darwin Harbour, same collection data as holotype, NTM W23766; Channel Island, same collection data, except coll. C. & B. Glasby, 28 October 2007, one specimen (NTM W23767); Channel Island, 'Town Hall', 12°33.74′S, 130°51.67′E, 10–20 m depth, coll. C. & B. Glasby, 21 September 2003, NTM W23760, coll. C. & B. Glasby, 9 September 2004 NTM W23759, NTM W23763; Channel Rock, 12°24.94′S, 130°47.04′E, 13 m, coll. B. Glasby, 5 September 2003, NTM W23761, NTM W23768; Stevens Rock, 12°29.102′S, 130°47.111′E, 14 m, coll. B. Glasby & party, 7 May 2002, NTM W23757, coll. B. Glasby & party, 22 August 2003, NTM W23758, NTM W23762; Sandy Island (aka Crocodile Island), 12°35.272′S, 130°52.262′E, 6 m, coll. C. & B. Glasby, 9 September 2004, NTM W23764; Sandy Island, 12°35.272′S, 130°52.262′E, 6 m, coll. B. Glasby & R. Willan, 5 June 2006, NTM W23765. Bynoe Harbour, Spencer Point, near Indian Island, 12°35.48′S, 130°31.29′E, NTM W23754. Wessel Islands, eastern Arnhem Land, off Rimbija Island, 3.2 km west off Cape Wessel, 11°45.82′S, 136°43.65′E, NTM W23756. Wigram Island, the English Company Islands, eastern Arnhem Land, 11°00.41′S, 136°43.65′E, NTM W23755.

Comparative material: Syllis ramosa, syntype BMNH 1885:12:1:150–154, Philippines, near Cebu, 95 fathoms, *Challenger* expedition; Japan, Sagami Bay, 100 fathoms, one specimen (NSM unreg.).

Table 1. Terminals used in the phylogenetic analysis and their GenBank accession numbers

Taxon	Locality	GenBank accession number	
		18S	*16S*
In-group			
Branchiosyllis exilis (Gravier, 1900)	Darwin, Australia	JF903583	JF903692
Branchiosyllis sp.	La Jolla, California, USA	AF474283	–
Brania arminii (Langerhans, 1881)	Cádiz, Spain	EF123831	–
Epigamia magna (Berkeley, 1923)	Washington, USA	AF474309	AF474263
Epigamia noroi (Imajima and Hartman, 1964)	California, USA	AF474310	AF474264
Erinaceusyllis hartmannschroederae San Martín, 2005	Sydney, Australia	EF123868	–
Eurysyllis tuberculata Ehlers, 1864	Banyuls, France	EF123833	–
Eusyllis blomstrandi Malmgren, 1867	Kaldbak, Faroe Islands	EF123887	EF123788
Exogone naidina Örsted, 1845	Wales, UK	EF123886	–
Haplosyllis sp.	Shark Bay, WA, Australia	JF903607	JF903698
Haplosyllis spongicola Grube, 1855	Banyuls, France	EF123837	EF123791
Myrianida convoluta (Cognetti, 1953)	California, USA	AF474303	AF474257
Myrianida pachycera (Augener, 1913)	California, USA	AF474304	AF474258
Nudisyllis pulligera (Krohn, 1852)	Port de la Selva, Girona, Spain	EF123873	–
Odontosyllis fulgurans (Audouin and Milne Edwards, 1834)	Port de la Selva, Girona, Spain	EF123882	EF123792
Odontosyllis gibba Claparède, 1863	Port de la Selva, Girona, Spain	EF123850	EF123793
Parahaplosyllis brevicirra Hartmann-Schröder 1990	Port Jackson, New South Wales, Australia	JF903679	JF903708
Parapionosyllis sp.	Banyuls, France	AF474287	–
Perkinsyllis augeneri (Hartmann-Schröder, 1979)	Sydney, Australia	EF123832	EF123794
Pionosyllis enigmatica (Wesenberg-Lund, 1950)	Tjärno, Sweden	EF123826	EF123795
Proceraea aurantiaca Claparède, 1868	Banyuls, France	AF474324	AF474278
Proceraea okadai (Imajima, 1966)	Washington, USA	AF474319	AF474273
Procerastea sp.	California, USA	AF474315	AF474269
Prosphaerosyllis xarifae Hartmann-Schröder, 1960	Galicia, Spain	EF123836	–
***Ramisyllis multicaudata* gen. et sp. nov.**	Darwin, Australia	JQ292795	JQ313812
Salvatoria clavata (Claparède, 1863)	Port de la Selva, Girona, Spain	EF123825	–
Salvatoria limbata (Claparède, 1868)	Port de la Selva, Girona, Spain	EF123872	–
Sphaerosyllis austriaca Banse, 1959	Port de la Selva, Girona, Spain	EF123884	EF123799
Sphaerosyllis boeroi Musco, Çinar and Giangrande, 2005	Port de la Selva, Girona, Spain	EF123856	EF123800
Sphaerosyllis hystrix Claparède, 1863	Port de la Selva, Girona, Spain	EF123880	–
Syllis armillaris (O.F. Müller, 1771)	Kaldbak, Faroe Islands	AF474292	–
Syllis compacta Gravier, 1900	Altea, Spain	EF123846–7	EF123806
Syllis corallicola Verrill, 1900	Port de la Selva, Girona, Spain	EF123875	EF123807
Syllis ehlersioides (Marenzeller, 1890)	Manazuru Peninsula, Japan	EF123841	EF123808
Syllis ferrani Alós and San Martín, 1987	Port de la Selva, Girona, Spain	EF123874	EF123809
Syllis gracilis Grube, 1840	Galicia, Spain	EF123876	EF123811
Syllis hyalina Grube, 1863	Port de la Selva, Girona, Spain	EF123851–2	EF123818
Syllis krohni (Ehlers, 1864)	Azores Islands, Portugal	EF123859	EF155920
Syllis marugani Aguado, San Martín and Nishi, 2006	Manazuru Peninsula, Japan	EF123862–3	EF123812
Syllis monilata (Imajima, 1966)	Manazuru Peninsula, Japan	EF123860–1	EF123819
Syllis okadai Fauvel, 1934	Manazuru Peninsula, Japan	EF123857–8	EF123814
Syllis vivipara Krohn, 1869	Galicia, Spain	EF123848–9	EF123815
Trypanosyllis coeliaca Claparède, 1868	Port de la Selva, Girona, Spain	EF123878	EF123816
Trypanosyllis zebra (Grube, 1860)	Shark Bay, WA, Australia	JF903677	JF903751
Trypanosyllis zebra	Banyuls, France	JF903676	EF123817
Virchowia clavata Langerhans, 1879	Banyuls, France	AF474314	AF474268
Out-groups			
Eunice pennata (Müller, 1776)	GenBank	AY040684	AF321418
Harmothoe imbricata (Linnaeus, 1767)	GenBank	AY340434	AY340463
Nereis pelagica Linnaeus, 1758	GenBank	AY340438	AY340470
Eulalia viridis (Johnston, 1829)	GenBank	AY996085	AY996064
Sigambra sp.	GenBank	AY340444	AY340481

Table 2. Segments at which branches develop in all individuals of *Ramisyllis multicaudata* **gen. et sp. nov.** with a head available for study

Specimen	Segment with first branch	Direction of first branch (anterodorsal perspective)	Segment with second branch	Segment with third branch	Segment with fourth branch
W23751	15	Left	–	–	–
LACM-AHF unreg	20	Left	30	41	–
W23752	17	Left	31	–	–
W23750	15	Right	17	–	–
W23745 (holotype)	17	Right	22	30	–
NTM W23790)	14	Left	18	26	38

The direction of the first branch only is given, as subsequent ones alternate.

Figure 1. *Ramisyllis multicaudata* **gen. et sp. nov.**, paratype NTM W23790 showing two orders of branching (branches all incomplete). Dc, dorsal cirrus; ph, pharynx; pr, proventricle; g, gut. Scale bar: 3.0 mm.

Etymology: Name derived from the Latin, *multus* for many, and *caudata*, feminine, for tailed.

Description: All specimens incomplete. Body width about 1 mm throughout; length varies depending upon path taken to a particular posterior end, up to 3–4 cm when stretched out; segments of several distinct morphologies. Holotype with five orders of branching producing 2^{n-1} or 16 terminal branches, where $n = 5$ branch nodes (specimen incomplete); the complete worm therefore probably had hundreds, possibly thousands, of terminal branches. First branch appears on the left or right side between segments 14 and 20, and branching develops in individuals with as few as 26 segments (Table 2; Figs 1 and 2A–C).

Prostomium with two pairs of eyes, anterior pair ventral and lateral to posterior pair; antennae articulated, of equal length, median one placed behind lateral ones; palps small, oval shaped, anteroventrally directed (Fig. 3A, B). Tentacular cirri articulated, of equal length. Pharynx slender, about one-quarter the width of proventricle, tooth absent in adults, present in juveniles (Fig. 4). Proventricle prominent, barrel shaped, lying between chaetigers 12–14 in holotype (Fig. 2A) and 11–17 (other material); it lies further forward (segments 3–5) in juveniles (Figs 2C and 4). Segments anterior to first
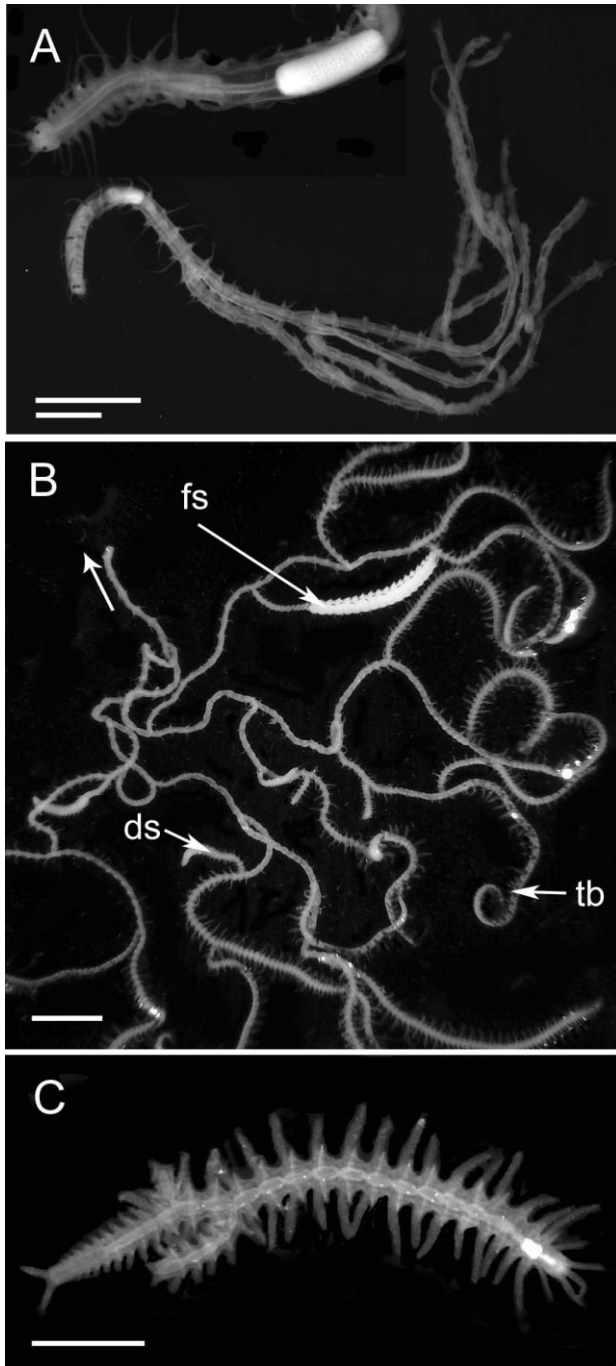
Figure 2. *Ramisyllis multicaudata* **gen. et sp. nov.** A, holotype, anterior end; B, non-type, mid-posterior body, showing terminal branches and developing female stolon – the arrow indicates the direction of the head; C, smallest individual (NTM W23750) showing branching, ventral view; fs, female stolon; ds, developing stolon; tb, terminal branch. Scale bars: A, 2.0 mm (upper), 3.0 mm (lower); B, 10 mm; C, 0.5 mm.
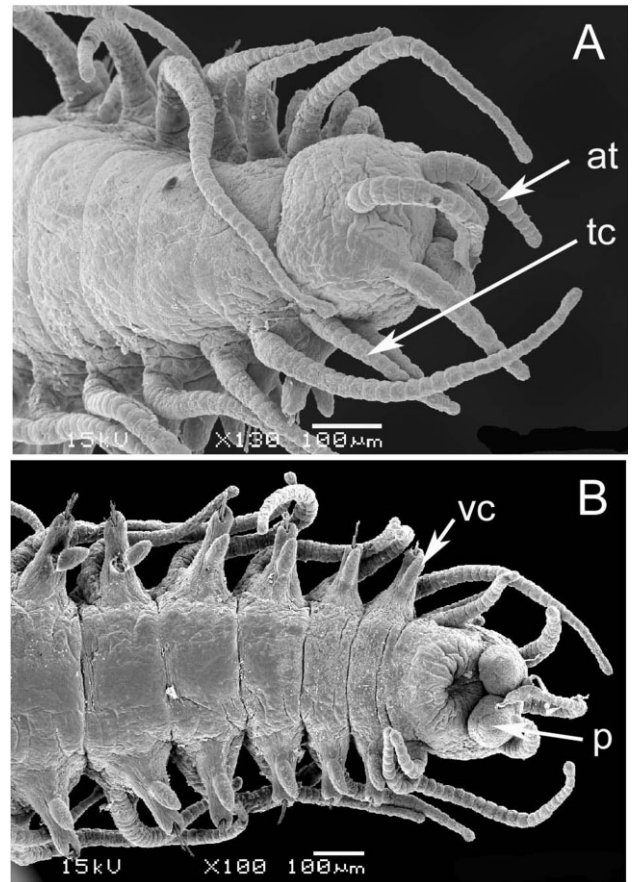

Figure 3. *Ramisyllis multicaudata* **gen. et sp. nov.** holotype, SEM anterior end. A, dorsal view; B, ventral view; at, antenna; p, palp; tc, tentacular cirrus; vc, ventral cirrus.
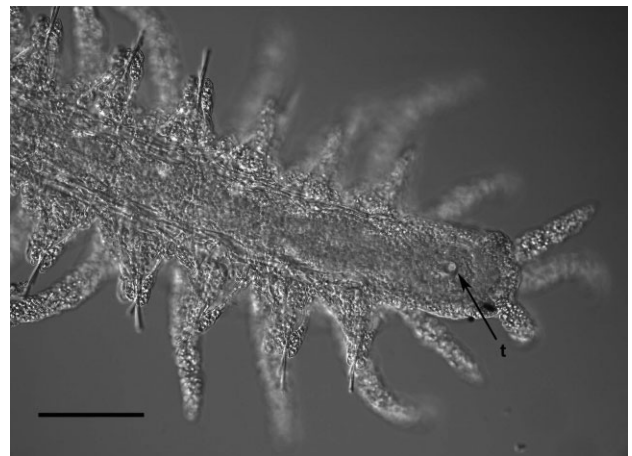

Figure 4. *Ramisyllis multicaudata* **gen. et sp. nov.** unbranched juvenile (NTM W23753), ventral view, Nomarski optics, showing presence of mid-dorsal tooth and anterior position of pharynx; t, tooth. Scale bar: 0.1 mm.
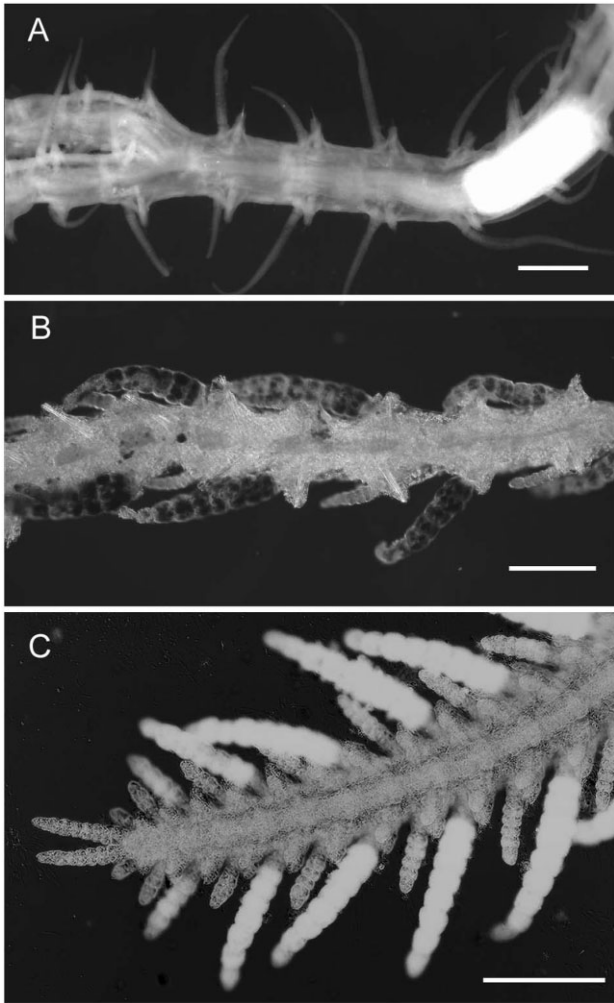
Figure 5. *Ramisyllis multicaudata* **gen. et sp. nov.** A, holotype, ventral view of anterior region showing asymmetrical dorsal cirri and first branch (upper); B, non-type from white form of *Petrosia* sp., mid-posterior body region showing asymmetrical dorsal cirri; C, non-type from purple form of *Petrosia* sp., posterior (external) body region showing alternation of enlarged and slender dorsal cirri and pygidium. Scale bars: 1.0 mm.

Figure 6. *Ramisyllis multicaudata* **gen. et sp. nov.** A, non-type (NTM W23752), light microscopy (Nomarski optics) image of branch point, mid-body, showing newly developed branch with tail end; B, non-type, SEM of branch point, mid-body region, showing form of mature branch. Scale bars: 1.0 mm.

branch node have elongate dorsal cirri with 17–30 articles. Dorsal cirri slender anteriorly, showing slight length–position alternation; pattern may be altered in post-proventricle segments (after segment 11 in holotype), resulting in dorsal cirri of markedly different sizes being borne on the two sides of a single segment (Fig. 5A, B); symmetrical alternation pattern can reappear posteriorly (Fig. 5C). Pygidial cirri articulated, resembling smaller dorsal cirri of posterior segments (Fig. 5C).

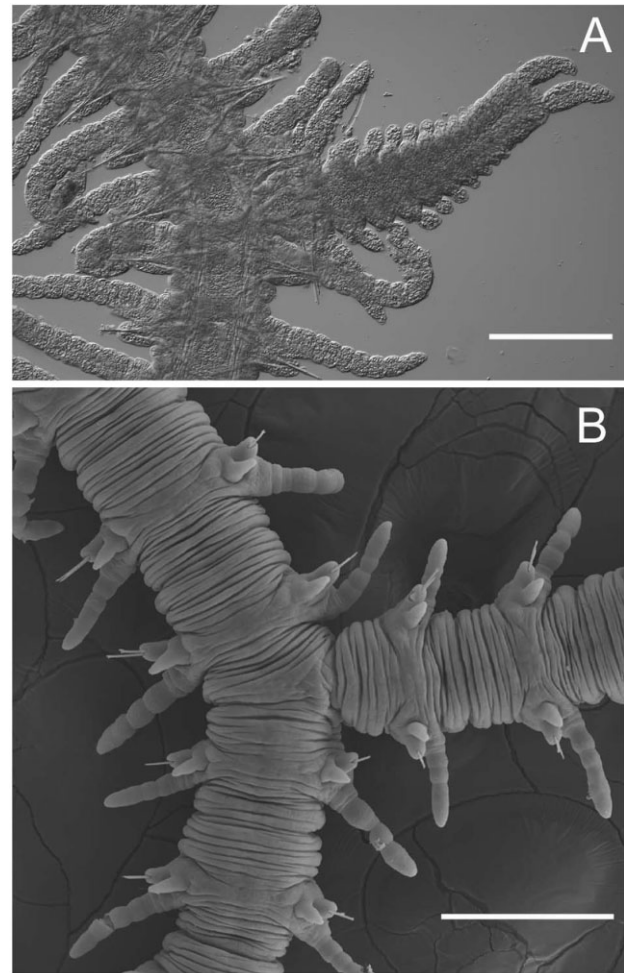Segment form and branching pattern differ depending on location in host. Segments in internal regions of host highly variable in length (200–700 µm; series of longer ones tending to span largest gaps in sponge tissue), largely devoid of pigment, dorsal cirri slender and short. First branch from segments 14–20 on right side (holotype) or left side (most other paratypes), and subsequent ones usually emerging on alternate sides of body (Table 2); branches emerging more or less at right angles from between parapodium and anterior face of posterior septal wall of originating segment (Fig. 6A, B). Posterior segments at or near surface of host shorter (about 100 µm in length), bearing dorsal cirri that include large, highly pigmented, thick ones that arch over the dorsal surface in preserved specimens (Fig. 7), and are stretched horizontally on the surface of the host in life (Fig. 11), alternating with shorter unpigmented cirri; branching remains alternate, although with fewer segments between
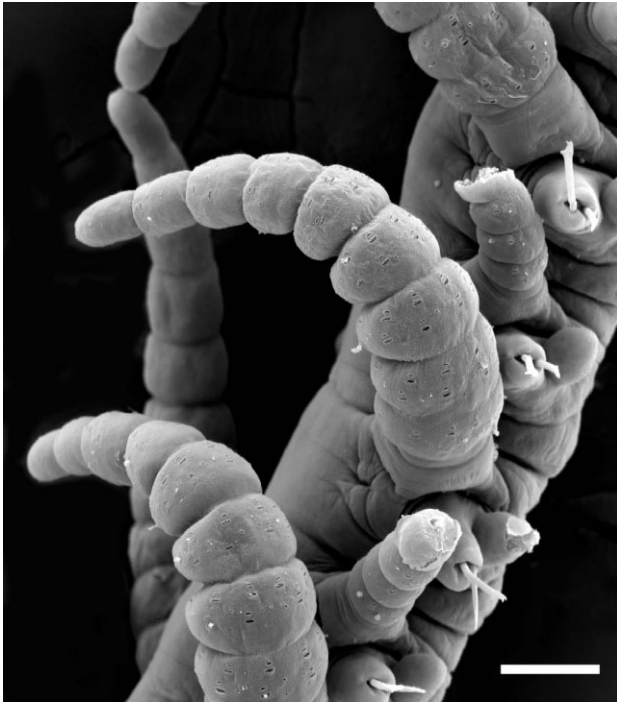
Figure 7. *Ramisyllis   multicaudata* gen. et sp. nov. Non-type, lateral view of posterior segments showing prominent arrays of pits and perforated plates. Scale bar: 50 μm.

branches; branching pattern tending towards pinnate in stolon-bearing regions of body (see below).

Each parapodium comprises an articulated dorsal cirrus, a small subconical unarticulated ventral cirrus, and slightly larger neuropodia, bearing two or three simple chaetae and a tapered, blunt-tipped acicula. Articles of most dorsal cirri covered with prominent arrays of pits and perforated plates (Fig. 7); they may occur in groups of three or four, up to about 24. Chaetae tomahawk shaped, bifid distally, prominent subdistal spur, and series of denticles between teeth and spur (Fig. 8A); chaetal shape constant along body.

Reproduction: Male and female stolons are produced de novo as reproductive structures, which is typical of gemmiparous schizogamy. However, unlike typical gemmiparity, in which stolon segments originate from a proliferation zone immediately in front of the stolonal pygidium (Garwood, 1991), the stolonal segments in *R. multicaudata* gen. et sp. nov. are produced at the end of a specialized short branch: the stolon stalk (Fig. 2C). Stolon stalks have between six and 13 segments; they develop toward the external (posterior) segment region (Fig. 2B), but are not observed to be exposed at surface in life; male stolons are similar in width to stalk, about half the width of egg-laden females, and have relatively better devel-

oped parapodia and musculature; the heads of both sexes have two pairs of well-developed dorsal (posterior) and ventral (anterior) eyes, and lack palps, antennae, and tentacular cirri (Fig. 9). The parapodia bear similar tomahawk-shaped chaetae as the parent stock, except that the angle and relative size of distal teeth differ slightly (Fig. 8B). Regeneration of the posterior end (pygidium) of parent-stock stalks may occur before detachment of the stolon, as suggested by what appears to be minute developing anal cirri (Fig. 9). We are unsure about where fertilization takes place, the fate of the stolons (and stolon stalks), and the form of the larvae (no larvae were found in the sponges examined). The smallest juveniles found ranged in size from 2.2–2.5 mm in length and 28–29 segments; they were unbranched. The smallest branched specimen was 2.7 mm in length with 26 segments along the primary axis (Fig. 2C; Table S2).

Distribution and habitat: Coastal waters of the 'Top End' of northern Australia from low water spring tide level to at least 20 m; endosymbiont of *Petrosia* sp. (purple and white forms).

Remarks: At the generic level the new taxon is most similar to *Parahaplosyllis* Hartmann-Schröder 1990, sharing with this taxon the presence of three antennae, small, oval-shaped palps that are free to the base, and tomahawk-shaped chaetae (Table 3). It differs from this genus in the dendriform body, alternating form of the dorsal cirri, in lacking a trepan, and in adults a pharyngeal tooth, and in having only a single type of parapodial chaeta (*Parahaplosyllis* has two); however, recent studies suggest that the presence or absence of a pharyngeal tooth is sometimes variable at the species level, as a result of ontogenetic loss (Aguado & San Martín, 2009 and references therein). Further comparisons of key features between the new genus and other syllid genera having simple chaetae are shown in Table 3.

At the species level, *R. multicaudata* gen. et sp. nov. bears an obvious resemblance to *S. ramosa*, especially in the dendriform body and the asymmetrical pattern of the dorsal cirri. The new taxon may be distinguished from previously described forms of *S. ramosa* in coloration, parapodial and chaetal morphology, and in having a greater diversity of segment types, as discussed below. However, it is likely that the name *S. ramosa* encompasses more than one species.

Illustrations of the type material of *S. ramosa* contain a number of enigmatic features (McIntosh, 1885: plates 34A and 8, the head) compared with the typical features of the genus *Syllis*. Most strikingly, the illustration shows no sign of a proventricle, usually considered to be a defining feature of the

Figure 8. *Ramisyllis multicaudata* **gen. et sp. nov.** A, chaetae from segment 8; B, chaeta from stolon. Scale bars: A, 5.0 µm; B, 3.0 µm.
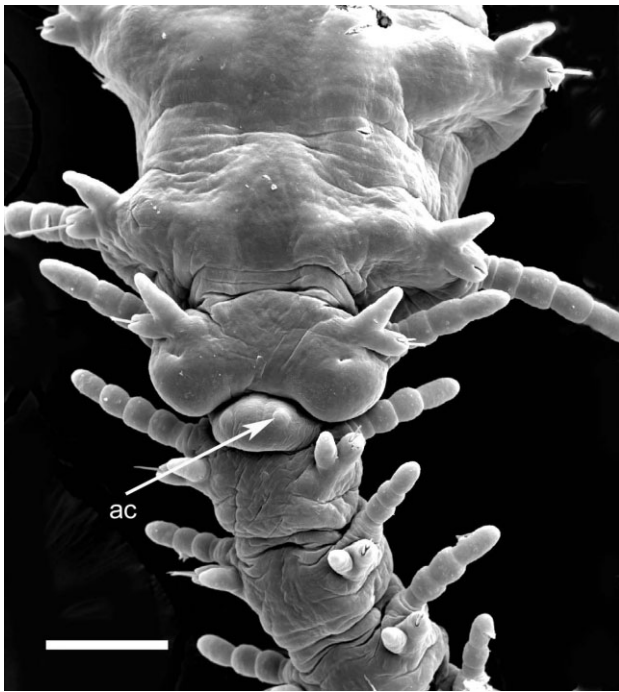

Figure 9. *Ramisyllis multicaudata* **gen. et sp. nov.** Anterior end of developing female stolon attached to peduncular segments of parent stock, ventral view. Note what may be minute developing anal cirri; ac, anal cirrus. Scale bar: 100 µm.

Syllidae (Aguado & San Martín, 2009); nor is the median antenna, typical of many genera, including the genus *Syllis*, indicated. Palps are not illustrated and the text suggests that they may be vestigial ('a minute and flattened lobe appears and it is possible that this is the homologue of the palpus'). Finally, neither figure nor text is clear on whether or not the four eyes typical of many syllids, including *Syllis*, are present. Izuka's (1912) description of the head of a Japanese specimen does not extend to the proventricle, of which no mention is made, but indicates that the head is that of a typical sylline syllid, including two pairs of eyes, and elongate, conical palps, which differ from the short ovoid palps of the new species and from McIntosh's illustration. As the *S. ramosa* material we received from Japan did not include a head, we cannot add to the description of the head nor confirm the accuracy of McIntosh's observations. The longer dorsal cirri of McIntosh's *S. ramosa* contain about 26 articles (28 in the Japanese specimens: Izuka, 1912), and the shorter dorsal cirri contain about 15 (both reports): many more than the cirri of the new species (Table S1). Furthermore, the chaetae of *S. ramosa* are more slender, hooked at the tip, and have a distinct articulation, compared with the simple, robust, tomahawk-shaped ones of *R. multicaudata* gen. et sp. nov.

Oka (1895) reported that, in Japanese material, branches form on an intercalary segment that appears between two existing segments, and grow out from this on both sides of the worm. McIntosh (1885) illustrated no examples of this type and explicitly stated that it was not evident in his material. He reports that branches may arise by the replacement of one parapodial dorsal cirrus with a zone of cell proliferation, which ultimately develops into a new branch of the worm. This form of development is also sketched and discussed by Okada (1937). We have not observed this process in any of our material: branching in *R. multicaudata* gen. et sp. nov. clearly occurs as an outgrowth of the body wall behind a parapodium; no dorsal cirri are involved (Fig. 6B) and no paired branches have been observed. Furthermore, the 'vestigial' palps described by McIntosh differ significantly from the more normal palps illustrated by Izuka (1912) from Japanese material.

Table 3. Comparison of features of **Ramisyllis gen. nov.** with other syllid taxa with simple chaetae, modified after Glasby & Watson (2001)

|	Body shape	Antennae (number)	Palps	Pharynx (armature)	Dorsal cirri	Chaetae	Aciculae (shape)
Alcyonosyllis Glasby & Watson, 2001	Not branched or flattened	3	Large, free basally	Subdistal dorsal tooth	Alternating thick and slender types; unarticulated	Two types (distally recurved, no teeth; and distally recurved, minute subdistal tooth)	Straight, tapered or blunt-tipped
Bollandiella Glasby & Krell, 2009	Not branched or flattened	0	Absent	Unarmed	All same thickness, irregularly wrinkled	Two types (large, distally recurved, no teeth; and flail-tipped)	Straight, tapered
Geminosyllis Imajima, 1966	Not branched or flattened	3	Large, free basally	Subdistal dorsal tooth and trepan	All same thickness, articulated	Two types (tomahawk and slender bifid types)	Straight, blunt tipped
Haplosyllis Langerhans, 1879	Not branched or flattened	3	Robust, triangular, fused basally	Subdistal dorsal tooth	All same thickness, articulated	One type (tomahawk type)	Bent at tip (usually)
Haplosyllides Augener, 1922	Not branched or flattened	3	Fused forming a single bilobed structure	Subdistal tooth (absent in adults)	All same thickness, unarticulated	Two different sizes (both tridentate)	Straight, blunt tipped
Parahaplosyllis Hartmann-Schröder, 1990	Not branched or flattened	3	Small, oval-shaped; free basally	Subdistal tooth and trepan	All same thickness, indistinct articulations	Two types (tomahawk, and long, bidentate)	Straight, tapered
Ramisyllis gen. nov.	Branched, not flattened	3	Small, oval-shaped; free basally	Subdistal dorsal tooth (absent in adults)	Posteriorly, thick and slender types alternate, clearly articulated	One type (tomahawk)	Straight, blunt-tipped
Trypanosyllis (Trypanobia) Imajima & Hartman, 1964	Flattened, not branched	3	Short, ventrally directed; free basally	Trepan only	All same thickness, articulated	One type, unidentate or bifid with subdistal spur	Straight, slender
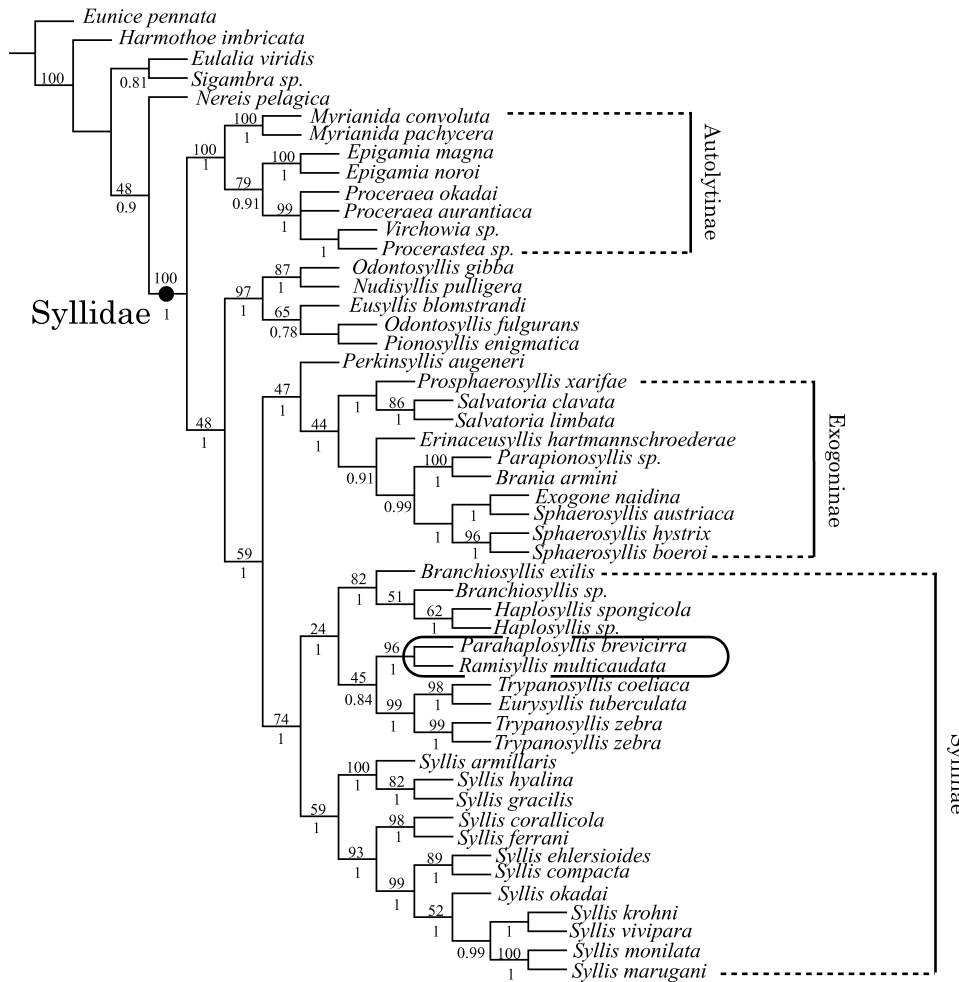
Figure 10. Strict consensus tree of the combined molecular data set analysed through maximum parsimony. Numbers above nodes represent the jackknife support values; numbers below nodes denote the posterior probabilities in the Bayesian inference analysis.

Okada (1937) notes that in his Japanese *S. ramosa* the stolon stalk is composed of a variable number of segments, all of which are typical in their structure. They thus differ from McIntosh's (1885: pl. 33, Fig. 11) description of the type material, in which the stolon stalks are formed of four segments with parapodia either incompletely developed or (in the two segments closest to the body) absent. Okada expresses doubt that the condition described by McIntosh actually occurs; McIntosh does not report how many such stalk–stolon combinations he observed. The segments of comparable stalks of *R. multicaudata* gen. et sp. nov. all contain well-developed parapodia.

The single photo published by Read (2001) of a headless portion of a branching worm collected from deep water off New Zealand looks heavier bodied than the other *S. ramosa*. Read observed that many stolons were present, but free of the stalks and 'congregated

in the hollow tube that forms the basal stalk of the sponge', and that their mode of egress from this location in the sponge is not apparent. The New Zealand specimens formed a reddish pigmented mass. Japanese specimens were also reported to be red in life (Imajima, 1966). Both the New Zealand and Japanese specimens were found in a species of the hexactinellid sponge *Crateromorpha*.

PHYLOGENETIC POSITION INFERRED FROM
MOLECULAR DATA

Parsimony analysis performed in TNT yielded two equally parsimonious trees, with 7510 steps. Results obtained from the MP and BI analyses resulted in highly congruent topologies (Fig. 10). After several DNA extractions and sequencing attempts, we were only able to get the first piece of *18S* for *R. multicaudata* gen. et sp. nov. Instead, the sponge sequences for

Figure 11. Living sponge, *Petrosia* sp. (purple form) photographed in the lab, with posterior ends of ***R. multicaudata* gen. et sp. nov.** emerging from surface pores and moving actively on the sponge surface. The sponge is about 90 mm in diameter.

the second and the third pieces were always obtained. However, the first piece of the large *18S* gene, in combination with *16S*, reveals hypotheses similar to those inferred from the morphology. The cladogram resulting from the combined data set confirms the close relationship between *Parahaplosyllis* and *Ramisyllis* gen. nov., both located within Syllinae, in a very well-supported clade, and sister to *Trypanosyllis* and *Eurysyllis* species. The *Ramisyllis–Parahaplosyllis* clade is defined by the presence of tomahawk-shaped chaetae. This kind of simple chaeta is derived from the compound ones, plesiomorphic in Syllidae, probably after a fusion process between the fang and the blade (Martin & Britayev, 1998; Aguado & San Martín, 2009; Lattig & Martin, 2009). A convergent process may explain the presence of very similar simple chaetae in other genera and species, such as *Haplosyllis* Langerhans, 1879 and *Syllis gracilis* Grube, 1840 within Syllinae, which are herein revealed as not closely related to the *Ramisyllis* gen. nov. and *Parahaplosyllis* clade (Fig. 10; Table 3).

The unrelated phylogenetic positions of *R. multicaudata* gen. et sp. nov. and *Syllis* species, where

S. ramosa might belong, indicates that a dendriform body has probably evolved more than once in Syllidae. Nevertheless confirmation of the phylogenetic position of *S. ramosa* is required, as this species has several features that are not typical of the genus *Syllis*. Therefore, whether the dendriform body represents a synapomorphy of a clade of syllids or an autapomorphy for each of *R. multicaudata* gen. et sp. nov. and *S. ramosa* requires verification.

BIOLOGICAL OBSERVATIONS

Inside the sponge the worm is extensively branched throughout the internal canals and cavities, with the single head located centrally and basally in the sponge and the multiple tails near its surface (Fig. 11). The worm could not be coaxed out of its host even when it was subjected to drops of formalin added to seawater surrounding the sponge. The distribution of the worm within the sponge is uneven. Although some areas have high densities, others have no worm at all. The number of worms with heads ranges from one to three per sponge; however, in the only sponge ($n = 30$) found to have more than one worm, one

specimen (NTM W23766) was clearly an adult occupying most of the space of the sponge, whereas the other two (NTM W23750, W23752) were short juveniles with only two orders of branching.

On the surface of the sponge, groups of posterior ends of the syllid emerge from larger openings, whereas smaller openings may have only one (Fig. 11). One small sponge had at least 100 posterior ends emerging from both the oscula and the ostia, with ten or more emerging from a single osculum. They move about actively on the sponge surface, but cannot move far from the opening through which they are attached to the rest of the worm. In life, the dorsal cirri of these emergent posterior segments bear a shining white pigment, and the cirri are held perpendicular to the axis of the body, making the worms quite visible. In formalin-fixed material, the pigment in the dorsal cirri turns purple; in ethanol-fixed specimens it is extracted and disappears.

Some clusters of posterior ends do not reach the surface; they are instead jammed together in small chambers inside the sponge, especially on the side attached to the substrate. These ends nonetheless produce pigment, which stains the chamber of preserved sponges purple. Thus, although the pigment may be protective in the ends that do reach the surface (and bright tropical sunlight), the pigment does not appear to be produced in response to light stimulus. We believe that these developing branches encounter dead ends in the sponge passages, and never reach the surface.

DISCUSSION

WORM–SPONGE RELATIONSHIP

Most polychaete species known to be endosymbionts of sponges belong to the family Syllidae (Martin & Britayev, 1998); only two species, *S. ramosa* and *R. multicaudata* gen. et sp. nov., show branching. The more common situation, particularly among the genus *Haplosyllis*, involves unbranched (vermiform) individuals that occur in dense populations, which reproduce either sexually by schizogamy or asexually (Lattig & Martin, 2009, 2011). Although the two morphological strategies may appear very different, the level of fecundity for the worms per sponge may be similar. In the branching form, high fecundity is achieved by the production of a large number of stolons from body segments well down the body – certainly beyond the fifth order of branching – whereas *Haplosyllis* species have far fewer stolons per adult, but the dense adult populations ensure similarly high fecundity. Similarly, the percentage of the sponge canal system occupied by the symbionts may be similar for branched and unbranched forms,

and both are likely to exert an influence on the sponge, including water flow through the canals.

Perhaps the most obvious difference between a branching and a non-branching strategy for an endosymbiont syllid is feeding and diet. Ingestion of food by *Ramisyllis* gen. nov. is certainly possible, especially considering that *Ramisyllis* gen. nov. has essentially the same feeding apparatus as other syllid endosymbionts. Possibly *R. multicaudata* gen. et sp. nov. feeds on cells in the sponge mesohyl. As we have found only a couple of spicules in the gut of many centimetres of branches, they could be selectively excluded, or simply too large to be ingested by the worm. It may also feed on the cyanobacteria and microalgae known to be associated with sponges (Brümmer *et al*., 2008). A preference for sponge tissue is indicated in other syllid sponge symbionts: both Pawlik (1983) studying the sponge exosymbiont *Branchiosyllis oculata* Ehlers, 1887 and Magnino & Gaino (1998) studying the sponge endosymbiont *Haplosyllis* cf. *spongicola* (Grube, 1855) from the western Indian Ocean (Tanzania) found that extracts of the worms have absorption spectra similar to those of the sponge from which they were removed, strongly suggesting that these annelids feed directly on the sponge tissue. However, it seems inconceivable that *Ramisyllis* gen. nov. could derive sufficient nutrients through its mouth alone to sustain such a large body; furthermore, its branching morphology would appear to limit movement of the head for finding new feeding areas. Among alternative possible food sources, dissolved organic material (DOM) seems most likely. Although we have no direct evidence of uptake of DOM, it is known that integumental nutrient transport is a widespread characteristic of soft-bodied marine invertebrates, including polychaetes (Wright & Manahan, 1989).

The relationship between *Ramisyllis multicaudata* gen. et sp. nov. and its *Petrosia* host is certainly symbiotic, and possibly a strict endosymbiosis, with all observed life stages of *Ramisyllis* occurring within the sponge. *Ramisyllis multicaudata* gen. et sp. nov. displays a high degree of host specificity: it has not been reported from any other sponge species in inshore waters of northern Australia, despite intensive surveys by the NTM over the last 25 years. In terms of infestation prevalence and intensity, every *Petrosia* sp. sponge examined in this study (about 30) was occupied by an adult worm, regardless of the size of the sponge (~5–12 cm in diameter), and two sponges contained an adult plus two or three juveniles.

Other indications of a strict endosymbiont (or parasite) are their simplified morphology, particularly the reduction of chaetal diversity and simplified jaws (Martin & Britayev, 1998). *Ramisyllis* gen. nov.

displays reduced chaetal diversity compared with its hypothesized sister taxon (*Parahaplosyllis*), and also displays ontogenetic loss of the pharyngeal tooth in adults.

TOWARDS A BRANCHING MODEL IN *RAMISYLLIS*

Seeking patterns in the branching process in *Ramisyllis* gen. nov. is hampered by the fact that to date we have not been able to examine an intact specimen that includes both the head and posterior ends with anal cirri. That said, we report a few facts that can be drawn from the incomplete material available.

Somatic branches arise from the primary or secondary, and further subsidiary, axes anywhere posterior to the pharynx (Fig. 1), and from any of the segment types described earlier. Their segments are similar in form and width to those of the adjacent parent axis. The gut also branches and retains continuity with that of the axis from which it branches (Fig. 1). In small specimens the new branches have only slightly fewer segments than the terminal branch of the primary axis. Lower order axes (primary, secondary, and tertiary) may have as many as 120 segments (including all interbranch sections); the interbranch sections range from three segments (usually to a stolon-producing branch) to 27 segments. The distance from the last branch point to the pygidium ranges from 10–80 segments, but is more often in the range of 60–80 segments (Figs 2B and 6A).

If we define an axis as a branch that produces at least one additional branch, most internal branches (probably not too near the surface of the sponge) produce a new axis. Branches that produce a stolon usually form in this region, comprise between six and 13 segments (not counting the stolon itself), and have not yet been found from a long series of short posterior-type segments that compose the somatic branches (terminal series) described below. The post-reproductive fate of the stolon branches is unknown. It is conceivable that they could continue to develop into a new axis, but no unequivocal examples have yet been found.

Although it seems logical, we cannot assume that the branches along an axis form sequentially, such that the furthest from the base of the axis is the most recent. Figure 2B (centre bottom) shows several young branches, one of which (furthest left) will probably produce a stolon (abruptly swollen towards the rear), whereas the fate of the other cannot be determined. Thus we know that at least stolon-bearing branches can form after the axis has already grown past the site of stolon-stalk appearance.

Based on these observations the following model of branching is proposed: somatic branching begins early in the life of *Ramisyllis* gen. nov., within a 'branching zone' near the pygidium of the primary axis. Each of these branches will probably establish a secondary axis, most of which will in turn establish tertiary and quaternary axes, and lead to four or more pygidia. The side of the worm that produces the next new branch is irregular, showing no pattern. At maturity, additional short branches develop behind the somatic branches and produce reproductive stolons. The fate of these stolon-producing branches after the separation of the stolon remains uncertain.

SYMMETRY

Annelids have classically been found to add segments from the rear by cellular proliferation in a PGZ (e.g. Anderson, 1973). The phenomenon of segmentation and its relationship to this process of terminal addition has been the subject of considerable recent interest (e.g. Seaver, Thamm & Hill, 2005; Seaver & Kaneshige, 2006). The branching of both *S. ramosa* and *R. multicaudata* gen. et sp. nov. indicates that a normal process of segment formation can be initiated laterally in the trunk segments of post-larval individuals. Other examples of somatic segment addition outside the PGZ in annelids occur during larval and early development: for example, in *Chaetopterus*, which develop some anterior segments after more posterior segments have already formed (Irvine, Chaga & Martindale, 1999); regeneration of anterior segments and structures in many groups (Bely, 2006); and asexual reproduction by paratomy (Williams, 2004). Polychaetous annelids are therefore particularly diverse among bilaterians in their mechanisms of segment addition.

Replication of the A–P axis through branching is an apparent violation of the general body plan of bilaterian animals, i.e. bilateral symmetry about an A–P midplane. Furthermore, the asymmetrical dorsal cirri pattern – segments of different-sized cirri on each side – also contributes to left–right side asymmetry. The duplication of the A–P axis, which has been found to occur naturally in developmentally anomalous nereidids of several species (Boilly, Boilly-Marer & Combaz, 1975), seems to have been incorporated into normal development in ancestors of a few contemporary worms. This process was observed over a century ago by Andrews (1892), who summarized reports of bifurcated annelids found occurring in nature until that time, including at least one case in a sylline syllid.

The observation that branching pattern differs between individuals of *R. multicaudata* gen. et sp. nov. is not unexpected, given that it occupies an asymmetrical host with irregular internal canal morphology. Similar symbiont–host form mimicry is

found in hermit crabs and their gastropod hosts, where the crab's body asymmetry is a result of their occupation of asymmetrical (usually dextrally spiralled) gastropod shells; however, in this case the asymmetry is determined early in life (larval stage), and is directional (Palmer, 1996). In the case of *R. multicaudata* gen. et sp. nov., branching and therefore asymmetry does not begin until about 26 segments, and is best described as random (*sensu* Palmer, 1996) because the first branch can originate from the left or the right side of the body, and the segmental position of subsequent branches differs between individuals. Perhaps a better analogy is with certain species of lobsters, shrimp, and crabs, which are also initially bilaterally symmetrical, but after moulting one or the other chela becomes massively developed. In these cases asymmetry is random; however, the cause of the asymmetry in the two groups probably differs – the massively developed claw of the crustacean is the result of differential use (Govind & Pearce, 1989), whereas the asymmetrical branching in *R. multicaudata* gen. et sp. nov. is likely to be the result of an environmental trigger, either from the host itself or perhaps related to the canal microhabitat, for example water currents. Under a host-mediated random asymmetry hypothesis we would expect that juvenile *R. multicaudata* gen. et sp. nov. isolated from their sponge host would fail to develop a branching morphotype. The possibility that free-living unbranched forms of *R. multicaudata* gen. et sp. nov. could exist outside the sponge is intriguing.

TOWARDS A CELLULAR AND GENETIC MECHANISM BEHIND BRANCHING

Seaver *et al*. (2005) studied the distribution of dividing cells during normal development in two polychaetes with different modes of development. From analysing mitotic activity [bromodeoxyuridine (BUdR) labelling] they found that both generated three early segments along the sides of the developing larva, whereas subsequent juvenile development involved segment production from the PGZ, traditionally attributed to growing annelids. The serpulid *Hydroides elegans*, however, starts producing cells from the PGZ after the juvenile has undergone a period of elongation without new segment formation. These segments start with what will soon become segment number 8. Later, it intercalates four new segments (ultimately numbers 4–7) into the thoracic portion of the body between the initial three laterally formed segments and the first PGZ segments. Although a specific pattern of cell labelling was not found in this intermediate area, these results provide another case of cell production and tissue prolifera-

tion in trunk segments of a polychaete without involving the participation of the PGZ. It suggests that a zone of cell production and tissue proliferation can be present and induced in normal trunk segments. In the case of *Ramisyllis* gen. nov. the distribution of new cell-producing areas appears to be random, as there is no detectable pattern in the segmental appearance of new branches.

De Rosa, Prud'homme & Balavoine (2005) made orthologues of the *caudal* and *even-skipped* genes from *Platynereis dumerilii* (Audouin & Milne Edwards, 1834), and studied their expression pattern in developing embryos, juveniles, and (mostly) in regenerating individuals. The studies of regenerating animals might be relevant to the question of determinants of branch formation in *R. multicaudata* gen. et sp. nov. They found a narrow zone of *caudal* expression corresponding to a periodically revealed zone of BUdR labelling in front of the pygidium of posteriorly regenerating *P. dumerilii*. Thus, the pygidium develops first, then a zone of segment formation appears anterior to it. The segment polarity gene *engrailed* seems to demarcate segment boundaries, the posteriormost of which is also the location of *caudal* and *even-skipped* expression in the zone of segment formation, anterior to the pygidium (Prud'homme *et al*., 2003). The expression of these genes is controlled by the *wnt* signalling pathway, and together they form a gene regulatory network (Chipman, 2010, and references therein). We suggest that branching in *R. multicaudata* gen. et sp. nov. occurs when the regulatory network involved in posterior growth is activated in the lateral region of normal somatic segments, resulting in cell proliferation. Drivers of selection for such a process could include allowing the worm to occupy a greater space within the sponge and increasing its reproductive potential.

It is not surprising that of all organisms with segmentally organized body parts – arthropods, annelids, and chordates – branching has only ever been found in two species of Syllidae. The Syllidae seem to be the only annelids – probably the only segmented animals – with the ability to produce reproductive individuals from newly produced segments (i.e. gemmiparity). A gemmiparous syllid bearing stolons is not that dissimilar in appearance to a branching syllid: in this sense a stolon may be viewed as a branch with gametes and a head. Indeed, our molecular results show *Trypanosyllis*, in which gemmiparity is prominent, to be closely related to *Ramisyllis* gen. nov. Therefore the genetic machinery required to produce individuals de novo from somatic segments is likely to be in place and active, at least in some groups of Syllidae. Gemmiparous syllids, including *Ramisyllis multicaudata* gen. et sp. nov. as a special case,

may therefore be ideal experimental organisms in which to study the genetic mechanisms behind branching and the consequent late onset of body asymmetry, a poorly known subject for all organisms (Levin & Palmer, 2007).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Table S1. Detailed comparison between features of **Ramisyllis multicaudata gen. et sp. nov.** and *Syllis ramosa*. The literature references are abbreviated as follows: I66, Imajima (1966); M79, McIntosh (1879); M85, McIntosh (1885); O95, Oka (1895).

Table S2. Raw morphological and meristic data for the type material, arranged by body width. The primary axis could not be determined on well-branched specimens (specimens 1, 9, and 10). 'Total chaetigers' includes tiny developing ones; the measurement of appendages was performed on one from the pair in best condition.

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