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Sperm ultrastructure of *Macrodasys caudatus* (Gastrotricha: Macrodasyida) and a sperm-based phylogenetic analysis of Gastrotricha

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Abstract

The spermatozoon of *Macrodasys caudatus* Remane, 1927 (Macrodasyida, Macrodasyidae) was studied and compared with both the spermatozoal patterns of *Macrodasys* sp. Ruppert 1978, and with preliminary observations of two species of *Urodasys*, the only other genus studied in the family. The spermatozoon of *M. caudatus* is similar to that of *Macrodasys* sp.: in both cells a mitochondrial helix surrounds the nucleus and the acrosome, and a monolayered striated cylinder encloses a conventional axoneme; the differences between the spermatozoa of the two species concern their acrosome organization. Preliminary data on the spermatozoa of *Urodasys anorektoxys* and *U. acanthostylis* (Macrodasyidae) are reported. Their spermatozoa greatly differ from the spermatozoon of *Macrodasys*, since they lack mitochondria and have unusual "fingerprint-like" chromatin condensation and peculiar acrosome morphology, suggesting a possible polyphyly of the Macrodasyidae. The spermatozoa of both *Macrodasys* species do not conform to the general plan described for Macrodasyida, mainly due to the reverse position of the mitochondrion, which in the other macrodasyidans is enclosed by a spring-shaped nucleus. A parsimony analysis was performed on a great number of spermatological data of 28 species from both orders: its results are congruent with those of the traditional systematics. A new set of autapomorphies characterizing the Gastrotricha sperm is proposed. Our analysis suggests that spermatozoal characters are useful in resolving monophyletic groups and broadening the basis of evidence in phylogenetic analyses of gastrotrichs.

Keywords: Gastrotricha, spermatozoa, phylogeny, morphology, meiofauna

Introduction

Gastrotrichs are microscopic (0.06-3 mm in length) aquatic worms which live freely in benthic and periphytic environments. The phylum includes about 690 species, which are traditionally grouped into two orders: Macrodasyida, with 240 strap-shaped species, all but two marine or estuarine, and Chaetonotida, with 450 bowling-pin-shaped species, two thirds of which are freshwater dwell-

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ers. Macrodasyida are oviparous hermaphrodites with cross-fertilization, whereas Chaetonotida are mostly parthenogenetic with the exception of the species of the hermaphrodite marine genera *Neodasys*, *Heteroxenotrichula* (except *H. pygmaea*), *Xenotrichula* and *Musellifer* (Ruppert 1991). Gastrotrichs are still insufficiently known, since they are small, delicate and difficult to manipulate and identify. Most of the genera and families currently known were described in the early 1900s by Remane (1927, 1936), but findings of new species are quite frequent, even at present (e.g. Hochberg 2003, Todaro & Rocha 2004). Since their discovery, the Gastrotricha have been recognized as one of the most enigmatic phyla of lower metazoans: their origin and phylogenetic relationships are still uncertain (Winnepenninckx et al. 1995; Wallace et al. 1996; Littlewood et al. 1998; Wirz et al. 1999; Baguña et al. 2001), whereas the in-group relations have begun to be clearer (Todaro et al. 2003). In the last twenty years many morphological studies, even supported by parsimony analyses (Travis 1983; Hochberg & Litvaitis 2000, 2001), have suggested phylogenetic hypotheses, overall confirming the taxonomic systematization proposed by Remane (1927). These studies, based on the anatomy of the body wall, digestive tract, muscular, reproductive and nervous systems, confirm the monophyly of the phylum, the two orders and most of the families, except Lepidodasyidae, Planodasyidae, Dactylopodolidae (Macrodasyida) and Chaetonotidae (Chaetonotida).

Since the ultrastructure of spermatozoa has proved to be useful for phylogenetic inferences among different metazoans (e.g. Ferraguti 2000 for euclitellate anellids), numerous ultrastructural investigations have been carried out during the last decade on sperm cells of 23 gastrotrich species, belonging to 17 genera and 8 families (Balsamo 1992; Fischer 1994,1996; Ferraguti & Balsamo 1995; Ferraguti et al. 1995; Balsamo et al. 2002; Hochberg 2003; Guidi et al. 2003a,b, 2004) and a basic sperm plan has been outlined for macrodasyidans: a long, corkscrew-shaped acrosome, often composed of two different portions and containing an internal striated tube, a spring-shaped nucleus surrounding one or more mitochondria and a flagellum made of an axoneme surrounded by a striated cylinder (Ferraguti & Balsamo 1995). On the contrary, no sperm basic model could be outlined for the Chaetonotida because of the high diversity of the spermatozoa of the seven hermaphroditic species investigated so far and belonging to 6 genera and 3 families (Hummon 1984; Balsamo 1992; Ferraguti & Balsamo 1994, 1995; Guidi et al. 2003b).

This study considers a number of spermatological data on species of the two orders, both from the literature and unpublished ones. We present here as new data the description of the sperm ultrastructure of *Macrodasys caudatus* Remane, 1927 (Macrodasyidae) and a reconstruction of spermatozoa of two additional macrodasyids, *Urodasys anorektoxys* Todaro, Bernhard & Hummon, 2000 and *U. acanthostylis* Fregni, Tongiorgi & Faienza, 1998 based on preliminary data (see Todaro et al. 2000; Pierboni et al. 2003) and unpublished data. In a comparative approach including species from both orders, we compare our new findings to the ultrastructural data obtained from the literature. Using sperm characters here, we attempt to construct a phylogenetic analysis at low taxonomic level within the gastrotrichs. The aim of this paper is to contribute to the understanding of the general ground plan of gastrotrich spermatozoa and to propose a new set of characters, the spermatozoal ones, for a better resolution of gastrotrich systematics and taxonomy.

Materials and methods

Specimen collection and microscopic study

Adult specimens were extracted from sandy sediments: *Macrodasys caudatus* and *Urodasys acanthostylis* (Macrodasyidae) from the Tyrrhenian Sea: Punta Ala, Tuscany and Cala Nave, Ventotene Island, respectively, while *Urodasys anorektoxys* comes from the anoxic bottoms of the Santa Barbara Basin (California, USA). The animals were fixed in SPAFG (Ermak & Eakin 1976). All the specimens were then washed in 0.1 M phosphate buffer (PBS) at pH 7.2 and postfixed in 2 % osmium tetroxide solution for one hour at room temperature. All fixatives were in PBS with 10 % sucrose. After a rinsing in PBS, the specimens were dehydrated in a graded acetone series, stained *en bloc* in uranyl acetate in 70 % acetone and embedded in araldite. Ultrathin sections were cut with a LKB Ultratome 2088V, contrasted with lead citrate and observed under a Philips 300 and a Zeiss 902 transmission electron microscope.

Phylogenetic analysis

The in-group and out-group taxa considered are listed in Table 1. The in-group comprises 28 species representing five families in the Macrodasyida (Dactylopodolidae, Macrodasyidae, Lepidodasyidae, Thaumastodermatidae and Turbanellidae) and two families in the Chaetonotida (Chaetonotidae, Xenotrichulidae). Since the sister group of the Gastrotricha is still uncertain (Todaro et al. 2003) and the sperm ground plan of any possible gastrotrich out-group within Platyzoa is too different to allow comparison, this study is restricted to a subgroup of Gastrotricha, to be able to establish apomorphic states of the sperm characters. Among all the species considered, the spermatozoon of *Neodasys ciritus* (Guidi et al. 2003b) was selected to root the tree: this species had already been reported by Remane (1961) as a possible link between Chaetonotida and Macrodasyida. In fact, on general morphological grounds, especially concerning the body plan and adhesive organs, *Neodasys* is considered to be the most primitive genus of the chaetonotidans (Tyler et al. 1980); this idea has recently been confirmed by the morphological phylogenetic analysis by Hochberg & Litvaitis (2000). The spermatozoon of *N. ciritus* looks like a "primitive" sperm (*sensu* Franzén 1955), due to the simple, short head, the low number of conventional mitochondria, the two centrioles and the $9\times2+2$ flagellum (Guidi et al. 2003b).

Thirty-tree spermatozoal characters have been considered in this study, all treated as unordered (Table 2). They concern different regions of the sperm cell: three are related to the general shape of the spermatozoon, twelve to the acrosome, five to the nucleus, three to the mitochondria and ten to the axoneme.

Phylogenetic analyses were carried out using PAUP (Phylogenetic Analysis Using Parsimony), version 4.0b10 for 32 bit Microsoft Windows (Swof-

Table 1. List of taxa and related references used in this study.

Order	Family	Species	Reference
Macrodasyida	Dactylopodolidae	*Dactylopodola baltica*	Fischer 1996
		Dactylopodola typhle	unpublished
		Xenodasys sp.	Pierboni et al. 2004 and unpublished
	Macrodasyidae	*Macrodasys* sp.	Ruppert 1978
		Macrodasys caudatus	present study
		Urodasys anorektoxys	Todaro et al. 2000; Pierboni et al. 2003 and unpublished
		Urodasys acanthostylis	Pierboni et al. 2003 and unpublished
	Lepidodasyidae	*Cephalodasys maximus*	Fischer 1994
		Mesodasys adenotubulatus	Balsamo et al. 1999
		Mesodasys laticaudatus	Ferraguti & Balsamo 1994
		Lepidodasys unicarenatus	Guidi et al. 2004
		Lepidodasys sp.	Guidi et al., 2004
	Thaumastodermatidae	*Pseudostomella etrusca*	Ferraguti & Balsamo 1995
		Tetranchyroderma sp. 1	Ferraguti & Balsamo 1995
		Tetranchyroderma sp. 2	Ferraguti & Balsamo 1995
		Tetranchyroderma papii	Ferraguti & Balsamo 1995
		Diplodasys ankeli	Ferraguti & Balsamo 1995
		Acanthodasys aculeatus	Guidi et al. 2003a
	Turbanellidae	*Turbanella ambronensis*	Ferraguti & Balsamo 1995
		Turbanella cornuta	Teuchert 1975
		Paraturbanella teissieri	Balsamo et al. 2002
Chaetonotida	Neodasyidae	*Neodasys ciritus*	Guidi et al. 2003b
	Chaetonotidae	*Chaetonotus maximus*	Balsamo 1992
		Lepidodermella squamata	Hummon 1984
		Musellifer delamarei	Guidi et al. 2003b
	Xenotrichulidae	*Heteroxenotrichula squamosa*	Ferraguti et al. 1995
		Xenotrichula intermedia	Ferraguti et al. 1995
		Xenotrichula punctata	Ferraguti et al. 1995

ford 2001). A heuristic search, using TBR algorithm, was performed with the following options: random addition sequence (ADDSEQ = RANDOM), saving all minimal trees found during the heuristic search (MULTREES = YES) and collapsing a branch if its minimum possible length was

Table 2. Morphological data matrix (0-6, character states; ?, unknown; –, not applicable).

Taxon	Character Number																																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Cephalodasys maximus	0	0	0	0	0	0	0	1	1	1	0	0	0	1	0	1	0	2	0	1	2	0	1	1	0	1	2	2	1	0	0	0	0
Lepidodasys sp.	0	0	0	1	1	–	1	?	0	1	0	0	0	2	1	1	1	2	0	1	2	0	3	1	0	0	0	–	–	–	0	0	0
Lepidodasys unicarenatus	0	0	0	1	1	–	0	?	0	1	0	0	0	2	1	1	1	2	0	1	2	0	3	1	0	0	1	–	–	–	0	0	0
Mesodasys laticaudatus	0	0	0	1	0	1	0	?	0	1	0	0	0	2	0	1	1	2	0	0	2	0	3	1	0	0	1	–	–	–	0	0	0
Mesodasys adenotubulatus	0	0	0	1	0	1	1	0	0	1	0	0	0	2	0	1	?	?	0	1	2	0	?	?	0	0	?	–	–	–	0	0	0
Acanthodasys aculeatus	0	0	0	1	0	0	0	2	0	1	0	0	0	2	1	1	0	2	0	1	2	0	1	1	0	1	2	2	1	1	1	0	0
Diplodasys ankeli	0	0	0	1	0	0	0	?	0	1	0	0	0	2	1	1	1	2	0	1	2	0	1	1	0	1	2	2	2	1	1	0	0
Pseudostomella etrusca	0	0	0	1	0	0	0	?	1	1	0	0	0	1	1	1	1	1	0	0	2	0	1	1	0	1	2	2	0	0	0	0	1
Tetranchyroderma sp. 1	0	0	0	1	0	0	1	2	1	1	?	?	0	2	1	1	1	1	0	1	2	0	1	1	0	1	2	2	0	0	1	0	0
Tetranchyroderma sp. 2	0	0	0	1	0	0	?	?	1	1	?	?	0	2	1	1	1	?	0	?	2	0	1	1	0	1	2	?	2	?	1	0	1
Tetranchyroderma papii	0	0	0	1	0	?	?	?	?	1	?	?	0	2	1	1	1	?	0	?	2	0	1	1	0	1	2	2	?	?	?	0	1
Urodasys anorektoxys	0	0	0	0	1	–	0	?	0	1	0	1	0	1	0	2	0	0	1	–	–	0	2	1	0	1	2	2	2	1	0	0	0
Urodasys acanthostylis	0	0	0	0	0	0	0	?	0	1	0	0	0	3	0	2	0	0	?	?	?	0	4	?	0	0	2	–	–	–	0	0	0
Macrodasys sp.	0	0	0	1	0	0	0	?	1	1	?	?	0	1	?	1	0	?	0	1	4	0	3	1	0	1	2	0	?	0	0	0	0
Macrodasys caudatus	0	0	0	1	0	0	0	3	1	1	1	?	0	1	1	1	1	0	0	1	4	0	3	1	0	1	2	0	?	0	0	0	0
Turbanella cornuta	0	0	0	1	1	–	1	?	1	1	0	1	0	2	1	1	1	0	0	1	2	0	3	1	1	0	0	–	–	–	0	0	0
Turbanella ambronensis	0	0	0	1	1	–	1	?	1	1	0	1	0	2	?	1	1	0	0	1	2	0	3	1	1	0	0	–	–	–	0	0	0
Paraturbanella tesseri	0	0	0	1	1	–	1	?	1	1	1	–	0	2	1	1	1	1	0	1	2	0	2	1	1	0	0	–	–	–	0	0	0
Dactylopodola baltica	0	0	0	–	–	–	–	–	–	–	–	–	0	4	1	1	0	3	0	0	2	0	2	?	0	0	2	–	–	–	0	0	0
Dactylopodola typhle	0	0	0	–	–	–	–	–	–	–	–	–	0	4	1	1	0	3	0	0	2	0	2	?	0	0	2	–	–	–	0	0	0
Xenodasys sp.	0	0	0	1	1	–	0	?	0	1	0	0	0	2	0	1	–	–	0	0	3	0	2	1	0	0	0	–	–	–	0	0	0
Neodasys ciritus	1	0	0	1	1	–	0	?	0	0	0	1	1	0	1	1	1	0	0	0	0	0	0	1	0	1	2	1	0	0	0	0	0
Chaetonotus maximus	2	1	1	–	–	–	–	–	–	–	–	–	1	0	1	?	0	3	1	–	–	1	–	–	–	–	–	–	–	–	–	0	0	0
Lepidodermella squamata	2	1	1	–	–	–	–	–	–	–	–	–	1	0	1	?	0	3	1	–	–	1	–	–	–	–	–	–	–	–	–	0	0	0
Musellifer delamarei	0	0	0	0	1	–	0	0	0	1	1	–	1	0	0	0	1	?	0	0	1	0	2	1	0	0	2	–	–	–	0	0	0
Heteroxenotrichula squamosa	0	0	0	0	1	–	0	0	0	1	0	0	1	0	1	0	1	1	0	2	1	0	2	0	0	0	2	–	–	–	0	1	0
Xenotrichula intermedia	0	0	0	0	1	–	0	0	0	1	0	0	1	0	1	0	1	0	0	2	1	1	–	–	–	–	–	–	–	–	–	0	1	0
Xenotrichula punctata	0	0	0	0	1	–	0	0	0	1	1	–	1	0	1	0	1	1	0	2	1	0	2	0	0	0	2	–	–	–	0	1	0

1. Sperm shape: 0, filiform; 1, commaform; 2, rod-like. **2. Vestigial spermatozoa:** 0, absent; 1, present. **3. Flagellum:** 0, present in spermatids or mature sperm; 1, absent. **4. Accessory fibres:** 0, present; 1, absent. **5. Striated cylinder:** 0, present; 1, absent. **6. Striated cylinder thickness:** 0, monolayered; 1, multilayered. **7. Axonemal microtubules:** 0, parallelly arranged; 1, helically arranged. **8. End-piece:** 0, thin; 1, bubble of cytoplasm; 2, hollow and thin; 3, twisted shaped. **9. Axonemal p.m.:** 0, not swollen; 1, swollen. **10. Centrioles:** 0, two; 1, one. **11. Cap-like structure:** 0, present; 1, absent or reduct. **12. Cap-like fibres:** 0, absent; 1, present. **13. Sperm head:** 0, spiral or helical; 1, straight. **14. Nuclear shape:** 0, straight; 1, spiral or slightly spiral; 2, ribbon-like; 3, complex spiral; 4, in alternate layers. **15. Nuclear diameter reduction:** 0, present; 1, absent. **16. Chromatin condesation:** 0, partial; 1, complete; 2, finger print. **17. Nuclear apex:** 0, convex; 1, flatt or slightly concave. **18. Nuclear base:** 0, with fossa; 1, flat; 2, slightly concave; 3, convex. **19. Mitochondria:** 0, present; 1, absent. **20. Mitochondria number:** 0, more than one and small; 1, one giant; 2, one small. **21. Mitochondrial pattern:** 0, randomly around head; 1, at nuclear base; 2, inside nucleus; 3, around connecting piece; 4, spirally around head. **22. Acrosome:** 0, present; 1, absent. **23. Acrosome shape:** 0, pear-shaped; 1, cork-screw; 2, cylindrical; 3, cylindrical and cork-screw; 4, helical. **24. Acrosome composition:** 0, homogeneous; 1, with different regions. **25. Acrosomal thick disks:** 0, absent; 1, present. **26. Acrosomal tubular structure:** 0, absent; 1, present. **27. Acrosomal material:** 0, regularly condensed at the base; 1, regularly condensed at the apex; 2, uncondensed. **28. Tubular structure organisation:** 0, continuous; 1, tubular-structure like; 2, ring-like. **29. Tubular structure shape:** 0, rectilinear; 1, rectilinear and twisted; 2, twisted. **30. Tubular structure withdrawal:** 0, absent; 1, present. **31. Perinuclear helix:** 0, absent; 1, present. **32. Para-acrosomal bodies:** 0, absent; 1, present. **33. Basal crystal:** 0, absent; 1, present.

zero (COLLAPSE = MINBRLEN). To evaluate the support for the tree topologies, the data set was analyzed by jackknifing (Farris et al. 1996). Five hundred replicates were subjected to a separate heuristic search; only Jack-knife frequencies ≥50% were reported. To test sperm characters in the light of morphological analysis, constrained analyses were performed, beginning with a constraint – CON1 – that forced the topology to reflect a previously published morphological estimate (Hochberg & Litvaitis 2000, 2001). The other constraint – CON2 – is based on CON1 but assuming monophyly for all the Macrodasyida families. The lengths and character changes of the most parsimonious solutions from each constraint analysis were then compared against the MP solution constrained on the topology of the strict consensus tree obtained and the Templeton test was run as implemented in PAUP.

Results

The spermatozoon of *Macrodasys caudatus*

The spermatozoon of *Macrodasys caudatus* is a filiform cell, about 75 µm in length, formed by an elongated head, which is about 24 µm in length, composed in sequence of the acrosome and the nucleus, the latter being surrounded by a single long helical mitochondrion. A conventional 9×2+2 flagellum, 51 µm long (Fregni 1998), is wrapped for its entire length in a striated cylinder, *sensu* Ferraguti and Balsamo (1994) (Fig. 1). The cone-shaped acrosome contains for its whole length an axial tubular structure surrounded by a sheath of homogeneous and moderately electron-dense material (Fig. 2A). The axial structure is straight except in its apical portion, about 5.45 µm long, which is twisted and penetrates basally into the nuclear apex for a short tract, of about 0.3 µm in length (Fig. 2B). The electron-dense sheath encloses also the nuclear apex for a length of about 0.35 µm (Fig. 2B), decreasing in thickness from the base to the acrosomal apex (Fig. 2D). The nucleus is an elongated, thin rod of fully condensed chromatin (Fig. 2C), about 0.25 µm in diameter and is slightly twisted. A single helicoidal mitochondrion encloses both the nucleus and the acrosome along their whole length, forming a coil with a pitch of about 0.6 µm and decreasing in thickness at the apex (Fig. 2C,D). The flagellum is composed of a 9×2+2 axoneme surrounded by a mono-layered striated cylinder (Fig. 2C,E,F). The nucleus and flagellum are connected to each other by a clasp-like structure (*sensu* Teuchert 1976) lying in a *fossa* at the nuclear base (Fig. 2C,E).

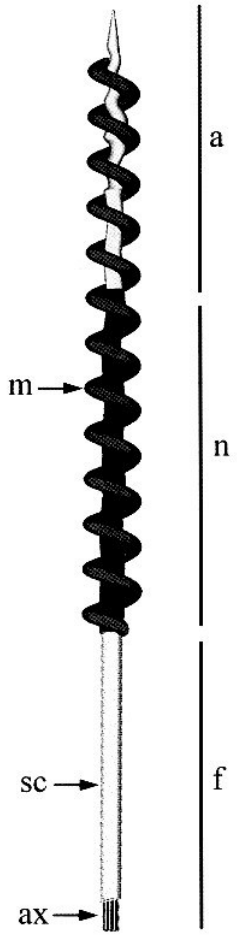


Fig. 1. Spermatozoon of *Macrodasys caudatus*. Three-dimensional reconstruction of a mature gamete. a = acrosome, ax = axoneme, f = flagellum, m = mitochondrion, n = nucleus, sc = striated cylinder.

Phylogenetic analysis of spermatozoal characters

The parsimony analysis of the 33 selected spermatozoal characters resulted in three most parsimonious trees (MPT), each with a length of 90 steps, a consistency index (CI) of 0.58 and a retention index (RI) of 0.74; the strict consensus

tree is shown in Fig. 3A. We arbitrarily decided to describe one of the three MPTs obtained, shown in Fig. 4A; in it, as in previous phylogenetic hypotheses (Hochberg & Litvaitis 2000, 2001), Thaumastodermatidae are monophyletic. As far as the Chaetonotida are concerned, *Xenotrichula punctata*, *X. intermedia* and *Heteroxenotrichula squamosa* group in a monophyletic Xenotrichulidae clade, which appears related to *Musellifer delamarei* as the sister group. *Chaetonotus maximus* and *Lepidodermella squamata* group together and their clade appears as the sister group of the monophyletic *Dactylopodola* genus, which is currently systematized into Macrodasyida.

A basal polytomy does not allow a better resolution among the Chaetonotida nor between the latter and the Macrodasyida. All macrodasyidan species, except *Dactylopodola baltica* and *D. typhle*, form a monophyletic Macrodasyida clade. The position of some Dactylopodolidae within the Chaetonotida (*Dactylopodola*) and of others at the base of the Macrodasyida clade (*Xenodasys* sp.) makes this family paraphyletic. Within macrodasyidans, the families Turbanellidae and Thaumastodermatidae are monophyletic. The Turbanellidae, represented here by three species, form a monophyletic clade close to the Macrodasyidae – Thaumastodermatidae assemblage; whereas within the Thaumastodermatidae, the closely related *Acanthodasys aculeatus* and *Diplodasys ankeli*, members of the monophyletic subfamily Diplodasyinae, make the subfamily Thaumastodermatinae (*Pseudostomella etrusca* and the *Tetranchyroderma* species) paraphyletic. The unexpected position of *Cephalodasys maximus* (Lepidodasyidae) close to the two *Urodasys* species (Macrodasyidae) makes both these families paraphyletic. Moreover, within the Lepidodasyidae, also *Lepidodasys* appears to be a paraphyletic genus, whereas the two *Mesodasys* species lie in a monophyletic clade. Among the more derived family Macrodasyidae, both *Urodasys* and *Macrodasys* are monophyletic genera.

The Jack-knife analysis only gives notable support to a few monophyletic taxa: among chetonotidans, the Xenotrichulidae and the group formed by *Chaetonotus maximus* and *Lepidodermella squamata*; among the macrodasyidans, the families Dactylopodolidae and Turbanellidae as well as the genus *Macrodasys*. The sister group relationship of *Musellifer delamarei* with the Xenotrichulidae is also supported. The Templeton test (Fig. 3C) points out that both the fully constrained solution CON1, which is 5 steps longer, and the CON2 solution, which is 6 steps longer, are not significantly different from the MP solution constrained on the topology of the strict consensus of the three MPTs obtained.

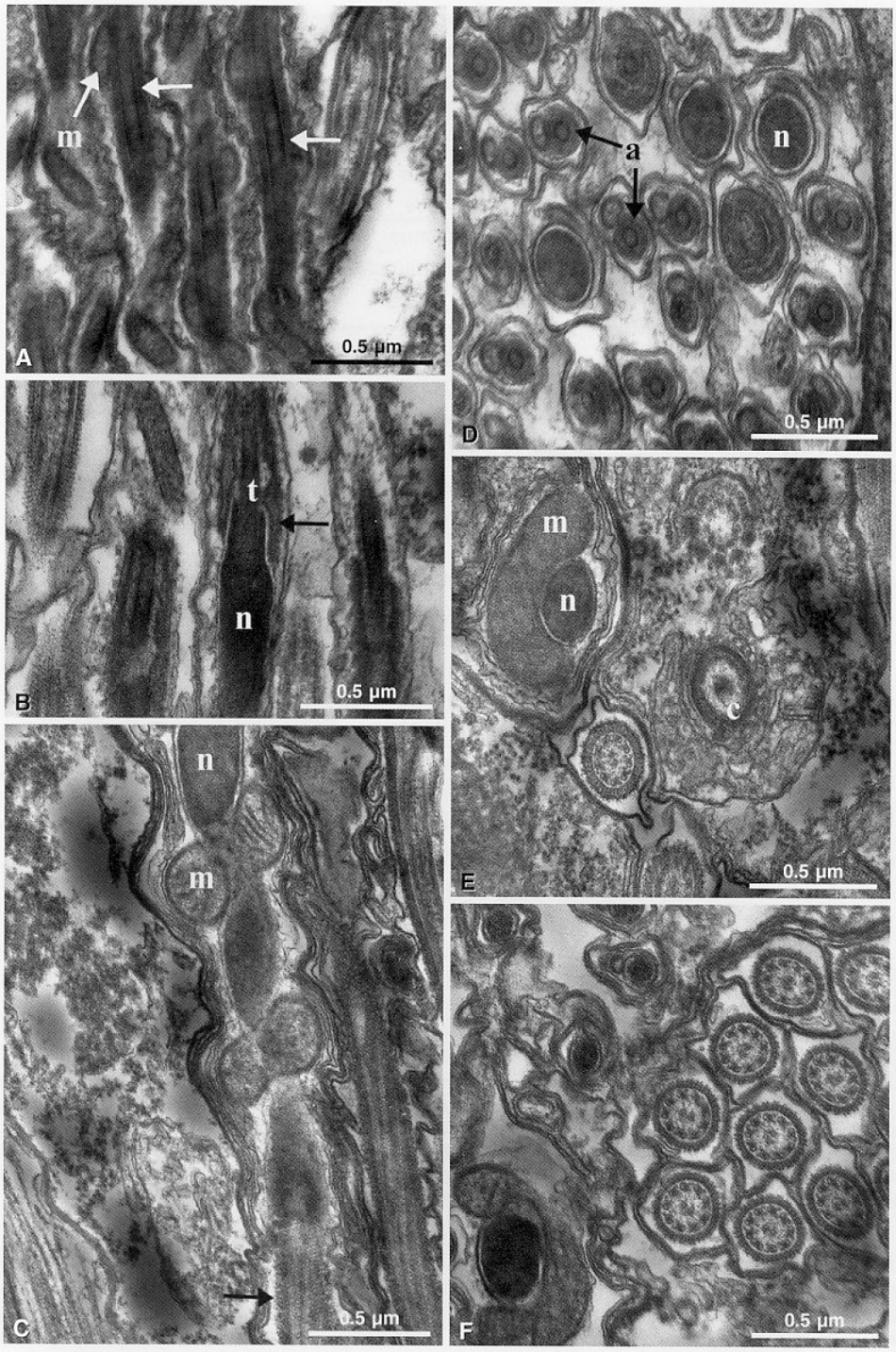
Discussion

The spermatozoon of *Macrodasys caudatus*

We only know from the literature a single spermatozoon of the Macrodasyidae, that of *Macrodasys* sp. (Ruppert 1978). Therefore, we will compare our findings on *M. caudatus* with those on *Macrodasys* sp., as well as with our unpublished observations on two species of *Urodasys*, the only other genus in the family. The acrosomal and nuclear morphologies of *M. caudatus* and *Macrodasys* sp. spermatozoa are similar. In both species the mitochondrial helix surrounds the nucleus and acrosome, leaving free only the apical acrosomal portion; and in both species the conventional axoneme is enclosed by a mono-layered striated cylinder. Major differences reside both in the morphology of the acrosomal apical portion, which is less twisted and much longer in *M. caudatus* than in *Macrodasys* sp., and the structure connecting the nucleus and acrosome, which in *M. caudatus* shows greater complexity than in *Macrodasys* sp., where it is a simple "collar of dense material". The overall close similarity of the spermatozoa supports the inclusion of both species in the same genus, the few differences observed confirming the existence of species-specific traits of the spermatozoa.

The spermatozoa of the *Macrodasys* species greatly differs from the basic sperm plan of macro-

Fig. 2. Mature spermatozoa of *Macrodasys caudatus*. **A.** Acrosome: the axial tubular structure and the sheath of homogeneous material, both surrounded by a long mitochondrion (m), are visible (arrows). **B.** Points of connection between acrosome and nucleus (n). The acrosomal tubular structure (t) withdraws into the nuclear apex whereas the sheath (arrow) encloses the nuclear apex. **C.** Basal nuclear portion and apical portion of the flagellum. The latter is wrapped by a striated cylinder (arrow). **D.** Cross-sections of nuclear (n) and acrosomal (a) regions. **E.** Clasp-like structure (c), connecting the nucleus to the flagellum, in cross section. **F.** Several flagella in cross section.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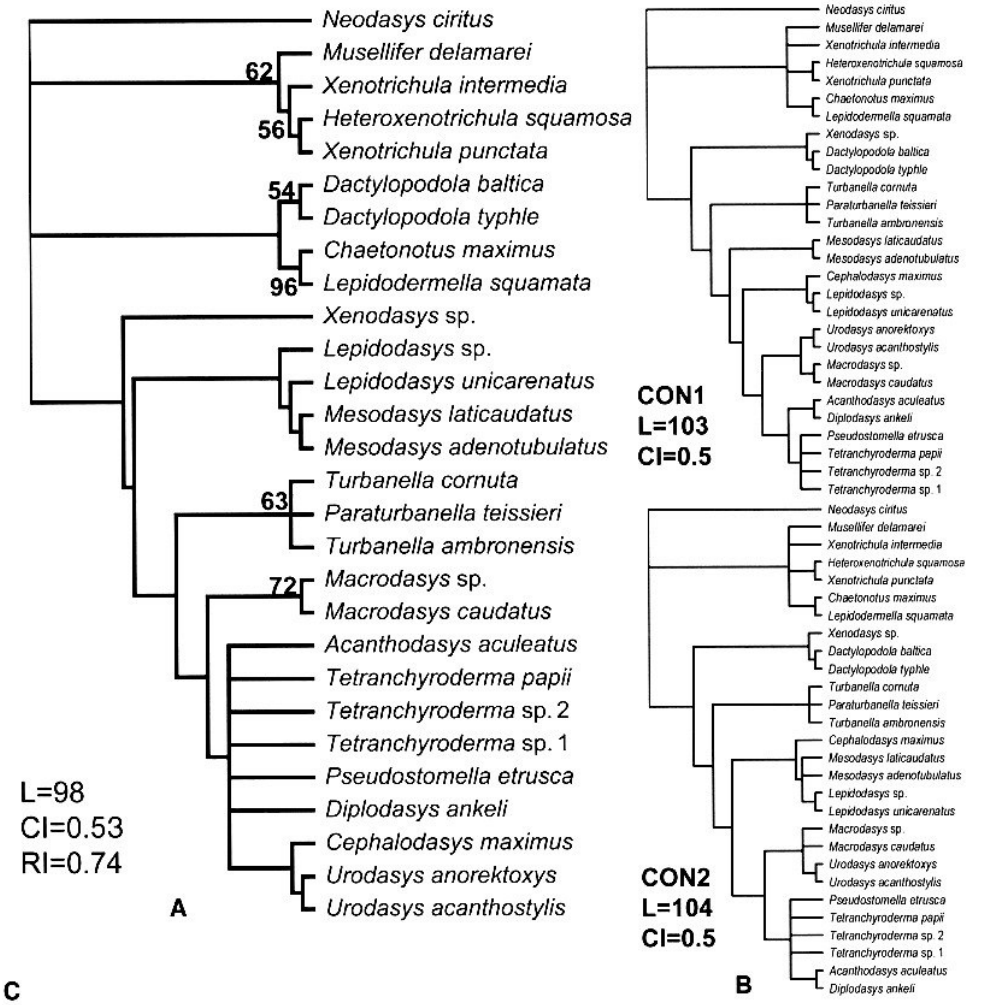
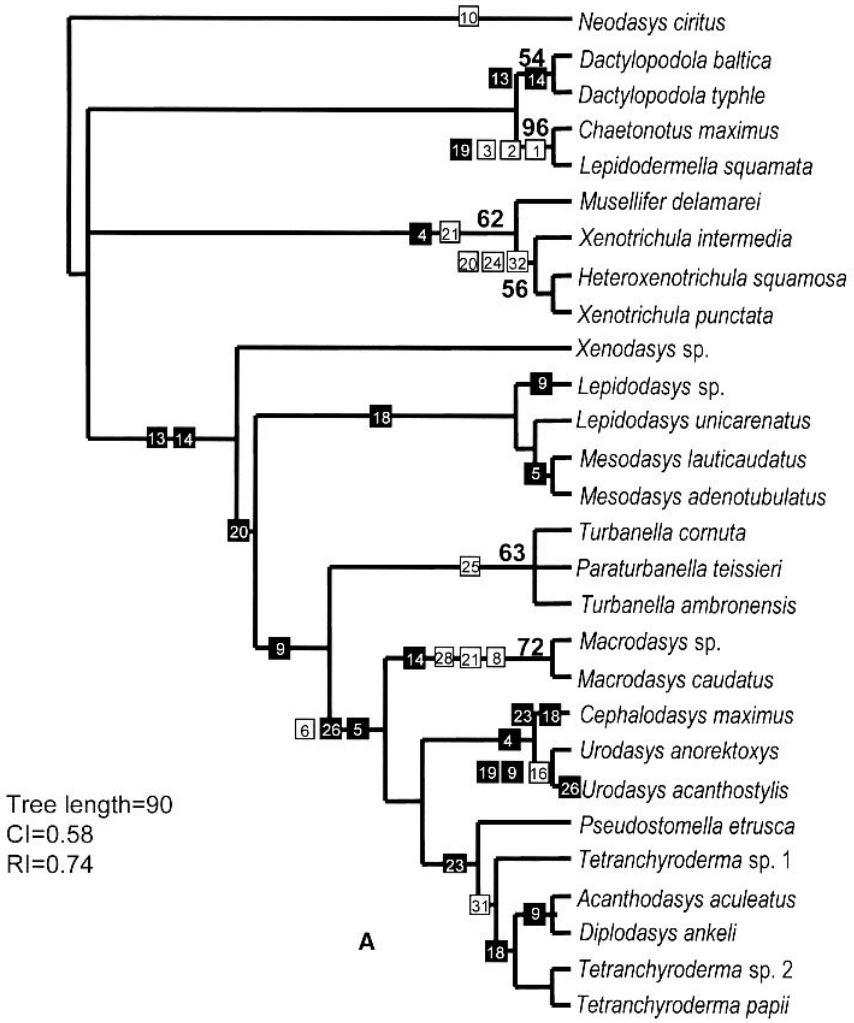


Constraint	Length	ΔLength	CI	P(Templeton)
consensus tree solution	98	-	0.53	-
CON1 – after Hochberg and Litvaitis, 2000	103	5	0.50	0.24
CON2 – as CON1, with all families monophyletic	104	6	0.50	0.13

Fig. 3. Constrained analysis. **A.** Strict consensus tree of the three MPTs obtained; number above branches are Jack-knife values. **B.** Constraint trees: CON1 and CON2 are constraint trees that force the topology, the first to reflect the morphological estimate of Hochberg & Litvaitis, 2000 (CON1), and the second also assuming monophyly for all macrodasyidan families (CON2). **C.** Templeton test. Results are compatible with the phylogeny based on CON1 and CON2. L = tree length; CI = consistency index; RI = retention index.

dasyidans, mainly due to the reverse position of the mitochondrion, which wraps externally both nucleus and acrosome (see also Ruppert 1978, 1991), whereas in the other macrodasyidans the mitochondrial axis is enclosed by a spring-shaped nucleus.

Our preliminary observations on *Urodasys anorektoxys* and *U. acanthostylis* reveal peculiar



B

CHARACTERS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Homology	1	2	3			6		8		10						16				20	21			24	25			28			31	32	
Homoplasy				4	5				9				13	14			17	18	19				23			26	27						

Fig. 4. A. One of the three MPTs obtained from the analysis of spermatozoal characters. Some of the sperm characters are mapped on the MPT (see Table below). Numbers above branches are Jack-knife values. **B.** Pattern of spermatozoal characters, as revealed by the parsimony analysis (See Discussion Section). Among the 33 sperm characters considered, 14 are autapomorphies (as black numbers in white squares in the Table and on the MPT) and 19 are homoplasies (as black squares in the Table and on the MPT). The homoplasies that support the tree topology are represented as white number in black squares in the Table and on the MPT.

spermatozoa in which the absence of the mitochondria is the most striking feature (Pierboni et al. 2003). Also the unusual "fingerprint-like" chromatin condensation and acrosome morphology, which is greatly different from *Macrodasys* as well as from the basic gastrotrich model, characterize both *Urodasys* species and suggest a possible polyphyly of the Macrodasyidae (Fig. 5A,B).

Phylogeny of Gastrotricha studied through spermatozoa

This is the first phylogenetic analysis of Gastrotricha based on sperm ultrastructure. The low consistency index value obtained reveals poor congruence among spermatozoal characters, thus reflecting the high variety of sperm ultrastructure observed among gastrotrichs (Balsamo et al. 1998). The gap between consistency index (CI = 0.58) and retention index (RI = 0.74) and the pattern of sperm characters as revealed by this parsimony analysis point out that most spermatozoal characters are homoplasic characters that fit the tree topology, i.e. secondary homologies with a low level of generality (De Pinna 1991). This means that most spermatological traits arose independently several times at the base of large groups during the evolution of gastrotrichs. These homoplasies, as shown by this analysis, have remarkable information content, supporting the deepest branches of the MPT obtained (see Fig. 4A): they increase the overall phylogenetic structure (Källersjö et al. 1999).

The fact that a large portion of spermatozoal characters is formed by homoplasic characters could also explain the instability of sperm characters – mainly at the deeper branches of the phylogenetic tree (Fig. 4A) – as emerged from the jackknife analysis. The poor jackknife support, and the polytomy within the Chaetonotida could be also due to the small sample of chaetonotidan species considered in this analysis. In fact, our study has covered only a fraction of the total character variation in the gastrotrich taxa, which amounts to 29 genera of the Macrodasyida and 26 genera of the Chaetonotida described so far. On the other hand, high character incongruence also characterizes morphological (Hochberg & Litvaitis 2000, 2001), as well as molecular (Todaro et al. 2003) data sets.

The results of this analysis are largely congruent with those obtained from traditional systematics, as confirmed by the Templeton test (Fig. 3C). With the exception of *Dactylopodola*, Macrodasyida appear to be resolved separately from the Chaetonotida. Out of the four families of Macrodasyida recognized as monophyletic on morphological grounds, spermatological data support the monophyly of the Turbanellidae and Thaumastodermatidae, the two most diverse gastrotrich families. Moreover, ultrastructure and morphology agree in recognizing the paraphyletic status of Lepidodasyidae, and on the nesting order of different macrodasyid families (Hochberg & Litvaitis 2000, 2001). Among the Macrodasyida, Dactylopodolidae (under exclusion of *Dactylopodola*) confirm to be the most basal family, followed by the paraphyletic Lepidodasyidae, the sister group of the assemblage including the monophyletic Turbanellidae and the derived sister families Macrodasyidae and Thaumastodermatidae. The paraphyly of Dactylopodolidae, suggested only by spermatozoal characters, is due to the different grouping of *Xenodasys* at the base of the Macrodasyida, and of *Dactylopodola* together with the freshwater Chaetonotida, respectively. The reason for this grouping could be the fact, that the sperm plan of *Dactylopodola* species diverges from that of all the other macrodasyidan sperm: no flagellum, a rod-shaped nucleus, and a peculiar organization of mitochondria and dense bodies (Fischer 1996; Guidi et al. unpublished data). Within Chaetonotida, the close relationship between Xenotrichulidae and *Musellifer delamarei* as sister groups is also supported by other morphological data and suggests to reconsider the affiliation of *M. delamarei* to the family Chaetonotidae. Sperm data also agree with molecular characters showing significant differences between the two orders (Manylov et al. 2004).

Character analysis

To discuss the pattern of sperm characters, the MPT in Fig. 4A was selected: in it, as noted above, the family Thaumastodermatidae is monophyletic. A sperm head spiral or helical (#13) is the major autapomorphy supporting the Macrodasyida, excluding *Dactylopodola*; it is an homoplasic character as to the whole in-group, due to the close relationship between *Dactylopodola* and parthenogenetic chaetonotids. Within macrodasyidans, Turbanellidae and Thaumastodermatidae are more homogeneous from a spermatological point of view than Lepidodasyidae and Macrodasyidae. The only autapomorphy supporting Lepidodasyidae, excluding *Cephalodasys maximus*, is a slightly concave nuclear base (#18); it is an homoplasy arosen independently in two other macrodasyidan groups (Fig. 4A,B). A single autapomorphy supports the monophyly of the Turbanellidae: the peculiar condensation of the inner acrosomal axis in thick regularly overlapping disks (#25). Major autapomorphies supporting the monophyletic

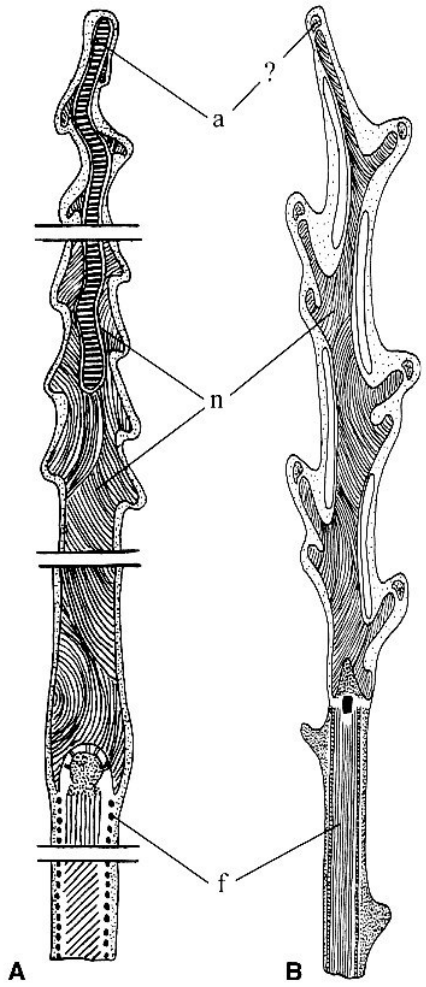


Fig. 5. Schematic drawings of the spermatozoa of *Urodasys anorektoxys* (**A**) and of *Urodasys acanthostylis* (**B**). a = acrosome, f = flagellum, n = nucleus.

Macrodasys are a characteristically twisted endpiece (#8), the mitochondria spirally wound around the sperm head (#21) and a homogeneous – not striated – acrosomal tube organization (#28). The single autapomorphy supporting monophyletic *Urodasys* is the peculiar "fingerprint" chromatin condensation (#16). The presence of accessory fibers (#4) groups *Urodasys* together with *Cephalodasys maximus*: but it is an homoplasic character also evolved independently at the base of the *Musellifer delamarei* + Xenotrichulidae assemblage. A corkscrew acrosome (#23) is the major autapomorphy supporting the Thaumastodermatidae; although in a larger framework it is an homoplasy, which arose independently in *Cephalodasys maximus*. The presence of a perinuclear helix (#31) is the single autapomorphy for all the Thaumastodermatidae, excluding *Pseudostomella etrusca*. Within the Chaetonotida, major autapomorphies supporting the Xenotrichulidae are the presence of a simple acrosome that is homogeneous in its inner composition (#24), flanked by para-acrosomal bodies *sensu* Ferraguti et al. (1995) (#32) and a single small mitochondrion (#20). As hypothesized by Guidi et al. (2003b), the basal position of mitochondria interpolated between the nucleus and tail (#21), a large accessory fiber surrounding the axoneme (#4) and the peculiar loose chromatin condensation (#16) are synapomorphies between the Xenotrichulidae and the chaetonotidan *M. delamarei*.

Synapomorphies between the post-parthenogenetic vestigial (#2) spermatozoa of *Chaetonotus maximus* and *Lepidodermella squamata*, both consisting of uncondensed chromatin enclosed in a cellular membrane, are the rod-shape (#1), and the complete loss of acrosome, and flagellum (#3). The complete loss of mitochondria (#19) is an homoplasic character, which also arose independently in *Urodasys* species (see Fig. 4A,B).

To summarize, our analysis has shown that spermatozoal characters may be important in resolving monophyletic groups and to broadening the basis of evidence in phylogenetic reconstruction. In the light of these results, a "total evidence" approach to gastrotrich phylogeny, including different data sets, molecular, ultrastructural and morphological, could be informative.

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