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The evolution of the reproductive system of *Urodasys* (Gastrotricha: Macrodasyida)

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Abstract. Macrodasyidan gastrotrichs are hermaphrodites with complex reproductive organs that function in sperm transfer and receipt, but homology among the organs of members of different clades remains undetermined, as does a broader understanding of evolutionary trends in the reproductive biology of macrodasyidans. In this study, we investigate the evolution of reproduction in *Urodasys*, a clade of 15 macrodasyidan species that shows variability in reproductive mode (hermaphroditic and parthenogenetic) and sexual anatomy. We use partial 18S rDNA sequence data from 30 specimens representing five described species, sequence data from one undescribed species in GenBank, and sequence data from a potentially new species found at Capron Shoal, Florida, to gain insight into the phylogeny of the clade and clarify evolutionary trends in reproductive modality. Based on a total of 33 specimens of seven potential species, we found that members of *Urodasys* can be separated into three clades reflective of different reproductive modalities: Clade I, species with paired male and female gonads but without accessory sexual organs; Clade II, species with a single left testis, paired ova, and accessory organs including a sclerotic stylet; and Clade III, parthenogenetic species without testes or accessory organs. In addition, we find that the potentially new species from Florida can form spermatophores, a condition shared with another species in Clade I. Herein, we describe this novel spermatophore-bearing species and discuss the significance of spermatophore formation in the genus.

Additional key words: Macrodasyidae, meiofauna, phylogenetics, spermatophore

Gastrotrichs are microscopic, vermiform invertebrates characteristic of both littoral and sublittoral sediments. To date, there are over 400 species of marine and brackish-water gastrotrichs described from around the globe (Hummon 2009; Hummon & Todaro 2010), approximately three-fourths of which are members of the Macrodasyida, a clade containing primarily simultaneous hermaphrodites with complex reproductive anatomies.

The typical macrodasyidan reproductive system includes a pair of anterior testes and one or two posterior ovaries, as well as two accessory sexual organs (Ruppert 1991). These accessory sexual organs, designated as the caudal and frontal organs after their relative positions within the gastrotrich body, are hypothesized to function, respectively, as a copulatory organ and seminal receptacle (Ruppert & Shaw 1977; Ruppert 1978; Guidi et al. 2009). This general reproductive plan, however, is highly variable in the clade, and homology among the various organs remains mostly undetermined.

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The genus *Urodasys* currently includes 15 described species, which can be distinguished based on body shape and the distribution and abundance of adhesive tubes, as well as the anatomy of their reproductive system. These species can be subdivided into three groups based on the presence or absence of various reproductive organs. Four species possess paired testes and ovaries but lack accessory sexual organs: *U. anorektoxys* Todaro, Bernhard & Hummon 2000; *U. apuliensis* Fregni, Faienza, Grimaldi, Tongiorgi & Balsamo 1999; *U. elongatus* Renaud-Mornant 1969; and *U. mirabilis* Remane 1926. Ten species posses a single testis, paired ovaries, and a single male accessory reproductive organ (copulatory bulb *sensu* Ruppert 1991) bearing a sclerotic stylet: *U. acanthostylis* Fregni, Tongiorgi & Faienza 1998; *U. bucinastylis* Fregni, Faienza, Grimaldi, Tongiorgi & Balsamo 1999: *U. calicostylis* Schoepfer-Sterrer 1974; *U. cornustylis* Schoepfer-Sterrer 1974; *U. nodostylis* Schoepfer-Sterrer 1974; *U. poculostylis* Atherton 2014; *U. remostylis* Schoepfer-Sterrer 1974; *U. spirostylis* Schoepfer-Sterrer 1974; *U. toxostylus* Hummon 2011; and

U. uncinostylis Fregni, Tongiorgi & Faienza 1998. The remaining species, *U. viviparus* Wilke 1954, is hypothesized to be parthenogenetic based on the absence of any testes or copulatory organs. Additionally, *U. viviparus* is the only gastrotrich known to give live birth.

Based on the reproductive modality of most other macrodasyidan gastrotrichs, Fregni et al. (1999) proposed that the plesiomorphic condition in *Urodasys* is simultaneous hermaphroditism, with the ancestor possessing paired testes, paired ovaries, and both a frontal and caudal organ. They hypothesized that the genus *Urodasys* could be divided into two evolutionary lines (Fregni et al. 1999). In one evolutionary line, the accessory reproductive organs were lost, followed by a sequential reduction and loss of the right and left testis. In the second evolutionary line, a complete regression of the right testis occurred followed by a loss of the left testis and then eventually of the frontal and caudal organs. The obligate parthenogenetic species, *U. viviparus*, may have evolved from either line.

In this study, we describe a potentially novel species of *Urodasys* from Florida based on a single specimen with an unusual reproductive modality, and investigate the phylogeny of *Urodasys* using partial 18S DNA sequences in order to test the hypotheses of Fregni et al. (1999) on the evolution of the reproductive organs within the genus.

Methods

Gastrotrichs were collected from sublittoral sediments at six locations from 2010 to 2013 (Table 1). All samples except those from Capron Shoal, Florida, were collected by hand via snorkeling or SCUBA. Capron Shoal sediments were collected via small anchor dredge (29×12 cm opening) from the Smithsonian Marine Station's R/V Sunburst. Once collected, samples from all locations were placed into plastic bags or buckets and examined within 5 d of collection.

Meiofauna was extracted from the sediments using an anesthetization–decantation technique with isotonic $MgCl_2$ and a 53-μm mesh (Pfannkuche & Thiel 1988). Animals were sorted with a Leica EZ4 stereomicroscope and mounted alive on microscope slides in isotonic $MgCl_2$. Individual specimens were identified and documented with a Zeiss A1 compound microscope equipped with DIC and a Sony Handycam digital video camera. Measurements were taken with an ocular micrometer. Following identification and documentation, individual specimens were fixed in 95% ethanol for later treatment. In the description, lengths and positions of organ systems are described in terms of percentage body units, where total body length from anterior (U00) to posterior (U100), excluding the tail, is 100 units. A full list of specimens with sampling date, location, and depth as well as GenBank accession numbers is presented in Table 1.

DNA extraction was performed using the DNEasy Mini Blood and Tissue kit (Qiagen, USA) according to the manufacturer's instructions. An ~800 base-pair region of the nuclear 18S gene was amplified by PCR using primers designed by Norén & Jondelius (1999) or by two newly designed primers: forward 5′-GACCGCGAATGGCTCATTAAA and reverse 5′-CCCGTCCGTCCCTCTTAATC. Sequencing was performed commercially by Eurofins MWG Operon (Alabama, USA), and trace files were manually edited.

The six-parameter general time-reversible (GTR) model with gamma distribution was determined as the best fitting model of sequence evolution using jModelTest2 (Guindon & Gascuel 2003; Darriba et al. 2012) with three substitution schemes. Model selection was computed using the Akaike information criterion (AIC; Akaike 1974). Phylogenetic analyses were preformed with both Maximum likelihood and Bayesian methods. Maximum likelihood (ML) analysis was performed in RaxmlGUI v1.31 (Silvestro & Michalak 2012) with 1000 fast bootstrap replicates. Bayesian analysis was performed in MrBayes 3.2.0 (Ronquist & Huelsenbeck 2003). Two runs with four simultaneous chains were run for 50,000,000 generations. Trees were sampled every 500 generations with a 25% burn-in period, and a consensus tree calculated following a 50% majority rule.

Results

Morphology of *Urodasys* sp. from Florida

The body was strap-shaped and 235 μm long, excluding the tail (Fig. 1). The long, slender tail was highly contracted and difficult to accurately measure, but was at least 160 μm in length. Body width stayed relatively uniform from the pharynx to the anterior trunk region, increased just anterior to the largest egg at U60, and tapered off to the tail. Widths at U25 (pharynx)/U70 (largest egg)/U90 (posterior trunk) were 26/28/25 μm, respectively. The pharynx was 119 μm long.

The epidermal glands were elliptical, 4–6 μm long and 3–4 μm wide, and located along the entirety of the body. The most anterior pair was present at

Table 1. List of specimens used in this study, collection data, and GenBank accession numbers where available.

Genus	Species	Country	Site name	Coordinates	Depth	Collection date	GenBank accession no.
Macrodasys	*caudatus*						AM231779
	buddenbrocki						AY963692
	achradocytalis	USA	Fort Pierce Inlet, FL	27°27′41″N 80°18′44″W	<1 m	8/2/2011	KM289032, KM289033
			Capron Shoal, FL; 3-mile station	27°28′47″N 80°13′38″W	14 m	8/9/2011	KM289034
Urodasys	*viviparus*	Little Cayman Island	Grape Tree Bay	19°41′58″N 80°09′07″W	4 m	6/25/2011	KM289036, KM289038, KM289039
			Point of Sand	19°04′59″N 79°57′51″W	2 m	6/28/2011	KM289042
			Jackson Wall	19°41′33″N 80°04′08″W	17 m	6/30/2011	KM289035, KM289037
			Owen Island	19°39′56″N 80°03′51″W	<1 m	7/2/2011	KM289043, KM289051, KM289055
			Mary's Bay	19°41′06″N 80°04′39″W	2 m	7/2/2011	KM289045
		Curaçao	Playa Jeremi	12°19′40″N 69°09′00″W	5 m	2/14/2012	KM289053
			Playa Parasasa	12°07′06″N 68°57′56″W	2 m	2/7/2012	KM289050
		Tobago	Angel Reef	11°18′08″N 60°31′27″W	26 m	8/3/2012	KM289040
			50/50	11°17′86″N 60°31′14″W	23 m	8/5/2012	KM289048, KM289049
			Highway to Heaven	11°20′15″N 60°38′36″W	33 m	8/9/2012	KM289041
		Brazil	Pereque, Ilhabela	23°48′45″S 45°22′07″W	<1 m	10/30/2012	KM289044, KM289046, KM289047, KM289052, KM289054
	poculostylis	USA	Capron Shoal, FL; 4-mile station	27°26′52″N 80°13′81″W	9 m	8/3/2011	KM289056–KM289058
	nodostylis	Belize	Trough near Carrie Bow Caye	16°48′13″N 88°04′61″W	7 m	1/20/2010	KM289059
	sp.	USA	Capron Shoal, FL; 3-mile station	27°28′47″N 80°13′38″W	14 m	3/20/2013	KM289060
	calicostylis	USA	Capron Shoal, FL; 3-mile station	27°26′52″N 80°13′81″W	14 m	8/9/2011	KM289063
			Capron Shoal, FL; 4-mile station	27°26′52″N 80°13′81″W	9 m	8/3/2011	KM289061, KM289062
	spirostylis	USA	Capron Shoal, FL; 3-mile station	27°28′47″N 80°13′38″W	14 m	3/20/2013	KM289064
	mirabilis	Tobago	50/50	11°17′86″N 60°31′14″W	23 m	8/5/2012	KM289065–KM289067
	Unknown						AY218102, DQ079912

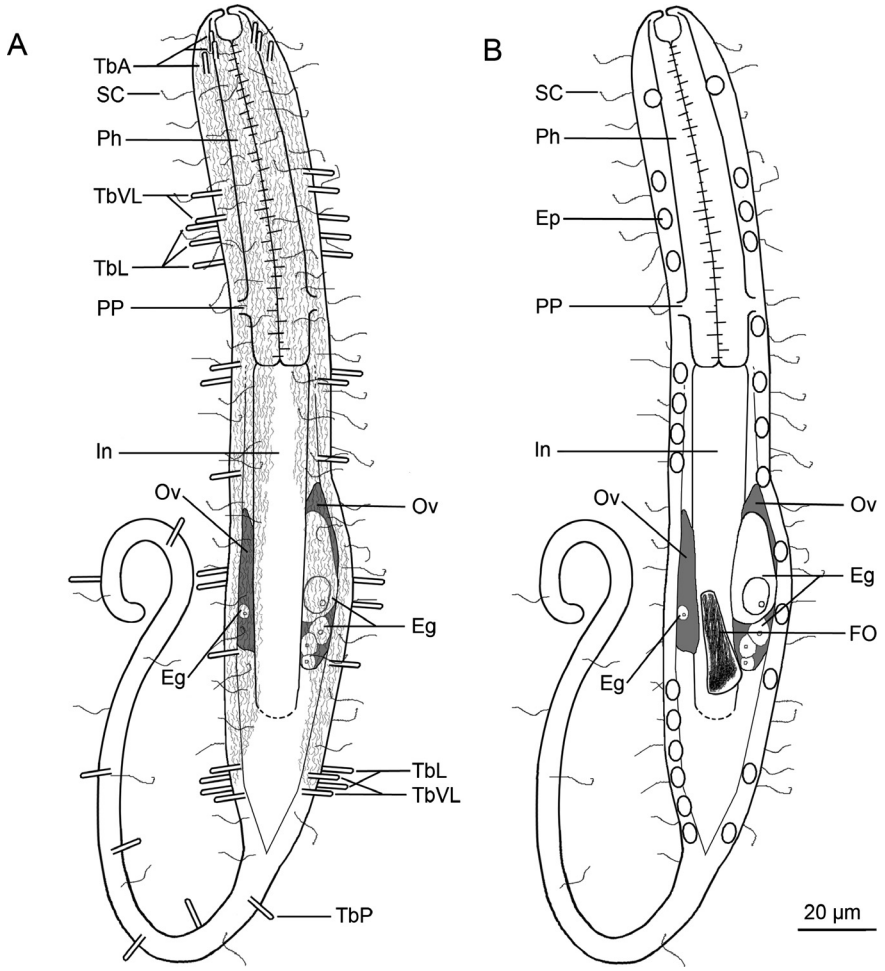

Fig. 1. *Urodasys* sp. Composite sketches of the (**A**) ventral part of the body and (**B**) the dorsal part of the body, showing internal anatomy. The sketches were based on a specimen mounted in dorsal view. Eg, egg; Ep, epidermal gland; FO, frontal organ; In, intestine; Ov, ovary; Ph, pharynx; PP, pharyngeal pore; SC, sensory cilia; TbA, anterior adhesive tubes; TbL, lateral adhesive tubes; TbP, posterior adhesive tubes; TbVL, ventrolateral adhesive tubes.

U10 and was positioned slightly more medially than the remaining glands. Three pairs of lateral epidermal glands were located in the pharyngeal region of the body from U20 to U32, and another epidermal gland occured on the left side only, in the pharyngeal region at U38. Eight epidermal glands were dispersed fairly evenly along the trunk on the left side. Ten glands were located in the trunk region on the right side of the body, and these were much more clumped, with four positioned between U45 and U60, and the remaining six glands grouped together between U83 and U100.

The ventral locomotory cilia field covered the area from just posterior of the mouth rim (at U02) to the pharyngeal–intestinal junction (around U40), where it divided into two ventrolateral bands that extended to U95. Sensory cilia, 11–16 µm long, were extremely numerous and scattered ubiquitously within the locomotory cilia field as well as sparsely on the tail.

Anterior adhesive tubes (TbA) were ~5 µm long. Two tubes per side were positioned in a column along the border of the pharynx from U02 to U03, and a third tube per side inserted slightly more laterally at U03 (Figs. 1 and 2B).

Ventrolateral adhesive tubes (TbVL) and lateral adhesive tubes (TbL) were each ~8 µm long. Two TbVL per side were located in the pharyngeal region, at U20 and U22 for the left side of the body and U22 and U25 for the right side of the body. Five additional TbVL per side were located in the trunk region at approximately U45, U55, U78, U94, and U95. Three TbL per side were grouped in the pharyngeal region between approximately U25 and U30. One TbL per side inserted just posterior to the pharyngeal–intestinal junction at U45. Four TbL

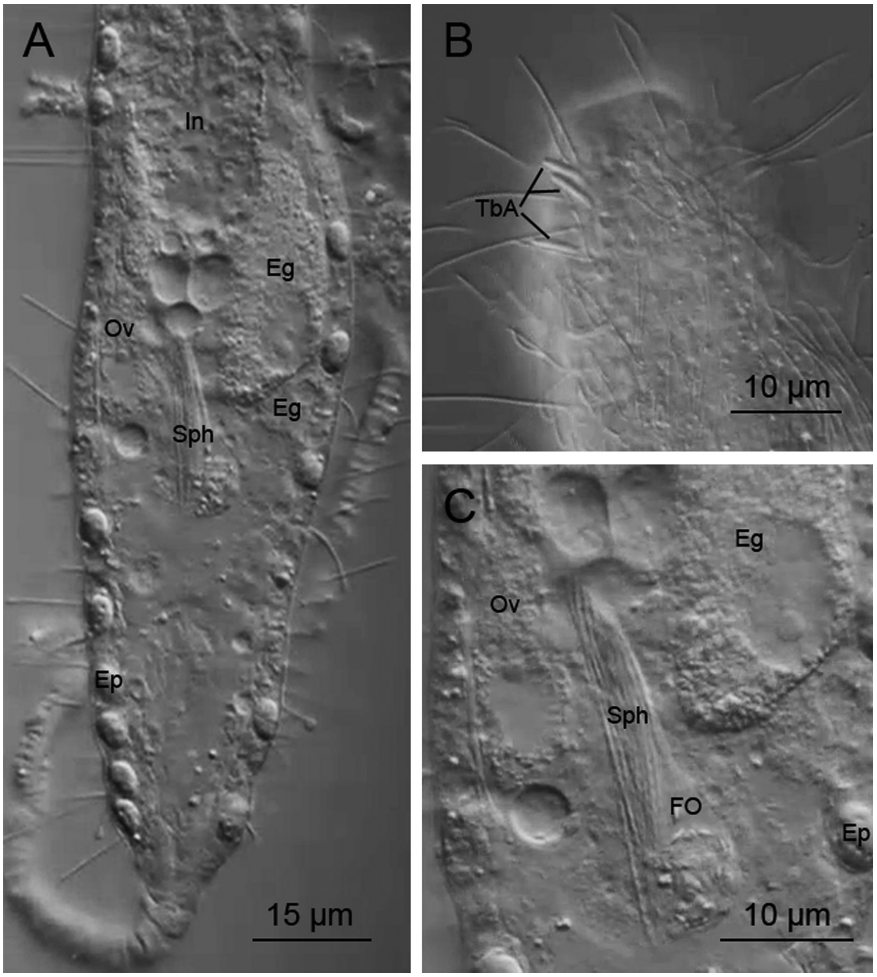

Fig. 2. *Urodasys* sp. viewed using DIC optics. **A**. Reproductive anatomy in the posterior trunk. **B**. Ventral view of the head, focusing on the adhesive tubes. **C**. The spermatophore in the frontal organ. Eg, egg; Ep, epidermal gland; FO, frontal organ; In, intestine; Ov, ovary; Sph, spermatophore; TbA, anterior adhesive tubes.

per side were arranged in two groups of two in the trunk region, with the first pair at U69 and U70 and the second pair at U94 and U95.

At least seven posterior adhesive tubes (TbP) were staggered along the length of the tail. However, this number may be an underestimate as the tail was highly contracted and partially obscured by detritus, which prevented an accurate count.

Testes and caudal organ were not observed. The female reproductive system consisted of paired ovaries extending frontally from U60. Ova matured from posterior to anterior, with the largest egg located between U62 and U75 and measuring 33 µm long and 22 µm wide. A spermatophore ~25 µm long was present dorsal to the posterior end of the intestine and medial to the most mature egg (Fig. 2). The spermatophore was somewhat cone shaped, with numerous tightly wound sperm. Individual sperm lengths were not measured.

Taxonomic remarks

Urodasys sp. differs from its congeners by its small adult body size (235 µm long compared to a minimum of 300 µm in *U. cornustylis* and *U. calicostylis*) and in the number and pattern of its adhesive tubes. For the latter, *Urodasys* sp. is generally most similar to *U. bucinostylis* based on the similar pattern of TbA. However, *Urodasys* sp. differs from *U. bucinostylis* in the number of TbVL (8 vs. 16) and TbDL (0 vs. 6). As typical for the genus, *Urodasys* sp. is most easily distinguished through the structure of its reproductive system.

Complete absence of males or male organs has only been documented in *Urodasys viviparus*, which is hypothesized to reproduce exclusively through parthenogenesis. *Urodasys* sp., while lacking any apparent testes or caudal organ, is unlikely to be parthenogenetic because of the presence of a sper-

matophore (Fig. 2). We take this as evidence that the potentially new species is probably a hermaphrodite, and the single specimen encountered had already spent its own sperm in a prior exchange with a partner, in return receiving allosperm as part of the pattern of mutual cross-fertilization. Alternatively, the potentially new species might be a sequential hermaphrodite, and our single specimen had yet to produce its own sperm. This appears to be an unlikely scenario as such a condition has not been previously described in *Urodasys*; however, sequential hermaphroditism is a condition frequently found in other macrodasyidans (Balsamo et al. 2002; Hummon 2010), including species of the related genus, *Macrodasys* (Hummon 2008).

While *U. viviparus* remains the only known parthenogenetic species in the genus, and can be identified by the total absence of male germ cells or accessory reproductive organs, all other species of *Urodasys* can be identified and placed into one of two groups proposed by Fregni et al. (1999) based on the presence of germ cells, their position in the body, and the structure of their accessory organs. The first group is united by the presence of paired testes and the absence of a frontal organ or caudal organ. The second group shows far more variability in the reproductive system, but in general consists of species with a single testis, paired ovaries, and a complex caudal organ with stylet. Within this group, a frontal organ has been described in only five species: *U. acanthostylis*, *U. calicostylis*, *U. cornustylis*, *U. poculostylis*, and *U. spirostylis* (Schoepfer-Sterrer 1974; Fregni et al. 1998; Atherton 2014).

The potentially new species tentatively differs from members of both groups by lacking testes altogether, although as indicated above, this may be the result of either prior mutual exchange or sequential hermaphroditism. If due to prior mutual exchange, then *Urodasys* sp. is more clearly linked to the second group of species by its possession of a frontal organ, even though a caudal organ is apparently absent and distinguishes it from all of these species.

Schoepfer-Sterrer (1974) indicated that at least one specimen of *U. cornustylis* was found lacking testes and with only a weakly developed muscle bulb portion of its caudal organ. Similar to *Urodasys* sp., this specimen had a ripe ovary and a frontal organ with sperm. However, the sperm within the frontal organ of this specimen of *U. cornustylis* was not contained in a spermatophore, nor has any spermatophore ever been recorded for *U. cornustylis*.

To date, only two species of *Urodasys* have been described to contain spermatophores: *U. poculostylis* (Atherton 2014) and *Urodasys* sp. from Florida. Spermatophores are well documented in other macrodasyidans including species of *Dactylopodola* (Teuchert 1968; Ruppert 1991; Kieneke et al. 2008b), *Neodasys* (Ruppert 1991; Guidi et al. 2003; Kieneke et al. 2009), and *Xenodasys* (Guidi et al. 2009), and presence of spermatophore formation in such potentially basal genera (Hochberg & Litvaitis 2001; Todaro et al. 2006) suggests it is a plesiomorphic characteristic of gastrotrichs (but see Kieneke et al. 2009).

Phylogeny

In this study, we provide new sequences from 33 specimens representing seven species of *Urodasys* (GenBank accession KM289035–KM289067). Twenty-one specimens were identified as *U. viviparus*, easily the most abundant species found; these were collected from Brazil, Curaçao, Little Cayman Island, and Tobago (Table 1). Eight specimens had paired ovaries, a single testis, and a caudal organ with stylet, and were identified as *U. calicostylis* (three specimens collected from Florida, USA), *U. nodostylis* (one specimen collected from Carrie Bow Caye, Belize), *U. poculostylis* (three specimens collected from Florida, USA), and *U. spirostylis* (one specimen collected from Florida, USA). Three specimens collected from Tobago had paired ovaries and testes only, and are tentatively identified as *U. mirabilis*. Finally, a single specimen of *Urodasys* sp. was obtained from Florida, USA (Table 1).

We also obtained two 18S sequences belonging to unidentified species of *Urodasys* from Genbank. These were the only 18S sequences available in Genbank for species of *Urodasys*. Although both represent unidentified species, Sørensen et al. (2006) indicated that the specimen used in their study (GenBank accession DQ079912) had paired testes without a caudal organ or stylet. No information on the identification or reproductive structures of the second *Urodasys* (GenBank accession AY218102) was provided (see Giribet et al. 2004).

While the identity of the sister group to *Urodasys* is still uncertain (see Kieneke et al. 2008a; Paps & Riutort 2012; Todaro et al. 2012), current classification indicates *Macrodasys* as the sister group to *Urodasys* (Hochberg & Litvaitis 2001). Thus, three species of *Macrodasys* were selected for the outgroup, including three specimens of *Macrodasys* cf. *achradocytalis* from Florida, USA, and *M. caudatus* Remane 1927 and *M. buddenbrocki* Remane 1924 downloaded from Genbank (see Table 1 for accession numbers).

Maximum likelihood (ML) analysis and Bayesian analysis (BA) yielded very similar results (Fig. 3). For

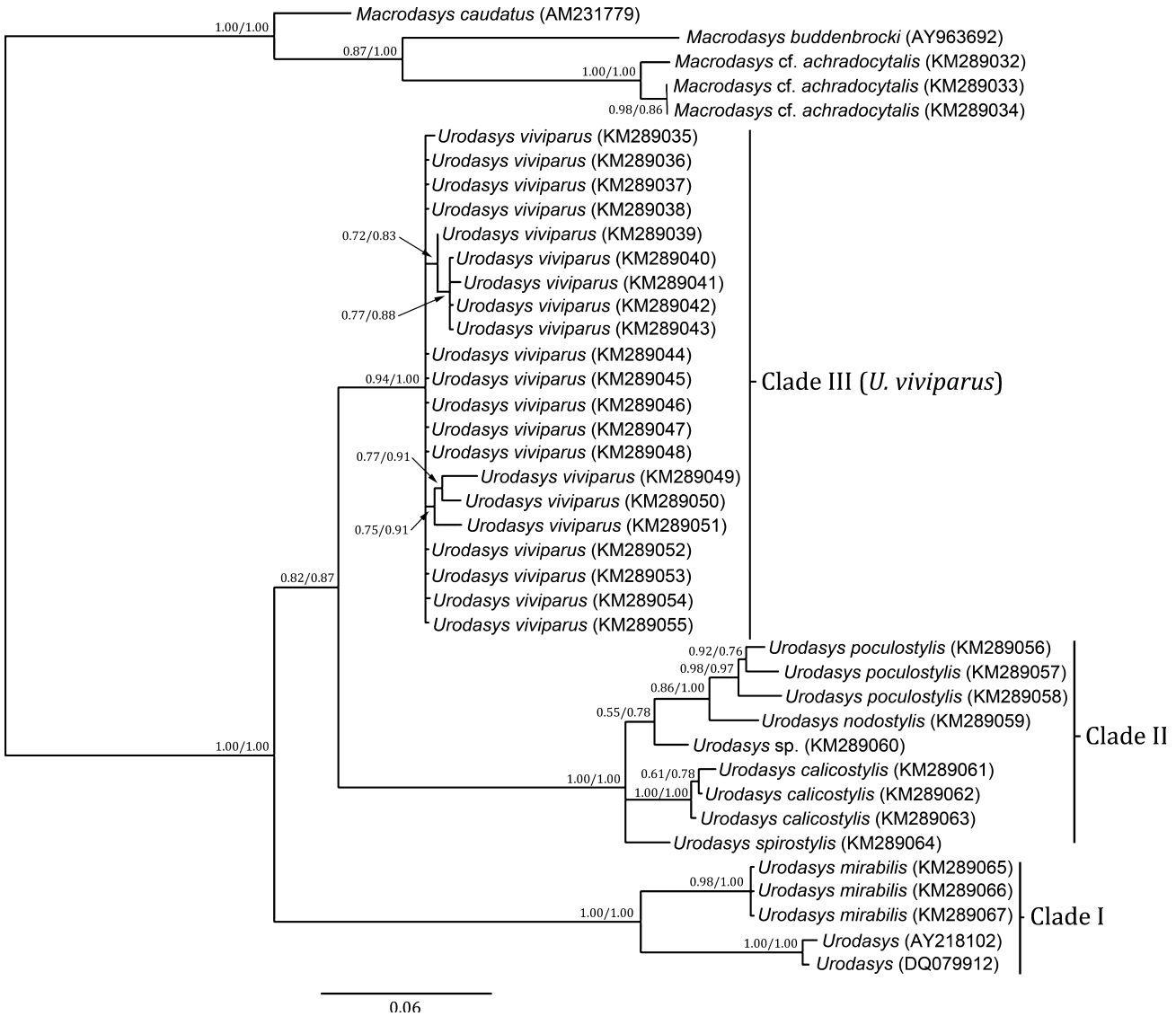

Fig. 3. Phylogenetic relationships of *Urodasys* inferred from Maximum Likelihood and Bayesian analysis of partial 18S DNA. Three species of *Macrodasys* are used as outgroups. Numbers at the nodes represent bootstrap support and posterior probability, respectively. GenBank accession numbers are given in parentheses after each taxon name.

both, specimens of the same species grouped together with high (>90%) bootstrap support and high posterior probability (>0.97) regardless of country of origin, and three *Urodasys* clades reflecting each of the three reproductive modalities received moderate to high support (bootstrap>80%, pp>0.85).

The first clade (clade I) in both analyses contained specimens of *Urodasys* with paired testes, paired ovaries, and without accessory reproductive organs (frontal and caudal organ). The three specimens of *Urodasys mirabilis* grouped with the two unknown species of *Urodasys* from Genbank, with 100% bootstrap support and 1.00 posterior probability.

A second group containing two clades, a parthenogenetic species (*U. viviparus*, clade III) and five species with a single testis, paired ovaries, and both accessory sexual organs (clade II), received moderate support values (bootstrap=82%, pp=0.87). In both analyses, the parthenogenetic *U. viviparus* and its sister taxon each received strong support for monophyly (bootstrap>94%, pp=1.00). Within clade II, an unresolved trichotomy of *U. calicostylis*, *U. spirostylis*, and the moderately supported (bootstrap=55%, pp=0.78) clade of (*Urodasys* sp. (*U. nodostylis*+*U. poculostylis*)) formed. The clade of (*U. nodostylis*+*U. poculostylis*) received high support (bootstrap=86%, pp=1.00).

Discussion

In this study, 18S DNA gene trees were created to understand reproductive evolution within *Urodasys*. In both analyses, *Urodasys* separated into clades reflective of reproductive modality and congruent with the two evolutionary lines proposed by Fregni et al. (1999). According to their hypothesis, the plesiomorphic sexuality modality of *Urodasys* is likely to be simultaneous hermaphroditism with paired accessory sexual organs; the two evolutionary lines have subsequently diverged from this hypothetical plesiomorphic condition.

The first clade of *Urodasys* (clade I) consists of *U. mirabilis* plus the two unidentified specimens from Genbank. Although neither specimen in Genbank was completely described, one of the two specimens (accession DQ079912) was mentioned as possessing paired testes without a caudal organ or stylet (Sørensen et al. 2006). The similarity between the 18S sequences of both unidentified specimens indicates that they are probably the same species. Thus, within the first evolutionary line of *Urodasys*, both accessory organs were presumably lost, resulting in species with paired testes and ovaries only (Fig. 4). How insemination is achieved in members of this clade remains unknown, but is likely to be similar to other macrodasyidans that also lack accessory reproductive organs (Balsamo et al. 2002).

The second group (clades II and III) can be best characterized as including those species of *Urodasys* that have lost at least a single testis during their evolutionary history (Fig. 4). Of the hermaphroditic species in this group (clade II), most contain paired ovaries, a frontal organ, and a copulatory bulb with a stylet. *Urodasys spirostylis*, *U. calicostylis*, and *U. poculostylis* best embody the characteristics of this particular clade as proposed by Fregni et al. (1999). *Urodasys nodostylis* is also included in clade II and fits the general pattern, albeit without a frontal organ (the "vagina mouthpiece" was later identified as a broken part of a copulatory stylet according to Ruppert (1991), although this has not been subsequently confirmed). Although *Urodasys* sp. from Florida was described from a single specimen lacking any male characters (either due to prior mutual exchange or sequential hermaphrodit-

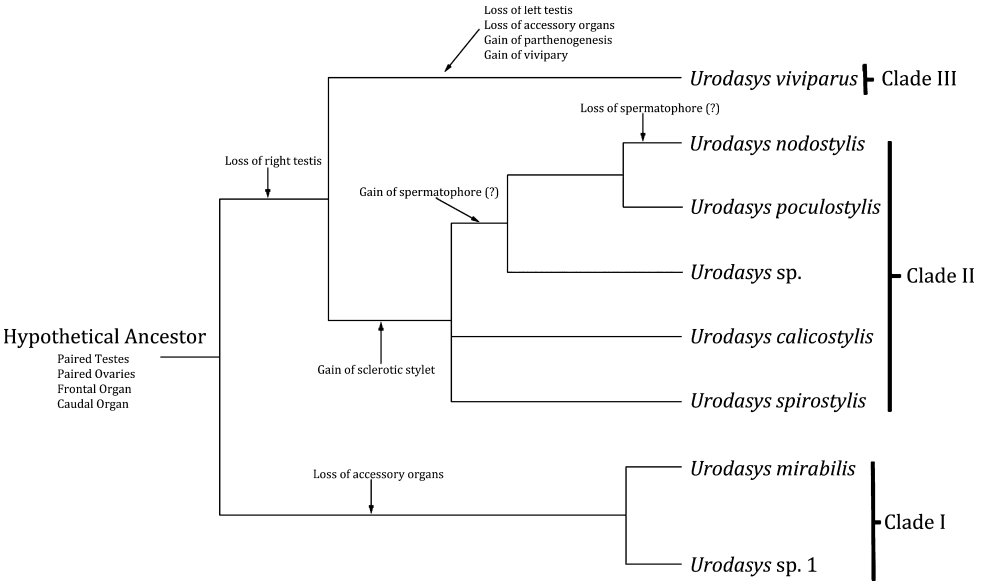

Fig. 4. Hypothesized transformations in the evolution of the reproductive system of *Urodasys*. If the ancestor contained paired testes, paired ovaries, and a set of two accessory reproductive organs, then clade I evolved through the loss of both the copulatory organ (caudal organ) and seminal receptacle (frontal organ). How insemination is achieved in members of this clade remains unknown. The second group is united by the shared loss of the right testis and is further divided into two clades. Species within clade II possess a complex stylet, which might be a synapomorphy of that grouping. In addition, spermatophore production appears to either be a synapomorphy of the ((*U. poculostylis*+ *U. nodostylis*) *Urodasys* sp.) clade that was then secondarily lost in *U. nodostylis* (shown in figure) or a homoplasy of *U. poculostylis* and *Urodasys* sp. (not shown if figure). *Urodasys viviparus* is the sole representative of clade III and evolved through the loss of all hermaphroditic capacity (loss of left testis+accessory reproductive organs). *Urodasys viviparus* also presumably gained the ability to generate embryos by parthenogenesis and became ovoviviparous.

ism, see above), the presence of a frontal organ is congruent with the general pattern of this clade.

The 18S gene tree suggests that *U. viviparus*, an obligate parthenogenetic species, shares a synapomorphic loss of a single testis with the hermaphroditic species within clade II (Fig. 4). *Urodasys viviparus* may then have diverged from clade II and subsequently lost all male sexual capacity (loss of left testis + accessory reproductive organs), leaving parthenogenesis the only viable sexual modality. When viviparity was gained remains unknown, but its singular presence in this species suggests that it is very likely an autapomorphy of *U. viviparus*.

Spermatophore formation is known to occur in genera such as *Dactylopodola* and *Neodasys* (Hochberg & Litvaitis 2001; Kieneke et al. 2008b, 2009) and therefore may be a plesiomorphic characteristic within Gastrotricha. However, as spermatophore formation is notably absent from all *Urodasys* species except *U. poculostylis* and *Urodasys* sp. from Florida, the condition appears to be derived within *Urodasys*. Interestingly, evidence of spermatophore production has never been reported for *U. nodostylis*, the closest sister to *U. poculostylis* and the third member of the ((*U. poculostylis*+*U. nodostylis*), *Urodasys* sp.) clade. Therefore, spermatophore production appears to either be a synapomorphy of that clade and was then secondarily lost in *U. nodostylis*, or perhaps convergently evolved in *U. poculostylis* and *Urodasys* sp. (Fig. 4).

The above comparison of the evolutionary scenario figured out by Fregni et al. (1999) with our results of the DNA sequence-based analysis of *Urodasys* has demonstrated a high degree of congruence between molecular and phenotypic characters. However, a full understanding of the evolutionary transformations will not be possible until genetic data for all species of *Urodasys* are available.

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