The muscular system of *Musellifer delamarei* (Renaud-Mornant, 1968) and other chaetonotidans with implications for the phylogeny and systematization of the Paucitubulatina (Gastrotricha)

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We studied comparatively the muscle organization of several gastrotrich species, aiming at shedding some light on the evolutionary relationships among the taxa of the suborder Paucitubulatina. Under confocal laser scanning microscope, the circular muscles were present in the splanchnic position as incomplete circular rings in *Musellifer delamarei* (Chaetonotidae) and *Xenotrichula intermedia* (Xenotrichulidae) and as dorsoventral bands in *Xenotrichula punctata, Heteroxenotrichula squamosa* and *Draculiciteria tesselata* (Xenotrichulidae); in the somatic position, *M. delamarei* shares the presence of dorsoventral muscles with all the Xenotrichulidae, in contrast with the remaining Chaetonotidae that lack these muscles. Maximum parsimony analysis of the muscular characters confirmed monophyly of Paucitubulatina and Xenotrichulidae, while the Chaetonotidae was paraphyletic, with the exclusion of *Musellifer*, which is the most basal genus within the Paucitubulatina. Xenotrichulidae is the sister taxon to Chaetonotidae, which in turn has *Polymerurus* as the most basal taxon. In general, the results agree with recent phylogenetic inferences based on molecular characters and support the hypothesis that, within Paucitubulatina, dorsoventral muscles are plesiomorphies retained in marine, interstitial, hermaphroditic gastrotrichs. Dorsoventral muscles were subsequently lost during changes in lifestyle and reproduction modality that took place with the invasion of the freshwater environment. This new information prompted us to reconsider the systematization of Chaetonotidae, proposing the establishment of Muselliferidae fam. nov. to include the genera *Musellifer* and *Diuronotus*. © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, **94**, 379–398.

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INTRODUCTION

The phylum Gastrotricha includes microscopic, acoelomate, worm-like invertebrates that are ever present among the meiobenthic communities of marine and freshwater systems (e.g. Balsamo & Todaro, 2002; Todaro *et al*., 2006a). Despite their ubiquity and relative abundance, the inter- and intraphyletic relationships of the gastrotrichs are still not completely understood; conflicts also exist between the current systematization and evolutionary hypotheses based

on morphology or molecules (see Todaro *et al*., 2003, 2006b,c). It should be emphasized that research on these animals is hindered as a result of their small and fragile bodies, which make collection and manipulation difficult; the situation is exacerbated by the shortage of taxonomic expertise which particularly plagues the 'minor' invertebrate phyla (Buyck, 1999; Bhadury *et al*., 2006).

In the current systematization, the *c*. 700 described species are subdivided into the two orders Macrodasyida and Chaetonotida, which are easily distinguished by a number of morphological, ecological and reproductive features; representatives of the two

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orders are so distinct in form that some authors have even doubted the common origin of the Gastrotricha (Rieger & Rieger, 1977; Ruppert, 1982). However, focused cladistic analyses based on morphological and molecular traits support the monophyly of the group (Hochberg & Litvaitis, 2000; Todaro *et al.*, 2006c). The order Chaetonotida includes most of the species (some 450 spp.) and its members share a fairly uniform organization, e.g. tenpin-shaped body, presence of solely posterior adhesive tubes (usually only two that form the so-called caudal furca), Y-shaped orientation of the pharynx lumen and the absence of pharyngeal pores (Remane, 1961). The divergence from the general chaetonotidan body design of the three known species of Neodasyidae (genus *Neodasys*), which in contrast have a worm-like appearance and possess several adhesive tubes along the body sides, is formally recognized by including these marine forms in the suborder Multitubulatina; the remainder of the Chaetonotida are reunited in the suborder Paucitubulatina (d'Hondt, 1971).

The Paucitubulatina encompasses six families, including the solely marine Xenotrichulidae, the marine and freshwater Chaetonotidae and the exclusively freshwater Dasydytidae, Dichaeturidae, Neogosseidae and Proichthydidae. The monophyly of the four freshwater families, as well as the likely derived position of these taxa along the Paucitubulatina branch, has never been questioned by systematists and has gained support from an inclusive cladistic analysis of the Gastrotricha based on 81 morphological and reproductive traits (Hochberg & Litvaitis, 2000). In contrast, the evolutionary relationships within and between the Xenotrichulidae (three genera, 23 spp.) and Chaetonotidae (13–14 genera, more than 370 species) are more troubled, as a result mostly, but not exclusively, of the status of the marine chaetonotid *Musellifer Hummon, 1969*.

This genus is cosmopolitan in distribution (*sensu* Sterrer, 1973) and includes three quite rare species that, by virtue of some of their characteristics that are perceived to be plesiomorphic (e.g. solely marine, hermaphroditic sexual apparatus, functional spermatozoa, etc.), are empirically considered to be basal within the family Chaetonotidae (Leasi, Virno-Lamberti & Todaro, 2006a). However, a recent molecular analysis found *Musellifer* to be basal among the Paucitubulatina, hence making the Chaetonotidae paraphyletic (Todaro *et al.*, 2006c). A similar indication resulted from the inclusive morphological analysis of Hochberg & Litvaitis (2000), although with low nodal support (bootstrap < 50%). Yet, a sister group relationship between Xenotrichulidae and *Musellifer* was hypothesized based on the similarity of the spermatozoa ultrastructure (Guidi *et al.*, 2003; see also Marotta *et al.*, 2005). Therefore,

new information is needed to better understand the phylogenetic alliances of *Musellifer* and, in a more general framework, the evolutionary relationships within the Chaetonotida Paucitubulatina.

Recent studies have indicated that suitable information in this regard might come from the thorough surveying of the muscular system (e.g. Hochberg & Litvaitis, 2003; Leasi *et al.*, 2006b).

It is well known that gastrotrichs rely chiefly on ciliary movement for their locomotion; however, the musculature plays a crucial role in general body movements, including longitudinal elongation and shortening, ventral flexion, head nodding, flexion of appendages, rapid escape behaviour and likely also contributes to the characteristic whole-body flexion observed during mating (Teuchert, 1968, 1978; Ruppert, 1978; Banchetti & Ricci, 1998; Hochberg & Litvaitis, 2001a, b; Todaro *et al.*, 2006b). For a long time, the muscular system, from the gross anatomical level to the ultrastructural one, has provided some insights into gastrotrich evolution (Ruppert, 1982; Travis, 1983; Ruppert, 1991). More recently, microscopical techniques using fluorescently labelled phalloidin in whole-mount specimens prepared for epifluorescence have revealed the complete muscular organization of gastrotrichs, supplying new, important information from which robust hypotheses about phylogenetic relationships have been inferred (e.g. Hochberg & Litvaitis, 2001a, b, c, 2003; Hochberg, 2005; Leasi *et al.*, 2006b).

The muscular system of species of Chaetonotida Paucitubulatina consists of band-like myo-fibres (single or in a bundle) in circular, longitudinal, helicoidal and dorsoventral orientations present in splanchnic (i.e. covering the alimentary canal) and somatic positions (Ruppert, 1975, 1991; Hochberg & Litvaitis, 2001a, c, 2003; Leasi *et al.*, 2006b). While longitudinal and helicoidal muscles are present in all chaetonotidans investigated so far, the same does not hold true for some components of the circular and dorsoventral musculature; yet the dorsoventral bands are the muscles that show the highest diversification among the taxa that possess them. It has been hypothesized that the dorsoventral bands are derivatives of the circular bands (Ruppert, 1975; Hochberg & Litvaitis, 2001c, 2003) and that further evolution of this muscle set took place with the invasion of freshwater ecosystems (Leasi *et al.*, 2006b).

In this evolutionary scenario, 'primitive' chaetonotidans should hold clues to the origins of particular muscle sets within the Paucitubulatina. Therefore, the aim of this study is to investigate the muscular system of *Musellifer delamarei*, a species considered basal within the Chaetonotidae. As knowledge of the muscular system of the most paucitubulatine taxa is still scant, a further aim of this study is

also to describe the musculature of a number of additional genera/species never before studied, and to provide novel information on the muscular system of three species that were previously surveyed with more conventional microscopical techniques.

In this study, new data derived from confocal laser scanning microscopy, together with information from previous studies, are coded in a phylogenetic data matrix and analysed cladistically in an attempt to shed light on the phylogeny of the Paucitubulatina, hoping that the phylogeny may help to clarify, in a functional perspective, the evolution of the muscular system in these animals.

MATERIALS AND METHODS

GENERAL

We used information regarding the muscular system, habitat and reproductive characteristics of 11 chaetonotidans and a single macrodasyidan species. Information regarding the muscular system of seven species: *M. delamarei*, *Aspidiophorus paramediterraneus*, *Chaetonotus* (*Chaetonotus*) sp., *Draculiciteria tesselata*, *Heteroxenotrichula squamosa*, *Xenotrichula*

intermedia and *Xenotrichula punctata* was obtained in this study. Data from the remaining five species were derived entirely from the literature. A full description of the musculature is only provided for the species that had not previously been investigated using epifluorescence techniques, with the two following exceptions. We do not provide a description of the musculature of *A. paramediterraneus,* as it does not differ substantially from the musculature of *Aspidiophorus marinus*, described based on a wide-field epifluorescence survey (cf. Hochberg & Litvaitis, 2003); however, we do provide a detailed description of the musculature of *X. intermedia,* as confocal microscopy unveiled several important traits that had not been reported previously for the species (Hochberg & Litvaitis, 2003). The full species list with habitat, region of collection and, when applicable, reference, is reported in Table 1. As the phylogenetic position within the Chaetontoidae of the monotypic, semipelagic, *Undula* (subfam. Undulinae Kisielewski, 1991), regarded as a recent derivative of some freshwater benthic forms (Kisielewski, 1991), appears to be irrelevant to the aim of the present research, it will not be discussed further.

Table 1. List of taxa, their current classification, collection details and references used in this study

Taxon	Habitat and region of collection	Source
Phylum Gastrotricha		
Order Macrodasyida		
Family Dactylopodolidae		
Dactylopodola baltica (Remane, 1926)*	Marine, Atlantic, USA	Hochberg & Litvaitis (2001d)
Order Chaetonotida		
Suborder Multitubulatina		
Family Neodasyidae		
Neodasys cirritus Evans, 1992	Marine, Atlantic, USA	Hochberg (2005)
Suborder Paucitubulatina		
Family Chaetonotidae		
Aspidiophorus paramediterraneus Remane, 1927	Marine, Mediterranean, Italy (40°16′N, 17°52′E)	Present study
Chaetonotus (*Chaetonotus*) sp.	Freshwater, Modena, Italy (44°30′N; 10°52′E)	Present study
Musellifer delamarei (Renaud-Mornant, 1968)	Marine, Mediterranean, Italy (40°16′N; 17°51′E)	Present study
Halichaetonotus aculifer (Gerlach, 1953)	Marine, Atlantic, USA	Hochberg & Litvaitis (2003)
Lepidodermella squamata (Dujardin, 1841)	Freshwater, USA	Hochberg & Litvaitis (2003)
Polymerurus nodicaudus Voigt, 1901	Freshwater, Modena, Italy	Leasi *et al*. (2006b)
Family Xenotrichulidae		
Xenotrichula intermedia Remane, 1934	Marine, Mediterranean, Italy (42°01′N; 14°52′E)	Present study
Xenotrichula punctata Wilke, 1954	Marine, Mediterranean, Italy (40°35′N; 08°15′E)	Present study
Heteroxenotrichula squamosa Wilke, 1954	Marine, Mediterranean, Greece (36°11′N; 28°07′E)	Present study
Draculiciteria tesselata (Renaud-Mornant, 1968)	Marine, Persian Gulf, Kuwait (28°3′N; 04°82′E)	Present study

*Species used as out-group in the phylogenetic analysis.

SURVEY OF THE MUSCULAR SYSTEM

Musellifer delamarei and the other marine taxa were narcotized and extracted from the sediment, using the narcotization/decantation technique with a 7% $MgCl_2$ solution, whereas the freshwater gastrotrichs were extracted and relaxed using a 1% $MgCl_2$ solution (Pfannkuche & Thiel, 1988). The relaxed specimens were incubated at 4 °C for 1 h in 4% formaldehyde [0.1 M phosphate-buffered saline (PBS); pH 7.4]; they were then washed several times with 0.1 M PBS, permeabilized for 1 h in a pre-incubation solution [0.2% Triton X-100, 0.25% bovine serum albumin (BSA) and 0.05% NaN_3 in PBS 0.1 M] and incubated in tetramethyl rhodamine iso-thiocyanate (TRITC)–phalloidin (Sigma) (8 µL 38 µM solution in 200 µL pre-incubation solution) for 1 h. The specimens, 3–8 for each species, were subsequently rinsed in PBS and embedded in Citifluor (Plano, Wetzlar) on microscope slides and surveyed using a Leica DM IRE 2 Confocal Laser Scanning Microscope. Series of optical sections were projected in one maximum-projection (MPJ) image or visualized as a simulated fluorescence projection (SFPJ) for a three-dimensional appearance. The positions of the muscles are expressed in reference to percentage body units; total body = 100 units (U). The muscle names follow the format presented by Ruppert (1975) with the following exceptions: the term 'Rückenhautmuskel' (Zelinka, 1889) is replaced with dorsodermal muscle; dorsoventral muscles are muscles inserted completely into the endocuticle (*sensu* Ruppert, 1991); partial dorsoventral muscles are muscles that show only some fibres inserted into the endocuticle.

PHYLOGENETIC ANALYSIS

Fourteen muscular and two bio-ecological traits were coded in 12 taxa (Tables 2 and 3). In the presence of inapplicable data we used a combination of reductive and composite character coding strategies (see Strong & Lipscomb, 1999). Eleven species representative of 10 genera and three families of the two suborders of Chaetonotida constituted the in-group whereas *Dactylopodola baltica,* a supposed basal macrodasyidan (see Hochberg & Litvaitis, 2001d), was used as the out-group, to determine character transformations within the Chaetonotida (Table 3).

Phylogenetic analysis was carried out using PAUP* (Swafford, 2001; version 4.0b10 for Macintosh) under the parsimony criterion. Parsimony analyses were run using a branch-and-bound search strategy; the

Table 2. Characters and character states used in the phylogenetic analysis

Number	Characters
	Longitudinal muscles
1	More then two pairs of somatic longitudinal myocytes: 0, absent; 1, present.
2	Longitudinal muscles inserted beside the mouth: 0, absent; 1, present.
3	Muscles in the furca/caudal lobes: 0, absent; 1, present.
4	Dorsodermal muscles: 0, absent; 1, present; 2, branched.
5	Posterior branching of the dorsal longitudinal muscles with insertion at the base of the furca: 0, absent; 1, present.
6	Pattern striation: 0, cross; 1, atypically cross; 2, oblique.
	Other splanchnic component
7	Helicoidal muscles span: 0, to the base of the pharynx; 1, to one-third of intestine; 2, past one-third of intestine.
8	Splanchnic muscles in the intestinal region: 0, absent; 1, present.
9	Type of muscles in the intestinal region: 0, complete circular; 1, incomplete circular; 2, complete dorsoventral; 3, incomplete dorsoventral; –, inapplicable.
10	Tip of the circular and/or dorsoventral muscles in the intestinal region: 0, simple; 1, branched; –, inapplicable.
	Other somatic components
11	Semicircular anterior bands at the ventral side of the pharynx: 0, absent; 1, present.
12	Somatic muscles in the intestinal region: 0, absent; 1, present.
13	Type of muscles in the intestinal region: 0, circular; 1, complete dorsoventral; 2, incomplete dorsoventral; –, inapplicable.
14	Three pairs of somatic dorsoventral muscles at the pharyngeo–intestinal junction: 0, absent; 1, present.
	Bio-ecological characters
15	Reproduction: 0, hermaphroditic; 1, parthenogenetic; 2, alternate.
16	Habitat: 0, marine; 1, freshwater; (01), represented in both.

Table 3. Characters matrix used in the phylogenetic analysis

Taxa	Characters															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Dactylopodola baltica	1	0	1	0	0	0	2	1	0	–	1	1	0	0	0	0
Neodasys cirritus	1	0	1	0	0	1	1	1	0	–	0	1	0	0	0	0
Draculiciteria tesselata	0	1	1	2	1	1	2	1	2	1	0	1	1	1	1	0
Heteroxenotrichula squamosa	0	1	1	2	1	2	2	1	2	1	0	1	1	1	0	0
Xenotrichula intermedia	0	1	1	1	1	2	2	1	1	1	0	1	2	1	0	0
Xenotrichula punctata	0	1	1	1	1	2	1	1	3	1	0	1	2	1	0	0
Aspidiophorus mediterraneus	0	1	0	2	0	2	1	0	–	–	0	0	–	0	2	(01)
Chaetonotus (Ch.) sp.	0	1	0	2	0	2	1	0	–	–	0	0	–	0	2	(01)
Halichaetonotus aculifer	0	1	0	1	0	2	1	0	–	–	0	0	–	0	2	0
Lepidodermella squamata	0	1	0	1	0	2	1	0	–	–	0	0	–	0	2	(01)
Musellifer delamarei	1	0	1	1	0	1	0	1	1	0	1	1	2	0	0	0
Polymerurus nodicaudus	0	1	0	1	0	2	1	1	2	1	0	0	–	0	2	1

characters, all unordered, were equally weighted. A bootstrap analysis was performed using 1000 replicates (branch-and-bound search) and summarized in a 50% majority-rule consensus tree.

RESULTS

GENERAL MUSCLES ARRANGEMENT

In the surveyed species, the muscles appeared to be arranged as a series of bands along the digestive tract and closely aligned with the other organ systems of the body. Confocal laser scanning microscopy (cLSM) revealed muscles in three orientations: circular, longitudinal and helicoidal; in some taxa, dorsoventral muscles were also present (i.e. *M. delamarei* and the Xenotrichulidae). Furthermore, other muscle arrangements characterized a number of the species studied (e.g. the anterior semicircular bands in the ventral side of the pharynx in *M. delamarei;* or the posterior branching of the dorsal longitudinal bands with insertions at the base of the furca in the Xenotrichulidae or the branching of the dorsodermal muscles in *D. tesselata*, *H. squamosa*, *A. paramediterraneus* and *Chaetonotus* sp.).

Circular muscles were always present along the pharynx, in the splanchnic position, where they lie to the inside of the longitudinal bands. *M. delamarei* and *X. intermedia* possessed circular muscles along the intestine only, but to the outside of the longitudinal bands as incomplete hoop-like rings.

Longitudinal muscles generally span the length of almost all gastrotrichs in dorsal, lateral, ventrolateral and ventral positions. Helicoidal muscles always enwrapped the circular and longitudinal splanchnic muscles with spirals ending at the pharyngeo–intestinal junction, as in *M. delamarei*, or at some point along the intestine, as in the remainder of

species. In the selected taxa that possessed dorsoventrally orientated muscles, *D. tesselata*, *H. squamosa* and *X. punctata* have them both in splanchnic and somatic position, while *X. intermedia* showed these muscles only in the somatic position. Despite the fact that the majority of the investigated taxa are functional hermaphrodites (i.e. the male apparatus includes normally developed spermatozoa), none of them shows musculature associated with reproductive tracts or with possible sexual accessory organs.

THE MUSCULAR SYSTEM OF SINGLE SPECIES

The muscular system of Musellifer delamarei
The musculature of *M. delamarei* consisted of bands in circular, longitudinal, helicoidal and dorso-ventral orientations (Fig. 1).

Circular muscles: Circular fibres (0.5 µm wide) were present in the splanchnic position along the pharynx and intestine, from the mouth (U2) to the anal region (U68). The pharyngeal wall (about 40 µm; U2-U24) was surrounded by at least 40 complete and equidistant hoop-like rings (cm). Along the intestine (70 µm, U25-U68), the circular muscles (icm; about 30 rings) that were incomplete (i.e. opened dorsally and ventrally), tightly wrapped the splanchnic longitudinal bands (Fig. 1A, C).

Longitudinal muscles: The entire digestive tube was flanked by thin, ventral (vm; 1 µm wide), ventrolateral (vlm; 1 µm wide) and dorsal (1 µm wide) longitudinal muscles (splanchnic component), surrounded in the intestinal region by incomplete circular muscles. Closer to the body wall, five pairs of thicker, apparently obliquely striated muscles were present
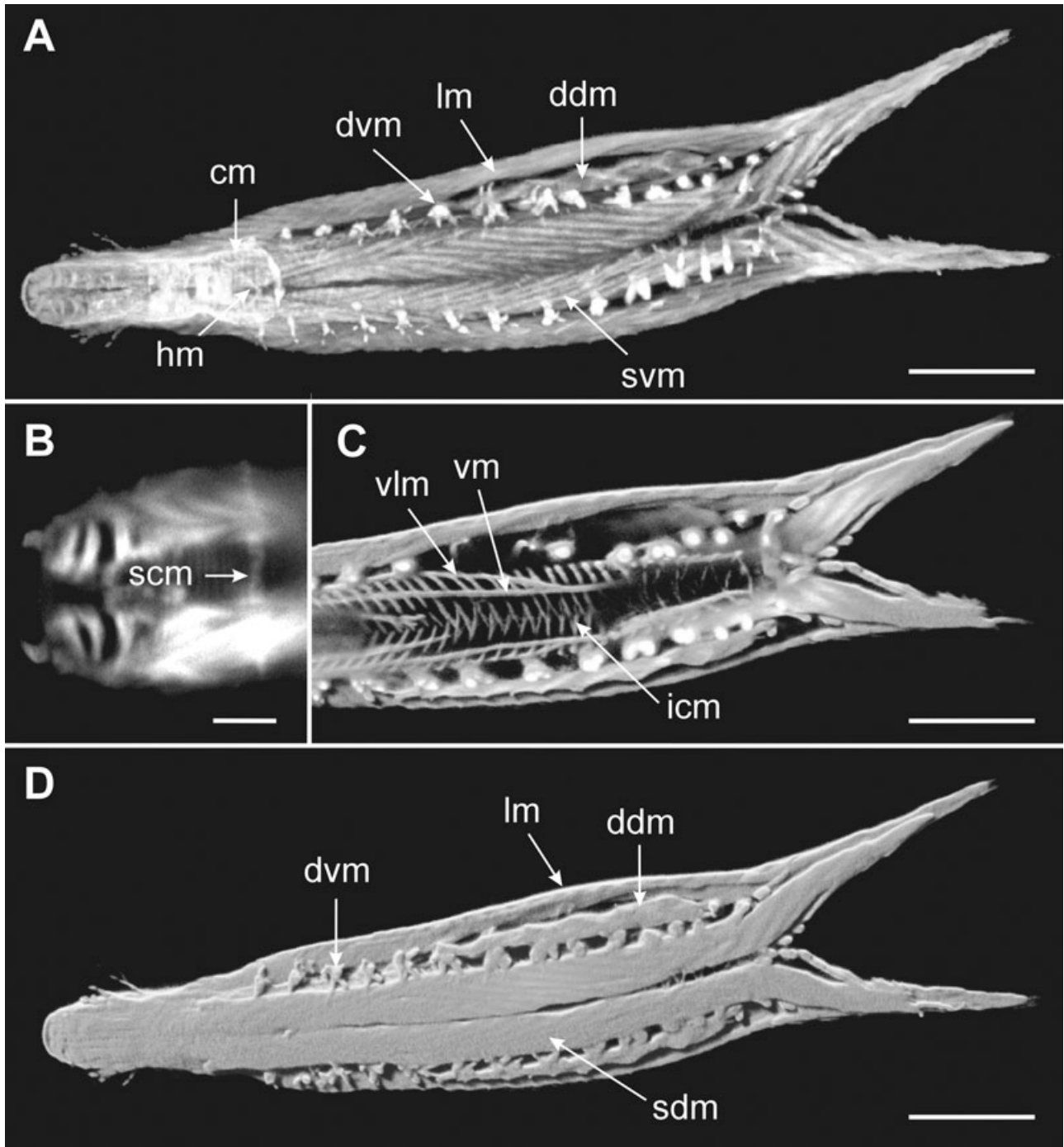
Figure 1. Musculature of *Musellifer delamarei* stained with TRITC–phalloidin: A, Z-projection of the whole mount displaying the general muscular arrangements from the ventral to dorsal view. B, close-up of the anterior end region showing the anterior semicircular band. C, internal region of the intestine displaying the incomplete circular muscles and the splanchnic ventral longitudinal bands (splanchnic dorsal longitudinal bands are not shown). D, SFPJ-projection of the whole mount in dorsal view, note the dorsoventral muscles. Scale bars: A–D, 20 μm. cm, circular muscles; ddm, dorsodermal muscles; dvm, dorsoventral muscles; hm, helicoidal muscles; icm, incomplete circular muscles; lm, lateral longitudinal muscles; scm, semicircular band; sdm, somatic dorsal longitudinal muscles; svm, somatic ventral longitudinal muscles; vlm, ventrolateral muscles; vm, ventral longitudinal muscles.

(somatic component). Two pairs were present in the ventral position (ventral: svm, 4 μm wide; ventrolateral: vlm, 2 μm) and two in the dorsal position (dorsal: sdm, 8 μm wide; dorsodermal: ddm, 4 μm wide). The pair of dorsodermal muscles, deriving from a branching of the dorsal longitudinal bands (at about U38), probably enclosed the eggs in the gravid specimens and merged again with the dorsal longitudinal bands at U68. Two muscles were present in the lateral position (lm; 5.5 μm wide). All the longitudinal muscles originated on the mouth rim and ended past the base of the furca (U75). Each muscle band, after merging with each other combined to form the furcal musculature (Fig. 1A, C, D). At the anterior end, the somatic longitudinal bands extended some thin lateral branching (0.5 μm wide) inserted on the basal lamina of the cuticle.

Helicoidal muscles: Two thin muscles (hm; 0.5 μm wide), helicoidally arranged, with respect to the longitudinal axis, surrounded only the splanchnic circular and longitudinal muscles. They crossed along the pharynx, only once in the ventral (U12) and once in the dorsal (U22) positions. No helicoidal muscles were present in the intestinal region (Fig. 1A).

Dorsoventral muscles: Dorsoventral muscles (dvm; 2.5 μm wide max) were only present in the somatic region from U20 and U70. Fourteen dorsoventral muscles were present on each side in all (Fig. 1A, C, D). At least three branches (0.5–1 μm wide) were present from the tip of each muscle. Each branch was inserted into the basal lamina of the cuticle, or maintained contact with the somatic longitudinal bands. Connections between the somatic dorsoventral and incomplete splanchnic circular muscles were not excluded. Some thinner dorsoventral muscles (5–8 muscles, 1 μm) were seen on the internal side of each furcal branch from U65 to U70 (Fig. 1C).

Other muscle arrangements: On the ventral side of the pharynx (U9), *M. delamarei* possessed a thin semicircular band (scm; 1 μm wide) as in most Macrodasyida (Fig. 1B).

Remarks: Based on transmission electron microscopy (TEM) observation, Ruppert (1991) indicated the muscles of *Musellifer sublittoralis* as being cross-striated; however, under cLSM, such a striation pattern, is not as clear in the musculature of *M. delamarei.* As, in other circumstances, cLSM has been able to vividly reveal cross-striation patterns (e.g. *Xenodasys eknomios*, Todaro *et al.*, 2006b), the failure in this case is probably because the muscles in *Musellifer* (true also for *Neodasys* and *Draculiciteria*) are atypically cross-striated (see Ruppert, 1991). In recognition of this in the phylogenetic data matrix, we scored the cross-striated pattern found in the muscles of some Chaetonotida and that typical of the Macrodasyida (e.g. *Dactylopodola*) differently.

The muscular system of A. paramediterraneus Remarks: The muscular system of *A. paramediterraneus*, in each component, did not appear to be different from that of *A. marinus* described by Hochberg & Litvaitis (2003).

The muscular system of Chaetonotus (Chaetonotus) *sp.* The muscular system was composed of only circular, helicoidal and longitudinal muscles.

Circular muscles: Circular muscles, internal to all the longitudinal bands, were present in the splanchnic position along the pharynx (cm; 0.5–0.8 μm wide; 35–40 rings). No circular muscles were present in the intestinal region (Fig. 2B).

Longitudinal muscles: Some splanchnic longitudinal muscles ran close to the digestive tube in ventral (vm; 1.5 μm wide), ventrolateral (vlm; 0.5 μm wide) and dorsal positions (dm; 1.5 μm wide). All these muscles were inserted anteriorly into the mouth rim, whereas posteriorly they merged with each other at the base of the furca, near the anal region. Two pairs of somatic longitudinal muscles were present in lateral (lm; 1 μm wide) and dorsodermal (ddm1; 1 μm wide) positions. Anteriorly, the lateral longitudinal muscles were inserted beside the mouth and posteriorly they were inserted behind the anus at the base of the furca. The dorsodermal muscles were seen as a branching of the dorsal longitudinal bands (U50) and they probably enclosed a region which contained the eggs. They merged again with the dorsal muscles at the base of the furca (U98). The dorsodermal muscles branched into thin and not easily visible longitudinal muscles (U60; 0.6 μm wide), both joining again at the base of the furca at U98.

Helicoidal muscles: Very thin helicoidal muscles (hm; 0.2 μm wide) wrapped around the digestive tract from the pharynx (U22) to the first half of intestine (U33), with two pharyngeal and three intestinal crosses (Fig. 2B).

Other muscle arrangements: At the base of the furca, a single muscular ring (am; 0.8–1 μm wide) was present; it is probably involved in the anal function (Fig. 2A). Posteriorly, a pair of dorsoventral muscles, like most other chaetonotidans, appeared to surround the ventral, ventrolateral and dorsal longitudinal muscles, but not the lateral and dorsodermal bands (dvm; 0.5 μm wide; Fig. 2A).
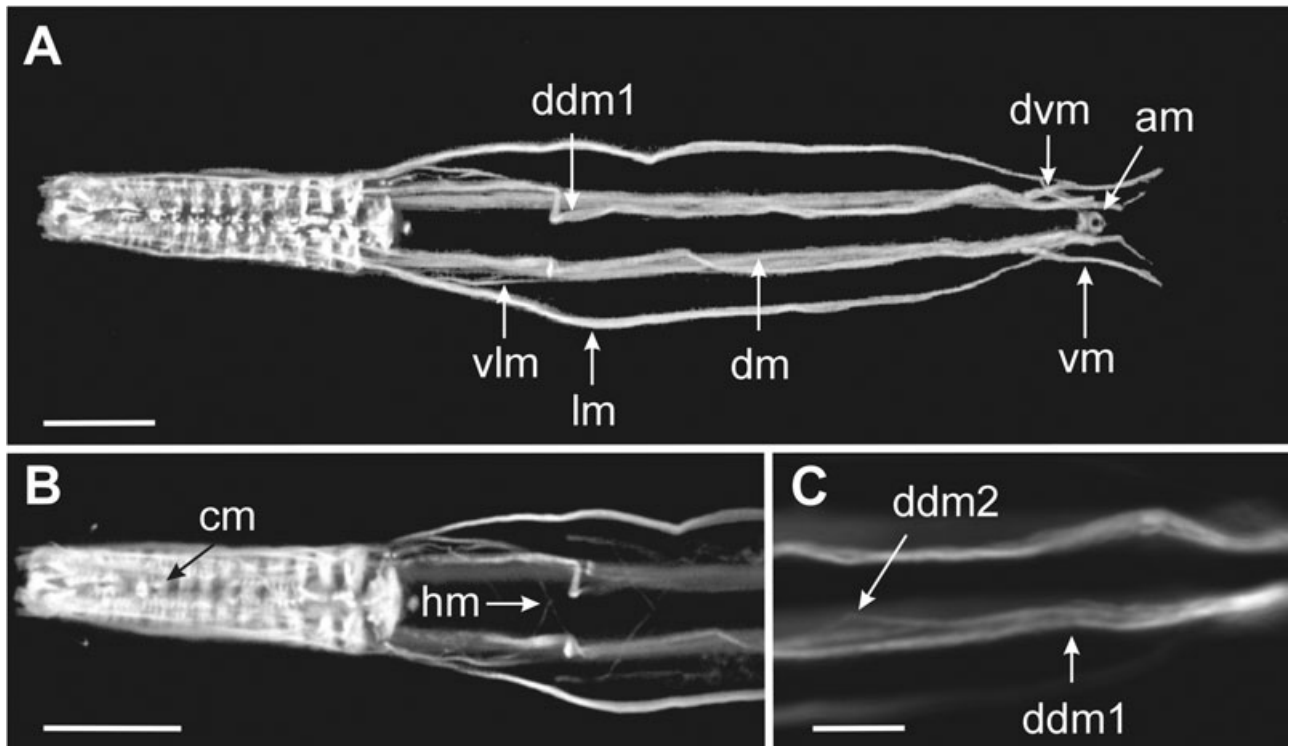
Figure 2. Musculature of *Chetonotus* (*Chaetonotus*) sp. stained with TRITC–phalloidin: A, Z-projection of the whole mount displaying the general muscular arrangements, B, anterior end. C, close-up of the dorsal longitudinal bands showing both of the dorsodermal muscles; note the absence of circular and dorsoventral muscles along the intestinal region. Scale bars: A, B, 20 μm; C, 10 μm. am, anal sphincter; cm, circular muscles; ddm1, dorsodermal muscles connected with dm; ddm2, dorsodermal muscles connected with dm1; dm, dorsal longitudinal muscles; dvm, posterior dorsoventral muscles; hm, helicoidal muscles; lm, lateral longitudinal muscles; vlm, ventrolateral muscles; vm, ventral longitudinal muscles.

Remarks: The muscular system of *Chaetonotus* (*Chaetonotus*) sp. in general mirrors that of other species of the same genus, as described by Hochberg & Litvaitis (2003), except for the absence of the muscle 'spikes' possessed by the other two species surveyed thus far. It should be emphasized, however, that this trait is unlikely to have phylogenetic significance as the presence/absence of these muscles has also been recorded among species of other genera. For instance, within *Lepidodermella*, 'spikes' have been observed in *Lepidodermella* sp.1 but not in *L. squamata* (Hochberg & Litvaitis, 2003).

The muscular system of Draculiciteria tesselata
Remarks: Survey under cLSM of the specimens from the Persian Gulf, confirming what was reported for the musculature of the North American specimens by Hochberg & Litvaitis (2003), allowed us to increase our knowledge of the muscular system of this species by adding some important information. In particular, in the Middle East specimens, the distal ends of the dorsodermal muscles appeared to be branched and,

most importantly, they possessed, like other Xenotrichulidae (see below), three additional dorsoventral bands in the proximity of and anterior to the pharyngeo–intestinal junction. Preliminary observations of some Mediterranean specimens indicated that the occurrence of these features can be generalized; therefore, in the data matrix we scored this trait accordingly.

The muscular system of Heteroxenotrichula squamosa
The muscles were present in circular, longitudinal, helicoidal and dorsoventral arrangements.

Circular muscles: Circular muscles (cm; 0.7 μm wide) were only present in the splanchnic position, as 20 individual rings overlying the wall of the pharynx below all the longitudinal bands (Fig. 3A).

Longitudinal muscles: Three pairs of splanchnic longitudinal muscles, flanking the entire intestine, ran in ventral (vm; 1.5 μm wide), ventromedial (vmm; 1 μm wide) and dorsal positions (dm; 0.8 μm wide).
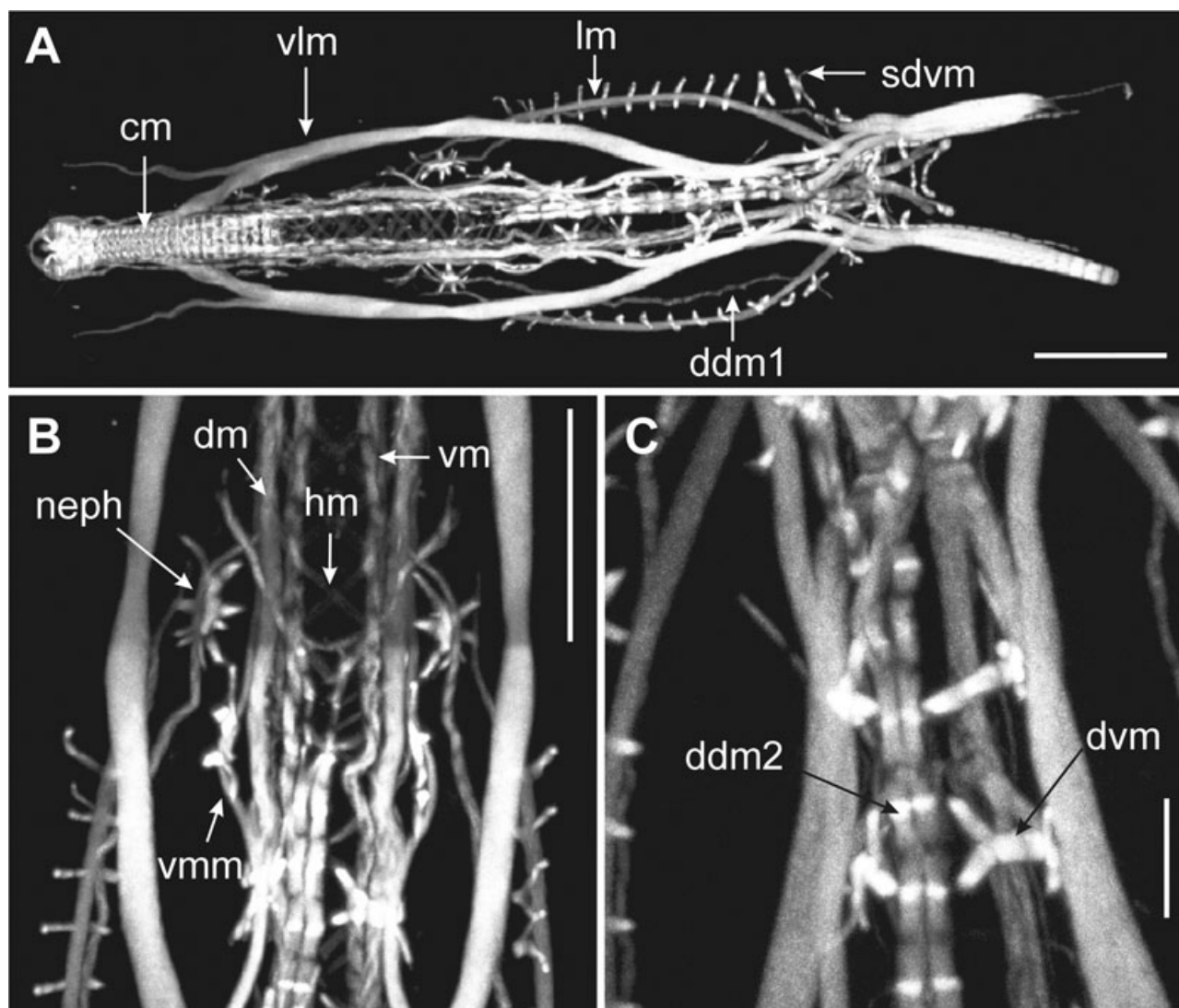
Figure 3. Musculature of *Heteroxenotrichula squamosa* stained with TRITC–phalloidin: A, Z-projection of the whole mount displaying the general muscular arrangements. B, close-up of the middle region; note the musculature associated with the nephrydial structures and somatic dorsoventral muscles. C, close-up of the splanchnic dorsoventral muscles. Scale bars: A, 20 μm; B, 10 μm; C, 5 μm. cm, circular muscles; ddm1, dorsodermal muscles connected with dm; ddm2, dorsodermal muscles connected with dm1; dm, dorsal longitudinal muscles; dvm, splanchnic dorsoventral muscles; hm, helicoidal muscles; lm, lateral longitudinal muscles; neph, nephrydial musculature; sdvm, somatic dorsoventral muscles; vlm, ventrolateral muscles; vm, ventral longitudinal muscles; vmm, ventromedial muscles.

Anteriorly, all these bands were inserted into the mouth rim and posteriorly they merged (U70) with each other near the anal region. More laterally, in the somatic position, four additional pairs of longitudinal bands were present; one pair in ventrolateral (vlm; 2.5 μm wide max) one in lateral (lm; 1 μm wide) and two pairs in dorsodermal positions. Anteriorly, the ventrolateral bands were inserted into the mouth rim, flanked the first half of the pharynx and expanded laterally along the body wall down to the anal region where they entered the furca. The two lateral bands were inserted laterally into the mouth rim, flanked the lateral body wall and posteriorly they entered the furcal branches. One pair of dorsodermal muscles (ddm1; 1 μm wide) originated from the dorsal longitudinal bands (U30), closely flanked the lateral muscle and merged again with the dorsal muscle posteriorly (U70). The second pair (ddm2; 1.8 μm wide) branched from the first dorsodermal fibre described above (U31) and flanked the dorsal body wall, merging with the dorsal longitudinal bands at U70 (Fig. 3A, B).

Helicoidal muscles: At least two pairs of muscles (hm; 1 µm wide), helicoidally arranged with respect to the longitudinal axis (50–55°), run from the pharynx (U10), to about halfway up the intestine (U50). Helicoidal muscles bounded only the ventral, ventromedial and dorsal longitudinal muscles with at least 10 dorsal and 10 ventral crosses (Fig. 3B).

Dorsoventral muscles: Dorsoventral muscles were present along the intestinal tract, close to the digestive tube (splanchnic position) and lateral to it (somatic position). In both positions, the muscles split into at least two extensions, each inserted on the basal lamina of the cuticle. In the splanchnic position, six bands (dvm; 1.5 µm wide) were present from U38 and U75. Ventrally, they extended between the ventral and ventrolateral longitudinal muscles, whereas dorsally they were external to the dorsal longitudinal bands. In the somatic position, *c.* 12 dorsoventral bands (sdvm; 0.4–0.7 µm wide) ran near to the lateral body wall. Ten bands (0.7 µm wide) followed one another (U38-U62), with insertion into the endocuticle between ventrolateral and lateral longitudinal bands, but nearest to the latter. The last two dorsoventral muscles (U64-U75; 0.7 µm wide) inserted externally to the lateral longitudinal bands, in the most lateral position. Two pairs of additional somatic dorsoventral muscles (0.4 µm wide) were present very close and anteriorly to the pharyngeo–intestinal junction (U26-U28); their extensions inserted laterally and near to the ventrolateral longitudinal bands. Posteriorly, three supplementary thin pairs of dorsoventral muscles (U80-U90 0.7–1 µm wide) were present in the internal region of the caudal furca (Fig. 3A–C).

Other muscle arrangements: At U35, two dorsoventrally orientated muscles were seen to be present; they extended into at least five branches in both ventral and dorsal positions (neph; 2.5–3 µm wide). Every branch was inserted into the basal lamina of the cuticle, external to the ventral and dorsal longitudinal muscles; these could be involved in nephridia functioning (Fig. 3B). At about U75, branching of the dorsal longitudinal muscles was seen; these branches ran to U80 where they are inserted into the base of the furca.

The muscular system of Xenotrichula intermedia
Muscular bands were present in circular, longitudinal, helicoidal and dorsoventral arrangements.

Circular muscles: Only in the splanchnic position, complete circular muscles (cm; 25–30 rings, 1 µm wide) surrounded the pharynx from the mouth to the pharyngeointestinal junction (U20). Along the intestinal tract, circular fibres (icm; 20 rings; 0.7 µm wide) were incomplete and opened onto the dorsal and ventral side. These latter fibres appeared to be branched at the end (Fig. 4A, B).

Longitudinal muscles: Longitudinal muscles were present in the splanchnic position, running close to the digestive tube and in the somatic position, lateral to the gut. In the splanchnic position, three pairs of muscular bands were present: one pair was present in a ventral position (vm; 2–2.5 µm wide), the two others in ventromedial (vmm; 1.5 µm wide) and dorsal positions (dm; 2 µm wide). Each band was inserted on the mouth rim, extended to the anal region (U70) and ended in the furca. The ventral longitudinal muscles parted at U50, creating a pore-like opening (about 2 µm wide), close to the region were the sperm are released.

Laterally, three additional pairs of longitudinal muscles were present: in ventrolateral (vlm; 2.5–3 µm wide), lateral (lm; 1 µm wide) and dorsodermal (ddm; 1 µm wide) arrangements. The ventrolateral longitudinal muscles, inserted anteriorly into the mouth rim, ran flanking the pharynx for half of its length (U15) and remain in proximity to the lateral body wall, reaching the maximum distance from the digestive tube at U50. In the anal region (U70) the two ventrolateral muscles were drawn together and merged with the ventral longitudinal muscles at the base of the furca. Laterally, the somatic longitudinal bands (vlm), inserted anteriorly into the endocuticle side by side with the mouth, run flanking the lateral body wall and merge with the musculature of the furca at U85.

The dorsodermal muscles, remaining close to the dorsal body wall and probably functioning to maintain the position of the eggs in gravid animals, began from each of the dorsal longitudinal muscles (U30) and joined (U80) with the musculature of the furca (Fig. 4A–C). At its anterior end, every longitudinal band extended at least a thin branch (0.5 µm wide) which was inserted into the endocuticle, laterally to the mouth.

Helicoidal muscles: Two pairs of thin muscles (hm; 0.5–0.8 µm wide), helicoidally arranged in opposite ways giving an angle of 50–55°, ran from approximately one-third of the pharynx (U10) to the first third of the intestine (*c.* U40). Helicoidal muscles surrounded the ventral, ventromedial and dorsal longitudinal bands. At least nine crosses on both the ventral and dorsal sides of the digestive tract (four crosses along the pharynx and five along the intestine, Fig. 4A) were present.

Dorsoventral muscles: Seven pairs of partial dorsoventrally arranged muscles (dvm; a maximum of 3 µm wide) were lateral to the intestine, in the somatic position, from U33 to U66. Each muscle, on each of its
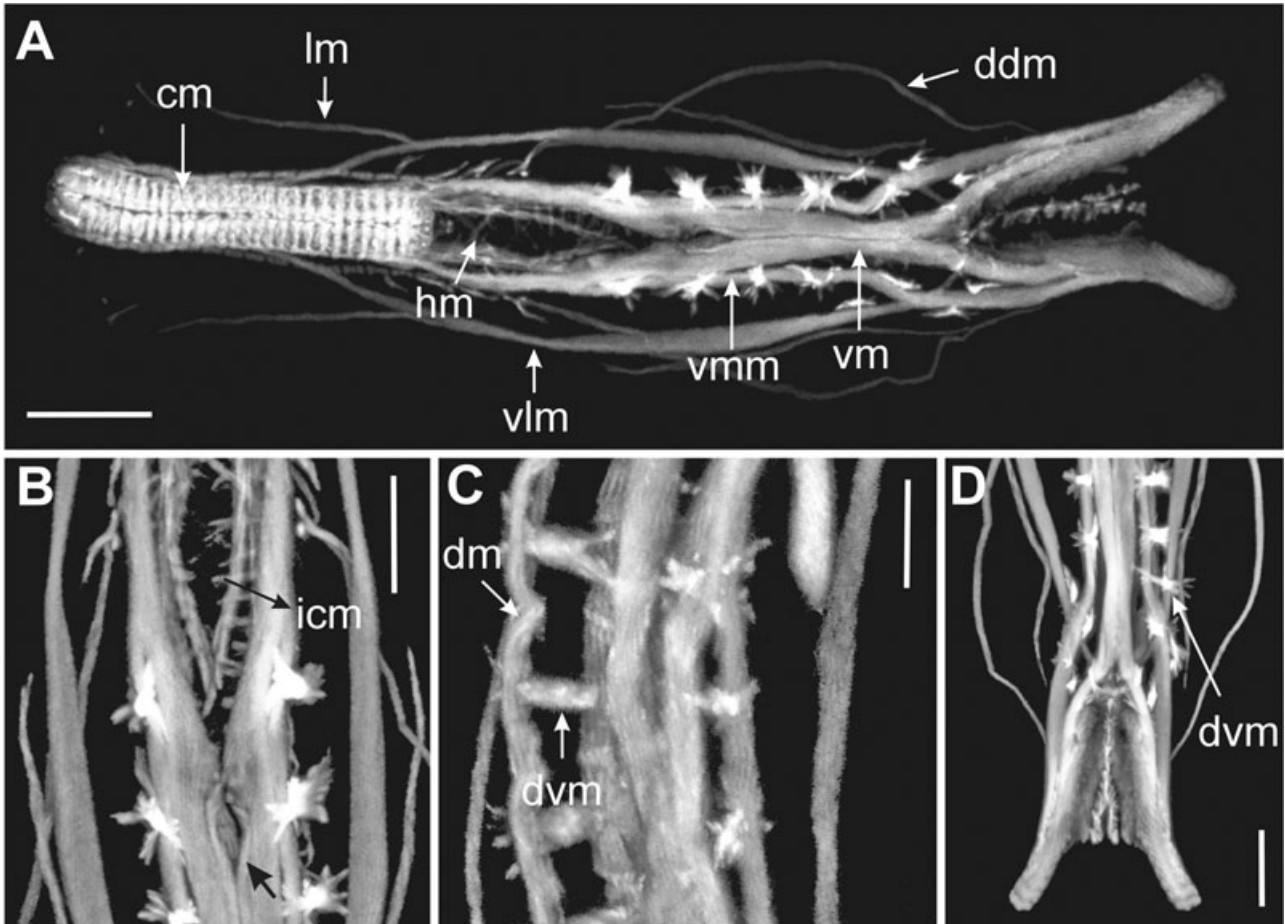
Figure 4. Musculature of *Xenotrichula intermedia* stained with TRITC–phalloidin: A, Z-projection of the whole mount displaying the general muscular arrangements. B, close-up of the middle region; note the internal incomplete circular muscles, the lateral dorsoventral musculature and the pore-like sexual male opening. C, close-up of the middle region, dorsoventral orientated. D, Z-projection of the posterior end displaying the dorsoventral muscles and the fibres associated with the furca. Scale bars: A, 20 μm; B–D, 10 μm. cm, circular muscles; ddm, dorsodermal muscles; dm, dorsal longitudinal muscles; dvm, dorsoventral muscles; hm, helicoidal muscles; icm, incomplete circular muscles; lm, lateral longitudinal muscles; vlm, ventrolateral longitudinal muscles; vm, ventral longitudinal muscles; vmm, ventromedial longitudinal muscles.

extremities, branched two to eight times on its dorsal and ventral aspect (each 0.3–0.5 μm wide) and was inserted into the basal lamina of the cuticle or joined with the nearest (but always internal) dorsal, ventromedial or ventrolateral longitudinal muscle.

Additionally, four different pairs of dorsoventral bands were present very close to the pharyngeo–intestinal junction, with the most anterior pair at the end of the pharynx (U19.5–U27; 1 μm wide; Fig. 4A–D). The thinnest dorsoventrally orientated muscles (five muscles; 0.5 μm wide) were present in the internal side of the furcal branches (U75-U95; Fig. 4C, D).

Other muscle arrangements: Posterior to the anal region, the dorsal longitudinal muscles extended two branches which were inserted into the endocuticle at the base of the furca (0.5 μm wide U75–U95).

Remarks: This survey of the *X. intermedia* muscular system under cLSM revealed traits that were not reported in previous observations of the species using wide-field epifluorescence (Hochberg & Litvaitis, 2003). The finding that the circular muscles in the splanchnic position along the intestine were open both dorsally and ventrally is particularly interesting, indicating that they should be considered as incomplete and not as complete circular muscles. Moreover, the somatic component indicated as incomplete by Hochberg & Litvaitis (2003) appeared clearly dorsoventral in orientation (although not completely inserted into the endocuticle) in our specimens. These discrepancies probably arise from the better resolution that can be achieved with confocal microscopy.

However, differences between specimens of the two investigated populations (Mediterranean vs.
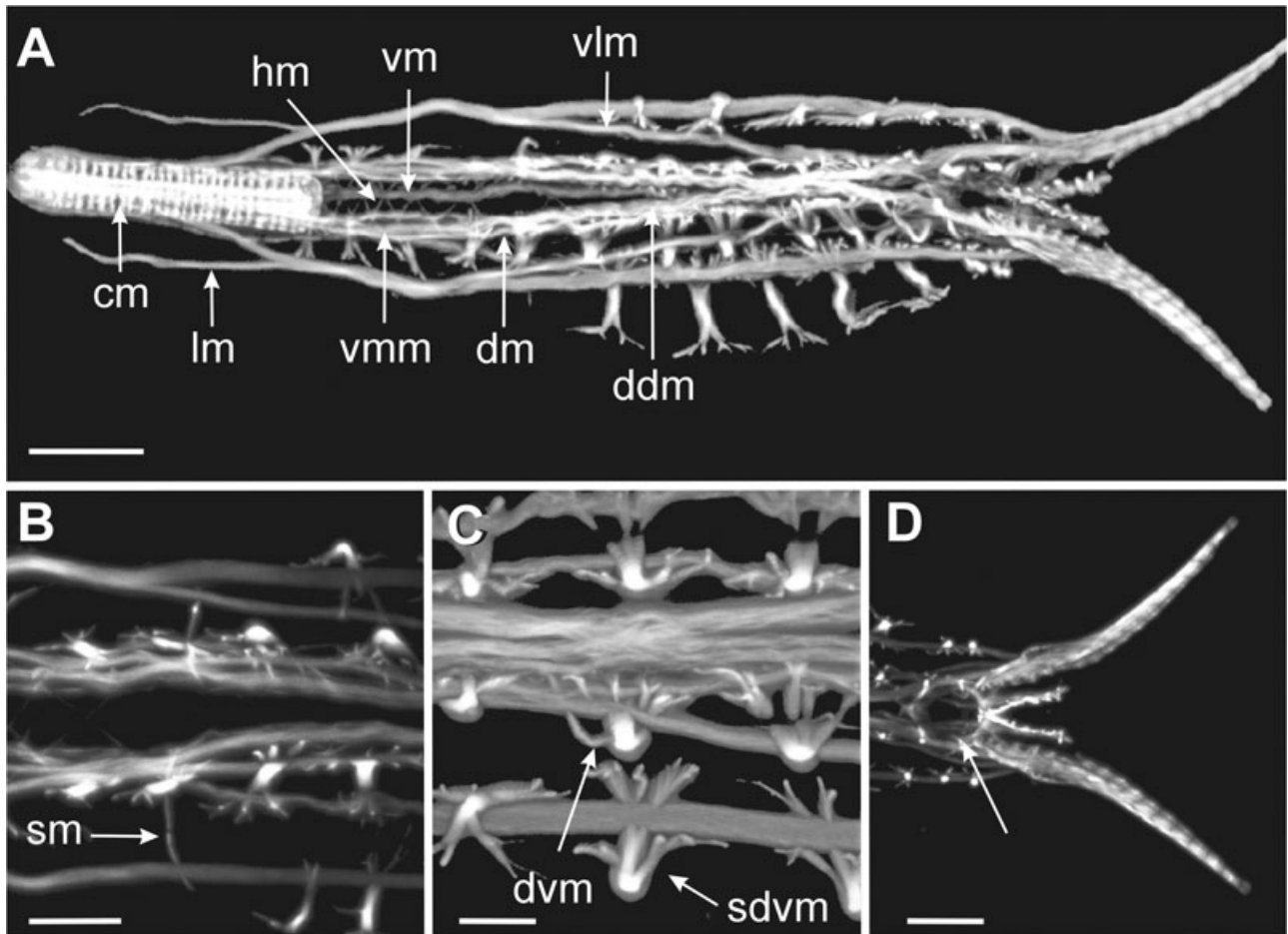
Figure 5. Musculature of *Xenotrichula punctata* stained with TRITC–phalloidin: A, Z-projection of the whole mount displaying the general muscular arrangements. B, close-up of the middle region displaying the dorsoventral muscles. C, close-up of the internal and lateral dorsoventral muscles. D, muscular fibres associated with the furca; note the pore in the anal region. Scale bars: A, 20 μm; B, D, 10 μm; C, 5 μm. cm, circular muscles; ddm, dorsodermal muscles; dm, dorsal longitudinal muscles; dvm, splanchnic dorsoventral muscles; hm, helicoidal muscles; lm, lateral longitudinal muscles; sdvm, somatic dorsoventral muscles; sm, musculature close to the sperm ducts converging; vlm, ventrolateral muscles; vm, ventral longitudinal muscles; vmm, ventromedial longitudinal muscles.

Western Atlantic) cannot be excluded. In this regard, it is worth mentioning that Todaro *et al.* (1996) found high genetic divergence between three geographically disjunct populations of the putative cosmopolitan *X. intermedia* and a hypothesis for granting each of them a different specific status was put forward. If further substantiated, recognition of morphological differences, at present undetected using the routine microscopy techniques in surveys of Gastrotricha (e.g. DIC and SEM), could strengthen the proposition.

The muscular system of Xenotrichula punctata
Muscular bands were present in circular, longitudinal, helicoidal and dorsoventral arrangements.

Circular muscles: Circular muscles (cm; 1 μm wide) were present only around the pharynx in the splanchnic position as 30 individual rings overlying the pharynx wall and internally to the longitudinal bands (Fig. 5A).

Longitudinal muscles: Six pairs of longitudinal muscles extended the length of the body, from the mouth to the posterior end of the caudal furca. The ventral (vm; 1.5 μm wide), ventromedial (vmm; 1.3 μm wide) and dorsal (dm; 0.8 μm wide) splanchnic longitudinal pairs were inserted anteriorly on the mouth rim and run to the posterior end close to the digestive tube. At the level of the anal region, the ventral muscles opened out slightly and they ended at

U75 in the caudal furca. The ventrolateral (vlm; 1.5 μm wide), lateral (lm; 2 μm wide) and dorsodermal (ddm; 1.3 μm wide) bands, ran out from the digestive tube. The pair of ventrolateral bands, inserted at the mouth rim, ran close to the pharynx wall for half of its length (U15) and opened out laterally, running close to the body wall. At the level of the base of the caudal furca, ventrolateral bands were again near to the digestive tube (U80) and were inserted inside the furcal branches. The somatic longitudinal bands (vlm) were inserted laterally to the mouth on the basal lamina of the cuticle and not run close to the digestive tube, whereas they flanked the lateral body wall, down to the caudal furca, sharing the other longitudinal bands.

Dorsodermal bands appeared as branches (U33) of the dorsal longitudinal muscles flanking the dorsal body wall, ending at U80 at the base of the furca where they merged with the dorsal longitudinal bands again. Dorsodermal bands may function in maintaining the position of the eggs (Fig. 5A).

Helicoidal muscles: Two pairs of thin fibres (hm; 0.3–0.5 μm wide), helicoidally arranged (50–55° with respect to the longitudinal axis), ran from about one-third of the pharynx length (U8) to halfway down the intestine (U50). They cross, dorsally and ventrally, at least five times along the pharynx and nine times in the intestinal region, externally to the longitudinal bands, with the exception of the lateral and dorsodermal muscles. No helicoidal muscles were seen from U37 to U43, where the sperm ducts converge (Fig. 5A).

Dorsoventral muscles: Partial dorsoventral muscles were present flanking the digestive tube (splanchnic position) and the lateral body wall (somatic position). In the splanchnic position, 10 dorsoventral bands (dvm; 2 μm wide U40-U80), branched into at least five extensions (each 0.4–0.7 μm wide). Some branching was inserted on the endocuticle (lateral to the dorsal and ventral longitudinal bands), while some joined with the dorsal, ventral and sometimes ventrolateral longitudinal muscles. Laterally, in the somatic position, eight dorsoventral muscles, flanking the lateral longitudinal muscles (1–2 μm wide U50-U80), were present on each side opposite the gut. All the lateral dorsoventral muscles had some myocyte extensions, which inserted on the endocuticle (laterally to the dorsal and ventrolateral longitudinal bands) or they joined the adjacent longitudinal bands (Fig. 5C).

Three additional pairs of muscles were present close to the pharyngeo–intestinal junction, external to the ventrolateral muscles but internal to the lateral longitudinal muscles (sdvm; 1 μm wide U25-U30).

Seven pairs of thinner dorsoventral muscles were present on the internal side of the caudal furcal branches. They started close behind the anal pore described below (0.5 μm wide; U80-U85). Four dorsoventral muscles (0.5 μm wide; U75-U80) encircled the anal pore.

Other muscle arrangements: Two thin muscle fibres (sm; 0.5 μm wide U40) extended from the lateral longitudinal muscle to the ventral longitudinal muscle on each side of the body (Fig. 5B). Their proximity to the site where the sperm ducts converge may have functional implications for reproduction. At the end of the intestine was a thin circular muscle (7 μm wide; U80) that may function as a sphincter around the anus. This muscle was surrounded by the four dorsoventral muscles described above (Fig. 5D).

PHYLOGENETIC ANALYSIS

The parsimony analysis of the 16 coded characters yielded a single most parsimonious tree (MPT, Fig. 6) of a length of 29 steps and the following index values: consistency index (CI) = 0.79, homoplasy index (HI) = 0.20, retention index (RI) = 0.85. maximum parsimony (MP) analysis found Paucitubulatina and Xenotrichulidae to be monophyletic, while Chaetonotidae was paraphyletic, as the chaetonotid *Musellifer* was found as the most basal genus within the suborder and the Xenotrichulidae sister to the remaining Chaetonotidae. The monophyly of the Chaetonotidae *sensu stricto* (i.e. all the Chaetonotidae except *Musellifer*) appeared extremely well supported (bootstrap = 99), with the indication of *Polymerurus* as the most basal taxon and a closer alliance between *Aspidiophorus* and *Chaetonotus*. Within Xenotrichulidae, the sister group relation between *Heteroxenotrichula* and *Draculiciteria* was found well supported. The position of *Neodasys* and the relationships between the two species of *Xenotrichula* are still unresolved.

DISCUSSION

GENERAL

The muscular system plays a crucial role in several aspects of the life of gastrotrichs; as these animals have a range of body shapes and sizes, occupy different habitats and display a variety of reproductive modalities, it is expected that the organization and extent of the musculature should reflect the evolutionary history of the different gastrotrich lineages. Consequently, a comparative study of muscular topography could provide insights for tracing the in-group phylogeny. Fluorescent phalloidin stains, in revealing a great variety of muscle organization that was not evident even at the ultrastructural level (e.g. Ruppert, 1991), proved to be extremely useful to obtain characters for exploring phylogenetic relation-
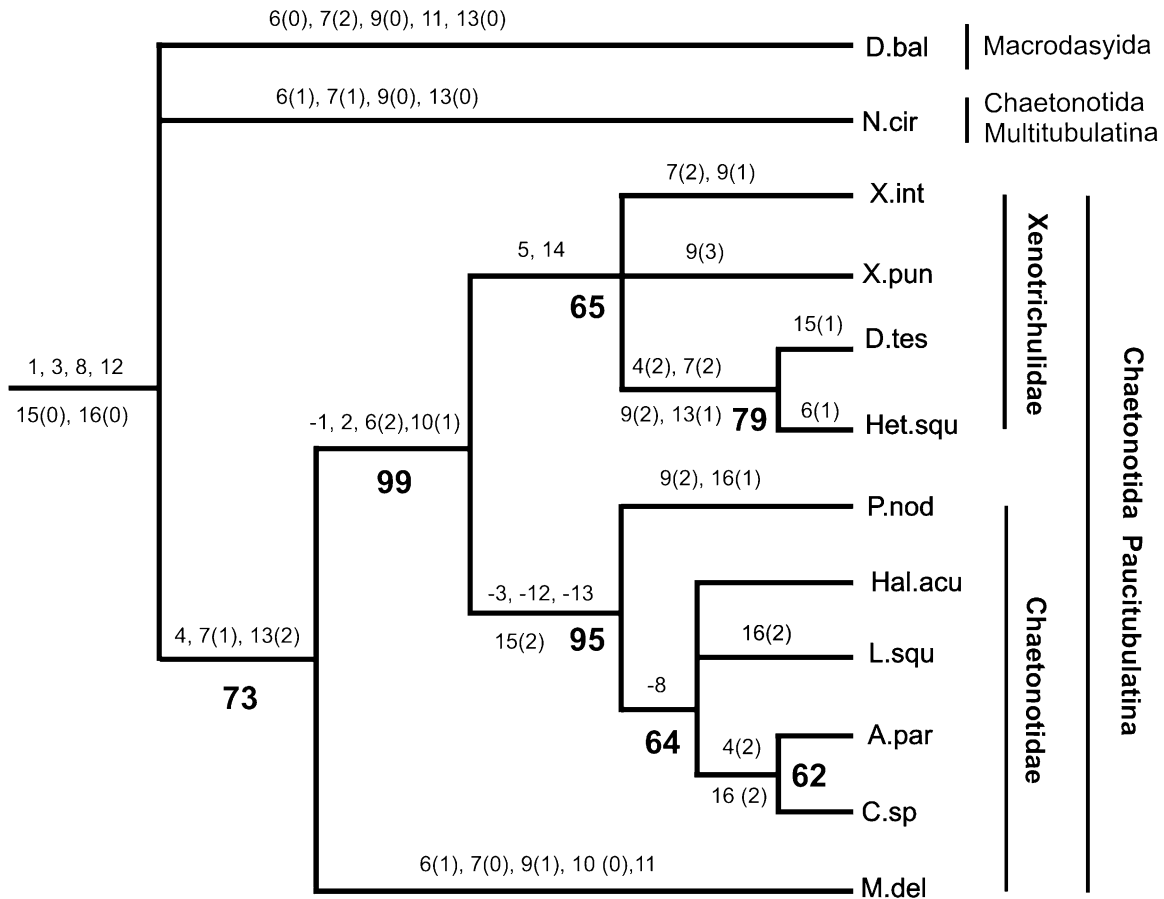
Figure 6. Single most parsimonious tree of 10 Paucitubulatina, one Multitubulatina and one Macrodasyida gastrotrichs based on 14 muscular and two bio-ecological characters. Values in bold represent bootstrap percentage based on 1000 replications (length 29 steps, consistency index 0.79, retention index 0.85); other numbers represent character states (see Table 2). A.par, *Aspidiophorus paramediterraneus*; C.sp, *Chaetonotus (Ch.) sp.*; D.bal, *Dactylopodola baltica*; D.tes, *Draculiciteria tesselata*; Hal.acu, *Halichaetonotus aculifer*; Het.squ, *Heteroxenotrichula squamosa*; L.squ, *Lepidodermella squamata*; M.del, *Musellifer delamarei*; N.cir, *Neodasys cirritus*; P.nod, *Polymerurus nodicaudus*; X.int, *Xenotrichula intermedia*; X.pun, *Xenotrichula punctata*.

ships. It is also because of the extensive use of such a technique that Hochberg (2005) could quite convincingly state that the plesiomorphic organization of the gastrotrich muscular system might likely have had the following layout: pharynx lined with splanchnic longitudinal muscles, circular muscles and helicoidal muscles, and midgut lined with inner longitudinal muscles, enwrapped by splanchnic circular and helicoidal muscles. The lateral region of the body contained the ventrolateral longitudinal muscle blocks enclosed by somatic circular muscles. In overcoming the restrictions of conventional epifluorescence microscopy such as limitations of the visual field, photo bleaching and low resolution along the Z axis, cLSM is perhaps the best method for surveying the musculature of whole-mount specimens. Consequently, cLSM should be regarded as the ultimate technique for corroborating previous records, while

detecting other possible departures from the basal muscle organization to be tested for phylogenetic significance.

Our survey confirms some of the previous information while bringing new light to the organization of the gastrotrich muscular system. For example, we confirm that the muscles are arranged as a series of bands along the digestive tract and are closely aligned with the other organ systems of the body. The bands are generally arranged in three orientations: circular, longitudinal and helicoidal, although in some taxa dorsoventral muscles are also present and other minor muscle bundles characterized a number of other species. However, our observations show that some muscular bands believed to be complete circular muscles are in fact incomplete (see below).

From functional and evolutionary perspectives, it is useful to comparatively discuss the present findings,

together with observations on other chaetonotidan taxa (e.g. *Neodasys*, Hochberg, 2005; *Polymerurus*, Leasi *et al*., 2006a) and a basal macrodasyidan such as *D. baltica* (Hochberg & Litviatis 2001d), in search of the evolutionary transformations of specific muscle groups, to uncover characters that may form potential synapomorphies for selected clades. In agreement with Hochberg & Litvaitis (2001d), it seems appropriate to compare patterns in two distinct body regions as follows.

COMPARATIVE MUSCLE PATTERNS ALONG THE PHARYNGEAL REGION

The general arrangement of muscles on the pharynx does not vary much among the 12 species studied. Circular muscle rings are abundant and surround the pharynx from its tip to the pharyngeo–intestinal junction, with no apparent differences among the surveyed species.

Longitudinal bands lie to the outside of the splanchnic circular muscles. In the splanchnic position, all the gastrotrichs possess longitudinal bands in dorsal, ventrolateral and ventral arrangements; in the somatic position, the bands are only lateral. *M. delamarei,* in contrast with the other species of Paucitubulatina, possesses additional somatic longitudinal bands in dorsal, ventrolateral and ventral positions. In general, longitudinal muscles are inserted at the anterior end on the mouth rim; however, lateral or ventrolateral bands may be inserted beside the mouth, as in most gastrotrich species, or behind the mouth at the site of the anterior adhesive tubes, as in *Dactylopodola.*

Helicoidal muscles in left- and right-hand spirals, surround the circular and longitudinal muscles of the pharynx. The number of helices may differ among species (e.g. one pair in *Musellifer* vs. two pairs in *Heteroxenotrichula*).

Dactylopodola baltica and *M. delamarei* are the only taxa possessing a semicircular band in the anterior ventral side of the pharynx, while *D. tesselata* is the only gastrotrich possessing dorsoventral muscles in a somatic position in the pharyngeal region. All the species of Xenotrichulidae possess three extraordinary bands of dorsoventral muscles near the pharyngeo–intestinal junction. These muscles lie between the 'usual' splanchnic and somatic components of the dorsoventral muscles and are quite evident in *X. intermedia* and *X. punctata*, but are not easily discernible in *D. tesselata* and *H. squamosa*.

COMPARATIVE MUSCLES PATTERNS ALONG THE INTESTINAL REGION

Among the 12 surveyed species, circular muscles are found in the intestinal region in only four taxa, *D. baltica*, *Neodasys cirritus*, *X. intermedia* and *M. delamarei*. All four species bear circular muscles in the splanchnic position, surrounding the intestine from the pharyngeo–intestinal junction to the anus. In *X. intermedia* and *M. delamarei*, the circular muscles appear as incomplete hoop-like rings; further, in *X. intermedia* these muscles are branched at the tips. In the somatic position, the circular muscles are present only in *Dactylopodola* and *Neodasys*.

Longitudinal muscles in the intestinal region appear to be continuations of those on the pharynx. All gastrotrichs, with the exception of species of Chaetonotidae, have longitudinal muscles extending past the base of the furca or the caudal lobes. However, only in species of Xenotrichulidae are two branches of dorsal longitudinal muscles present that are inserted at the base of the furca.

Helicoidal muscles are present in the intestinal region of all the gastrotrichs except *Musellifer*, which possesses helicoidal muscles terminating at the pharyngeo–intestinal junction.

Dorsoventral muscles exist in all species of Xenotrichulidae, in *M. delamarei* and in *Polymerurus nodicaudus*. In the splanchnic position these muscles were present in *D. tesselata, H. squamosa*, *X. punctata* and among the Chaetonotidae in *P. nodicaudus*. All these muscles are branched at the tips; while in most species all the resulting branches insert on the cuticles, in *X. punctata* some of them join with the nearby longitudinal muscles; consequently, in this species this musculature should be considered as partially dorsoventral.

In the somatic position, all species of Xenotrichulidae and *M. delamarei* possess dorsoventral muscles, all having branched tips. In *Musellifer* and in the two species of *Xenotrichula* these muscles are partially dorsoventral, as not all of the branches insert into the endocuticule; in fact, some elements connect with the adjacent longitudinal muscular bands and in *Musellifer* and *X. intermedia* also connect with the splanchnic component of the longitudinal and circular muscles.

PHYLOGENY

The parsimony analysis of the 12 species and 16 coded characters yielded a single most parsimonious tree (MPT, Fig. 6); the fairly high consistency index value obtained (CI = 0.79), reveals good congruence among the muscular characters, thus reflecting the rather homogeneous muscular architecture observed among the Gastrotricha. There is little homoplasy in the muscle characters, as revealed by the high retention and consistency indexes.

MP analysis found Paucitubulatina and Xenotrichulidae to be monophyletic, while Chaetonotidae is

paraphyletic relative to Xenotrichulidae because of the separate and more basal position of the chaetonotid *M. delamarei*. Tree topology showing the monophyletic Paucitubulatina with *Musellifer* as the most basal taxon also resulted from previous comprehensive analyses based on morphological or molecular characters (Hochberg & Litvaitis, 2000; Todaro *et al.*, 2006c). While monophyly of Paucitubulatina has never been questioned, a basal position of *Musellifer* along this evolutionary branch of the Gastrotricha conflicts with the current systematization. The muscular architecture of *M. delamarei* indicates a deeper divergence of this taxon from the other chaetonotids (but also from the Xenotrichulidae) and provides, together with the presence of a functional hermaphroditic sexual apparatus and the primitiveness of its habitat (i.e. solely marine), elements for a systematic revision of the current Chaetonotidae, i.e. calling for the establishment of a new family, the Muselliferidae fam. nov. to include *Musellifer* and its likely sister taxon, the recently described *Diuronotus* Todaro, Balsamo & Kristensen, 2005. A diagnosis of the new family is provided below, along with an amended diagnosis of Chaetonotidae.

Previous analysis based on morphology also yielded a monophyletic Xenotrichulidae (Hochberg & Litvaitis, 2000), an evolutionary scenario not supported by the study based on molecules, which showed *Draculiciteria* nested instead within a subset of Chaetonotidae (Todaro *et al.*, 2006c). It should be pointed out, however, that such a result may be as a result of contaminated sequences or taxonomic misidentification of deposited sequences (see Todaro *et al.*, 2003, 2006c). The well-supported sister group relation between *Heteroxenotrichula* and *Draculiciteria* based on the analysis of the muscular characters seems to support this hypothesis.

The present analysis found Xenotrichulidae to be sister to the remaining Chaetonotidae; moreover, the monophyly of the Chaetonotidae *sensu stricto* (i.e. all the Chaetonotidae except *Musellifer*) appeared to be extremely well supported (bootstrap value = 96), with the indication of *Polymerurus* as the most basal taxon (bootstrap value = 62; Fig. 6).

Apart from the disputable phylogenetic position of *Draculiciteria* (see above), a sister group relationship between Xenotrichulidae and Chaetonotidae appears to be supported by the analysis based on comparisons of the nucleotide sequences of the 18S rDNA genes (Todaro *et al.*, 2006c). In contrast, the comprehensive morphological analysis of Hochberg & Litvaitis (2000) found the monophyletic Xenotrichulidae only allied with some of the paraphyletic Chaetonotidae (*Chaetonotus, Aspidiophorus* and *Halichaetonotus* among others); however, the low bootstrap values (< 50%) at the nodes involved and the disagreement with the

current systematization left little confidence on the latter results (e.g. paraphyly of the Chaetonotidae *s.s.*). Current systematization is compatible with sister group relationships between Xenotrichulidae and Chaetonoidae *s.s.* and even more so in the revision we are proposing.

A basal position of *Polymerurus* within the Chaetonotidae *s.s.* as resolved by the present study contrasts with the previous hypothesis of Kisielewski (1991). In an attempt to trace evolutionary pathways within Chaetonotidae using the structure and organization of the body cuticular covering, Kisielewski came to the conclusion that a cuticular armature made up of keelless, spined scales and consistency between the dorsal and ventral sides should be considered as the plesiomorphic condition. Consequently, he indicated that the then newly established freshwater genus *Lepidochaetus*, possessing a plesiomorphic body cuticle, represents the ancestral line from which other Chaetonotidae, such as *Polymerurus, Lepidodermella* and *Chaetonotus,* arose.

While there is no doubt that the type and organization of the body cuticle bears a special place within the evolutionary history of gastrotrichs, it may be paradoxically difficult from this trait alone to obtain a strong, reliable phylogenetic signal given its vast diversity at both gross anatomical and microscopical levels (Rieger & Rieger, 1977; Ruppert, 1991), which surely embeds homoplastic phenomena. In any case, a basal position of *Polymerurus* within the Chaetonotidae *s.s.* is compatible with an evolutionary scenario inferred from the structure of the cuticle (i.e. plesiomorphic dorsal and ventral body cuticle), but receives additional support from the presence of dorsoventral muscles (see Leasi *et al.*, 2006b). The basal position of *Polymerurus* within the Chaetonotidae therefore argues for a more derived position of *Lepidochaetus*; in fact, recent preliminary observations (F. Leasi & M.A. Todaro, unpubl. data) of *L. zelinkai* (Grünspan, 1908) reveal an absence of dorsoventral muscles, similar to the condition for derived members of Chaetonotidae.

ORIGIN AND EVOLUTION OF THE DORSOVENTRAL MUSCLES

From our survey of the muscular system of seven species of Chaetonotida, the circular and dorsoventral elements appear to exhibit the widest variety of conditions; furthermore, it seems that within the Chaetonotida, the diversification of these muscles in the trunk region is tightly bound to the natural history paths followed by these animals, in space and time (see below).

It has been hypothesized that the somatic circular muscles formed via lateral branches of splanchnic

circular muscles and the dorsoventral muscles found in some Chaetonotida are evolutionary derivatives of complete circular bands (Ruppert, 1975; Teuchert & Lappe, 1980); with regard to the latter point, Hochberg & Litvaitis (2001c, 2003) went even further, envisioning an evolutionary decoupling between the splanchnic and the somatic components. Our study, demonstrating that dorsoventral muscles are not exclusive to the Xenotrichulidae as previously thought (e.g. Ruppert, 1979; Hochberg & Litvaitis, 2003; but see Leasi et al., 2006b), supports this vision while reinforcing it. Figure 7 summarizes the

hypothetical evolutionary scenario of the circular–dorsoventral musculature obtained by visually mapping information on these muscles on the more comprehensive phylogenetic tree.

In this evolutionary framework, we hypothesize that the dorsoventral muscles originated via the dorsal and ventral openings of the complete circular muscles combined with a gradual reduction and their progressive ventral and dorsal insertion into the cuticle. The occurrence of muscles in orientations such as complete circular, incomplete circular, incomplete-dorsoventral and dorsoventral is testi-
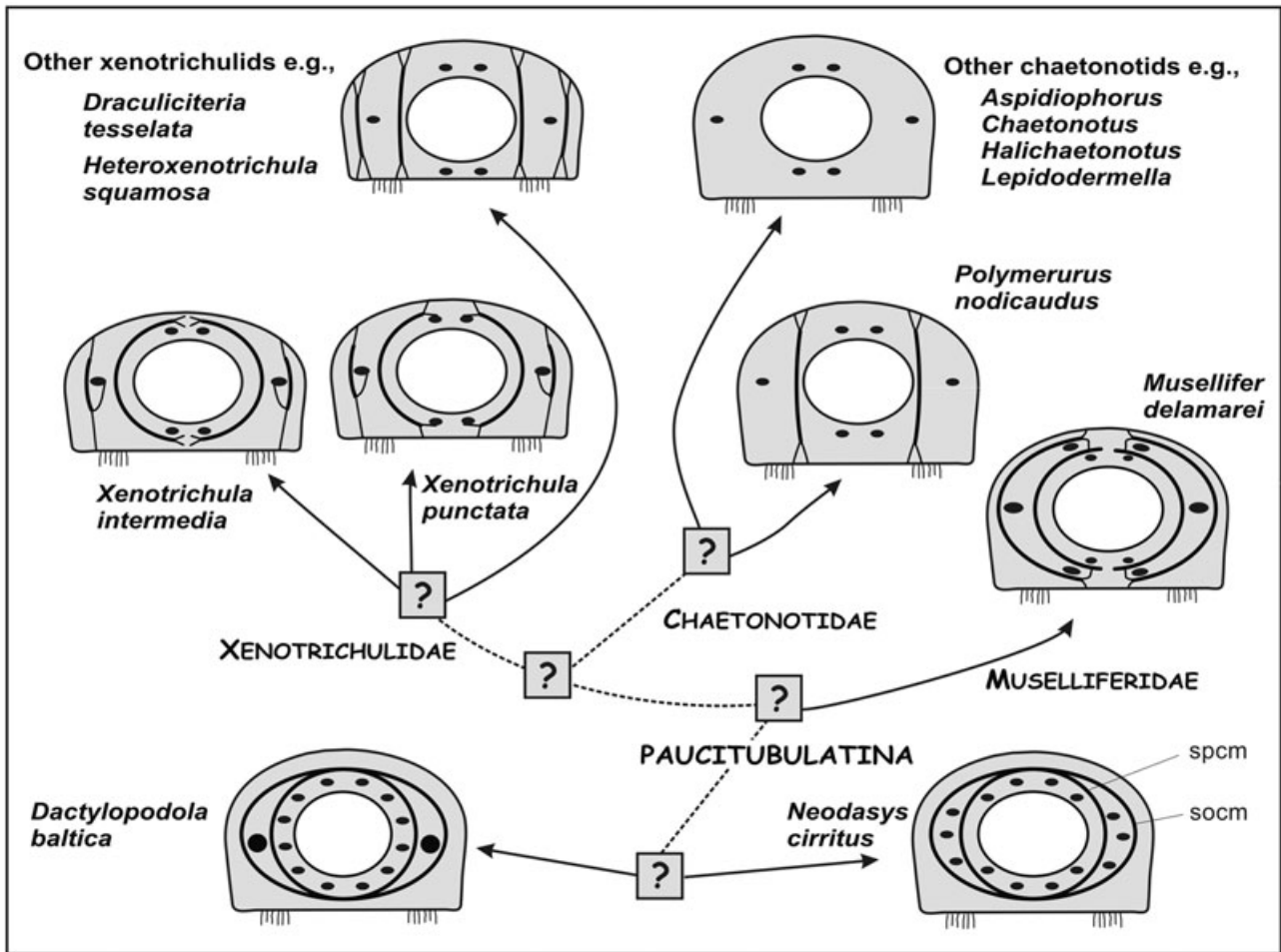

Figure 7. Schematic representation of the hypothetical evolutionary sequence of changes in the orientation of circular muscles of the intestinal region, seen in cross-section. Dark circles represent longitudinal muscles. In this framework, species of Macrodasyida (*Dactylopodola*) and the Chaetonotida Multitubulatina (*Neodasys*) bear the plesiomorphic condition represented by complete circular muscles. In the early Chaetonotida Paucitubulatina, the muscular rings began to open up both dorsally and ventrally; members of the Muselliferidae fam. nov. in the splanchnic position bear evidence of the earliest evolutionary steps (i.e. incomplete circular muscles in *Musellifer*). Further evolution of these muscles sees their gradual reduction and a progressive ventral and dorsal insertion into the cuticle. While in the marine, hermaphroditic Xenotrichulidae these muscles are retained and the shape modified for interstitial life and sexual reproduction, in the Chaetonotidae, they are eventually lost as these gastrotrichs experienced a switch to an epibenthic–periphytic life and adopted parthenogenesis during the invasion of the fresh water ecosystems. socm, somatic circular muscles; spcm, splanchnic circular muscles.

mony to this transformation process (Fig. 6). Furthermore, this process evidently took place early in chaetonotidan evolution and is variable among the major lineages (Fig. 7). For instance, while the absence among our records of the putative first evolutionary step into the somatic position (i.e. none of the investigated taxa possess incomplete somatic circular muscles) may indicate that the process started earlier in this position (perhaps prior to the first Paucitubulatina branching), our data seem to indicate that the rate of evolutionary change has been much faster in somatic than in splanchnic muscles, as in most cases the somatic muscles coexists with the splanchnic muscles in an earlier evolutionary condition.

While the adaptive nature of the evolutionary changes that involved these muscles remains largely uncertain, the phylogenetic framework put forward by our study may provide some new insights. Ruppert (1975, 1979) and Hochberg & Litvaitis (2001c, 2003) hypothesized that the main function of the dorsoventral muscles is to compress the body in the dorsoventral plane and antagonize longitudinal muscle contraction, facilitating defecation and expulsion of parthenogenetic eggs through the body wall. Leasi *et al.* (2006b), noticing that dorsoventral muscles are found mostly in the very flat, hermaphroditic taxa that live in the sand (i.e. Xenotrichulidae), but are missing in the parthenogenetic flask-shaped epibenthic–periphytic gastrotrichs, such as species of Chaetonotidae *s.s.*, indicated a primarily adaptive role for these muscles in interstitial life and sperm transfer. Indeed, the presence of the first evolutionary steps of the dorsoventral muscles in *M. delamarei,* along with the absence of any muscular sexual accessory organs in the hermaphroditic taxa, give support to the hypothesis that these muscles originated in marine interstitial hermaphroditic gastrotrichs and were reduced and finally lost during changes in lifestyle (interstitial vs. epibenthic–periphytic) and reproduction modality (hermaphroditic vs. parthenogenetic) that took place with the invasion of the fresh water environment.

DIAGNOSES

Chaetonotidae Gosse, 1864 (emended)
Paucitubulatina having body 60–770 μm long. Head rounded, furnished dorso-frontally with cephalion, laterally with one or two pairs of pleurae and ventrally with hypostomion. Head tentacles lacking but small cephalic papillae occasionally present. Body ends in form of caudal furca that bears a pair of terminal adhesive tubes; adhesive tubes occasionally absent (*Undula*). Cephalic ciliature in the form of one or two paired lateral tufts, or, occasionally, a paired undu-

lated transverse row (*Undula*). Ventral locomotory ciliature consists of a paired band of cilia extending along the whole body and being parallel to the body axis. Ciliary bands tend occasionally to be interrupted. Dorsal sensory bristles in the number of 1–3 (usually 2) pairs, the rearmost ones set on scales with double keel or (rarely) double spine. Body covered, at least partly, with simple scales, spined scales, pedunculated scales or scales bearing lamellae, or, occasionally, with a layer of soft and uniform cuticle. Ventral area between ciliary bands covered with simple, spined or pedunculated scales, or naked; however, at least a pair of perianal scales always present. Oral opening terminal or subterminal, in the form of a ring that consists of simple or articulated rods. Four to five pairs of longitudial muscles; helicoidal muscles extending past the pharyngeo–intestinal junction; circular muscles restricted to the pharyngeal region; dorsoventral muscles occasionally present, in splanchnic position, along the intestinal region (*Polymerurus*). Parthenogenic, usually with hermaphroditic (functional?) phase at the end of lifespan. Eggs maturing dorsally. Freshwater, marine and brackishwater. Benthic, periphytic and occasionally semipelagic. Two subfamilies. Chaetonotinae Kisielewski, 1991 (type subfamily) and Undulinae Kisielewski, 1991. Chaetonotinae include 10 genera: *Chaetonotus* Ehrenberg, 1830 (type–genus), *Arenotus* Kisielewski, 1987, *Aspidiophorus*, Voigt, 1903, *Fluxiderma* d'Hondt, 1974, *Heterolepidoderma* Remane, 1926, *Ichthydium* Ehrenberg, 1830, *Lepidochaetus* Kisielewski, 1991; *Lepidodermella* Blake, 1933, *Polymerurus* Remane, 1926, *Rhomballichthys* Schwank, 1990. Undulinae includes a single genus: *Undula* Kisielewski, 1991 (type–genus).

Muselliferidae fam. nov.
Paucitubulatina having body 173–533 μm long. Head weakly marked, drawn out into a muzzle surrounded by a ciliary band; cephalion, pleurae and hypostomion absent; head tentacles lacking. Body ends in form of caudal furca that bears a pair of terminal adhesive tubes (*Musellifer*) or two pairs (*Diuronotus*) of adhesive tubes. Cephalic ciliature in the form of a ciliary area which covers the muzzle, occasionally (*Diuronotus*) scattered sensory cilia of widely varying length are present. Ventral locomotory ciliature forms a continuous field from the posterior edge of the mouth to the second third of the pharynx then splitting into two thin bands that extend two-thirds of the length of the trunk region. Dorsal sensory bristles absent. Body completely covered with spined scales (*Musellifer*) or keeled scales (*Diuronotus*) with no differences between dorsal and ventral sides. Oral opening terminal or subterminal, in the form of a ring strengthened by teeth-like cuticular ridges. Eight pairs of longitudinal muscles; helicoidal muscles ending at the

pharyngeo–intestinal region; circular muscles present in the pharyngeal region and limited to the splanchnic component in the intestinal region; dorsoventral muscles present along the intestinal region in the somatic position (*Musellifer*). Hermaphrodite with paired, round to sac-like testes, lateral to the intestine; thread-like sperm; eggs maturing dorsally. Marine, infaunal or interstitial. Two genera: *Musellifer* Hummon, 1969 (type-genus) and *Diuronotus* Todaro, Balsamo & Kristensen, 2005.

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