ORIGINAL PAPER

Meiofaunal cryptic species revealed by confocal microscopy: the case of *Xenotrichula intermedia* (Gastrotricha)

Francesca Leasi · M. Antonio Todaro

Abstract The phylum Gastrotricha includes about 750 species of meiobenthic marine and freshwater species that are often widely distributed. The microscopic size, short life cycle, low motility of adults, and the absence of larval stages normally required for dispersal raise doubts about the putative cosmopolitan distribution of many of gastrotrich species. The phenomenon of cosmopolitanism is acknowledged for all major meiobenthic taxa ("the meiofauna paradox") and can be explained, at least in part, with the existence of sibling species, so far identified primarily by molecular analysis. In this paper, we report the discovery of sibling species in the marine chaetonotidan *Xenotrichula intermedia* using confocal laser scanning microscopy (CLSM). A total of 40 specimens collected from two geographically separate populations, the Mediterranean (Adriatic Sea) and the Arabian Gulf (Kuwait), were investigated. Fifteen specimens of each population were studied in vivo with a contrast interference microscope (DIC) in order to obtain the main morphometric parameters; ten other animals (five in each population) were fixed and marked with fluorescent phalloidin for the observation of their muscular systems under CLSM. The metrics and meristic data of the two populations fall within the range of measures recognized for the species. Pairwise comparisons (*t*-test) in general did not reveal statistically significant differences between the traits of specimens belonging to the two populations; moreover, multivariate analyses (cluster- and MDS analysis) were unable to separate clearly the Mediterranean from the Arabian specimens. In contrast, an examination of their muscular systems revealed clear dissimilarities between the two geographic groups. In particular, while the Italian specimens possessed incomplete circular bands and dorsoventrally orientated muscles that are partially inserted into the basal lamina of the cuticle, in both splanchnic- and somatic positions, their Arabian counterparts showed partial dorsoventral bands and complete circular muscles that surround the entire body of the animal in the same corresponding positions. Additional differences in the position of helicoidal bands (present in a more anterior location in the Italian specimens) are noted between Mediterranean and Arabian specimens. Since the investigated animals share a similar interstitial habitat, a working hypothesis connects the differences in the musculature to possible differences in the reproductive modality of specimens between the two populations.

Introduction

The phylum Gastrotricha contains meiobenthic marine and freshwater micrometazoans (0.06–3 mm in body length) as part of the Lophotrochoza clade (molecular data, see Todaro et al. 2006b; Dunn et al. 2008) or as sister taxon to the Ecdysozoa (mostly morphological data, see Zrzavý 2003). The phylum consists of two orders: (1) Macrodasyida, with mostly marine, hermaphroditic species, and (2) Chaetonotida, with marine- and freshwater species that may be hermaphroditic and/or parthenogenetic. Until now, about 750 species of gastrotrichs, which includes 280 species of Macrodasyida and 470 species of Chaetonotida, have been described from a wide range of regional areas

F. Leasi (✉)
Department of Biology, University of Milan, Milan, Italy
e-mail: francesca.leasi@unimi.it

M. A. Todaro
Department of Animal Biology,
University of Modena and Reggio Emilia, Modena, Italy

and habitats (Hummon 2007; Priyalakshmi et al. 2007; Todaro et al. 2009); however, many areas and biotopes the world over still remain unexplored. Consequently, our knowledge of the biodiversity, zoogeography, and evolution of these organisms is far from complete. For example, gastrotrichs are poorly known from the coastline of the Arabian Gulf, but our recent surveys revealed the presence of the cosmopolitan morphotype, *Xenotrichula intermedia* Remane, 1934 (Todaro et al. 2007).

Interstitial species of Chaetonotida (Xenotrichulidae) are known from northern Europe, across the Mediterranean Sea, the Atlantic- and Gulf coasts of USA, India, and Somalia (Todaro et al. 1996). According to Sterrer (1973), species whose presence is reported in two or more oceans, including connected seas, can be defined as cosmopolitan. However, because of shared life-history traits such as a short life cycle, a relatively low number of offspring, the general absence of a pelagic larval stage, and a relatively limited swimming ability of the adults, the meiofaunal species (particularly the interstitial forms) would be expected to have restricted geographical ranges; their "cosmopolitan" distribution came to be known as the "meiofauna paradox" (Giere 1993). Explanations to the contrary were proposed to substantiate such a wide distribution: vicariance associated with the Gondwana distribution (Sterrer 1973) or different dispersal mechanisms (Gerlach 1977).

Actually, the crux of the debate over the presumed cosmopolitan distribution of meiofaunal taxa concerns species identification. Since the beginning of the 19th century, microinvertebrate taxa were recognized through morphological data. However, critics have questioned the reliability of the identifications of species from geographically distant areas, especially when made by different investigators using different methods (often low-resolution microscopy) and, probably, their personal "instincts" to affiliate specimens with a given taxon. Further, the number of specimens investigated is often too low to allow meaningful comparisons within and among populations. Notwithstanding these considerations, some careful morphological analyses have shown composite assemblages of different species within certain taxa to subsist over a presumed wide geographic range (Huys 1992; Evans 1994; see also Hummon and Todaro 2007). On the other hand, further investigations have confirmed that cosmopolitanism appears to be a widespread phenomenon among some meiofaunal groups (Hummon 1994; Westheide et al. 2003; Fenchel and Finlay 2004; Todaro and Rocha 2004).

Since the advent of molecular taxonomy, there is increasing evidence that many small aquatic and marine invertebrates—once believed to be single, cosmopolitan species—are actually complexes of cryptic species (e.g. Westheide and Schmidt 2003 on polychaetes; Casu and Curini-Galletti 2004, 2006 on flatworms; Gomez et al. 2004

on harpacticoids; Suatoni et al. 2006 on rotifers; Baker and Giribet 2007, Baker et al. 2007 on Cycliophora). The apparently cosmopolitan gastrotrich *X. intermedia* has been investigated as the molecular level as well. Todaro and co-workers have shown that despite an identical external morphology, populations of *X. intermedia* from the Western Atlantic, the Gulf of Mexico, and the Mediterranean Sea are characterized by different mitochondrial haplotypes with a genetic divergence among populations up to as much as 11%, thereby casting doubts about the taxon's monophyly (Todaro et al. 1996). Based on results reported above, it seems that when species complexes are difficult or impossible to differentiate morphologically, species recognition has to be based on molecular markers (Westheide et al. 2003; Gomez et al. 2004; Baker et al. 2007). Ultimately, molecular analysis would seem the most authoritative technique for the identification of meiofaunal sibling species (e.g. Rocha-Olivarez et al. 2001; Casu and Curini-Galletti 2004; Suatoni et al. 2006).

However, it has been shown that the application of high-resolution microscopy techniques may reveal additional, taxon specific-, morphological characteristics that have so far escaped recognition by traditional light microscopy. For instance, the application of fluorescently labelled molecules to whole mount specimens prepared for confocal laser scanning microscopy (CLSM) have revealed the complete muscular organization of many microinvertebrates (Hochberg and Gurbuz 2007; Ruchel and Müller 2007; Leasi and Ricci 2008), providing important information on the evolutionary relationships among taxa (Leasi et al. 2006; Todaro et al. 2006a; Leasi and Todaro 2008). As the general muscular arrangement appears to be a conservative characteristic (i.e. taxon specific), differences in muscles organization may result in useful approaches to discriminate between species (Hochberg and Litvaitis 2001a, b; Leasi et al. 2006).

The muscular system of species of Xenotrichulidae consists of band-like myofibres in circular-, longitudinal-, helicoidal-, and dorsoventral arrangements present in splanchnic- (i.e. surrounding the alimentary canal) and somatic positions (Ruppert 1991; Hochberg and Litvaitis 2003). Circular- and dorsoventral elements appear to exhibit the widest variety of conditions and it seems that, within Gastrotricha, at the ordinal level, the diversification of these muscles in the trunk region is believed to be linked to the ecology and reproductive modalities of the different taxa (Leasi et al. 2006; Leasi and Todaro 2008).

So far, the muscular system of *X. intermedia* has been described in two populations—one from the East coast of the USA and the other from the Adriatic coast of Italy—using, respectively, wide-field epifluorescence microscopy (Hochberg and Litvaitis 2003) and confocal laser scanning microscopy (Leasi and Todaro 2008). Some slight differences

in the muscular organization of the two populations have been highlighted by Leasi and Todaro (2008). While the differences noted, particularly those regarding the organization of the circular- and dorsoventral muscles, have been ascribed to the different resolutions existing between the two microscopic techniques, the possibility that the discrepancies between the populations could be real was not completely ruled out.

The potential existence of sibling species in *X. intermedia*, first detected via molecular analysis (Todaro et al. 1996), and later, partially substantiated via fluorescence analysis of muscular characters, caused us to widen our investigation of this cryptic species complex. In a larger framework, morphological traits, even if detectable by non-conventional microscopic techniques (e.g. CLSM) are by far less time consuming and less expensive to record and use for taxonomic purposes than molecular markers; moreover, they can also offer tangible confirmation of molecular results that recommend for division of the species.

Here, we focus on two putative populations of *X. intermedia*, one from the Mediterranean Sea and the other from the Arabian Gulf, of which we compare some morphometric traits easily detectable using conventional light microscopy (i.e. DIC) as well some morphological characteristics that can be studied through non-conventional microscopy. In the case we present, we concentrate on the muscular system observed with CLSM.

Materials and methods

The Mediterranean population was sampled on 20/03/2007 from the intertidal zone of Petacciato Marina, Campobasso, Italy (Adriatic Sea: 42°02′16.47″N; 14°51′02.08″E) whereas the Arabian population was collected on 22/04/2007 from the intertidal zone of the southeast coast of Falaika Island, Kuwait (Arabian Gulf: 29°23′38.58″N; 48°24′07.37″E). After collection, the samples were stored at 20°C and brought as soon as possible to the laboratory in Modena, Italy. Gastrotrichs were extracted daily from the sediments using the narcotization-decantation technique and prepared for analysis within 10 days from the day of field collection (Todaro and Hummon 2008).

Measurements of 16 main body traits were obtained from 15 living-, relaxed adult specimens from each population. To this end, individual specimens were transferred singly by glass micropipette to a slide and examined, and observations recorded under a Nikon Eclips 90i microscope equipped with DIC (Nomarski) optics and a DS-5Mc Nikon digital camera; morphometric data were obtained directly or derived from photographs using the UCT-2U Nikon software.

Comparisons between the two populations were performed using both univariate- (*t*-test, Sigma Stat v. 3.11,

Systat software, Inc.) and multivariate analyses (Cluster Analysis and MDS, Primer 6 v. 6.1.6, Primer-E, Ltd).

For the comparison of the muscular systems, five relaxed specimens of each population were incubated on ice overnight in freshly made 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4); subsequently, they were washed several times with 0.1 M PBS and permeabilized for 1 h in a pre-incubation solution containing 0.2% Triton X-100, 0.25% bovine serum albumin and 0.05% NaN_3 in 0.1 M PBS followed by incubation in tetramethylrhodamine B isothiocyanate (TRITC)–phalloidin (Sigma) (2 μL of a 38-μL solution in 50 μL preincubation solution) for 1 h. The specimens were then rinsed in PBS and embedded in Citifluor (Plano, Wetzlar) on microscopic slides and surveyed using a Leica DM IRE 2 confocal laser scanning microscope. A series of optical sections were projected in one maximum-projection (MPJ) image or visualized as a simulated fluorescence projection (SFPJ) for a three-dimensional image. The positions of the muscles are expressed with reference to percentage body units; total body = 100 units (U).

The names of the muscles follow the format presented by Hochberg and Litvaitis (2003) with the following exceptions reported also in Leasi and Todaro (2008): the term "Rückenhautmuskel" (Zelinka 1889) is replaced with dorsodermal muscle; dorsoventral muscles are muscles inserted completely into the endocuticle (Ruppert 1991); partial dorsoventral muscles are muscles that show only some fibres inserted into the endocuticle.

Results

Metric and meristic traits

Fully mature (hermaphroditic) specimens of *X. intermedia* ranged from 192.6 to 204.0 μm in total body length (Figs. 1a, b, 2a; Table 1). The smallest value was found in the Arabian population, and the largest value in the Italian animals. The length of the pharynx varied from 47.2 to 59.3 μm, and both lengths were found in the Arabian population. The length of the furcal appendages varied from 30.6 to 37.1 μm with the extremes noted in the Kuwait and Italian specimens, respectively. The shortest (11.3 μm) adhesive tubes were found in the Italian population while the longest (15.4 μm) in the Arabian specimens (Table 1). Regardless of the population, the cuticular coverings of the specimens consisted of typical pedunculated scales, arranged on the dorsal side in 15–21 columns containing 45–50 scales each. In both populations, the inner margin of the furca was always covered with five scales (Table 1).

Pairwise comparisons (*t*-test) performed on each of the above characteristics, as well as on nine additional

Fig. 1 *Xenotrichula intermedia*; specimens from the Italian population. **a** Habitus, dorsal view. **b** Habitus, ventral view. **c** Z-projection of the whole mount displaying the general muscular arrangements. **d** Close-up of the middle region; note the splanchnic incomplete circular muscles and the somatic dorsoventrally orientated musculature. **e** Close-up of the dorsoventral muscles in lateral view. *Cm* circular muscles, *ddm* dorsodermal muscles, *dm* dorsal longitudinal muscles, *dvm* splanchnic dorsoventral muscles, *f* furca, *hm* helicoidal muscles, *icm* incomplete circular muscles, *lm* lateral longitudinal muscles, *p* pharynx, *vlm* ventro-lateral longitudinal muscles, *vm* ventral longitudinal muscles, *vmm* ventromedial longitudinal muscles. **a**, **b** DIC optics, **c–e** CLSM. *Scale bars* **a–c** 50 μm, **d**, **e** 25 μm
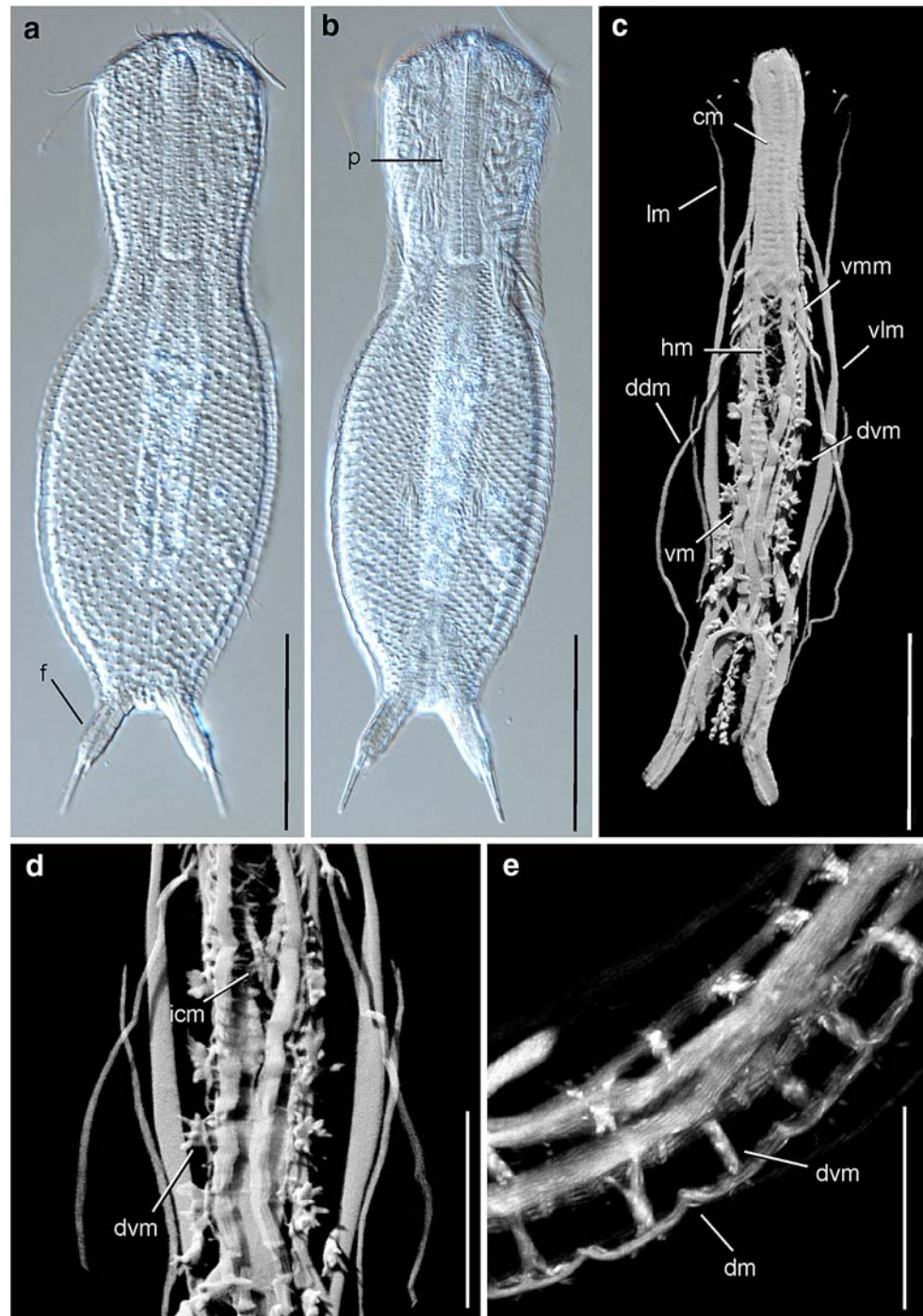

morphometric parameters, did not yield significant differences among the specimens of the two populations, except for the number of dorsal columns of scales and the number of scales in the dorsal median column. The number of dorsal columns of scales was significantly lower ($P < 0.001$) in the Kuwaiti specimens (mean = 15.6) than in the Italian worms (mean value = 19.6 μm). Furthermore, the number of scales in the dorsal median column was significantly lower in the Arabian population (mean value = 48.0) than in the Mediterranean counterpart (mean value = 51.0).

The multivariate analysis (Cluster Analysis and Multi-Dimensional Scaling) carried out could not readily distinguish individual specimens from the two different locations (Bray-Curtis similarity >95%; Fig. 3).

Muscular system of *X. intermedia* (Italian population)

All the five specimens showed identical muscular architecture. For the purpose of this paper, a short account of the arrangement of the muscles is given below; for more

Fig. 2 *Xenotrichula intermedia*; specimens from the Arabian population. **a** Habitus, ventral view. **b** Z-projection of the whole mount displaying the general muscular arrangements. **c** Close-up of the middle region; note the splanchnic dorsoventral- and the somatic complete circular musculature. *Cm* circular muscles, *ddm* dorsodermal muscles, *dvm* splanchnic dorsoventral muscles, *f* furca, *hm* helicoidal muscles, *lm* lateral longitudinal muscles, *p* pharynx, *scm* somatic circular muscles, *vlm* ventrolateral longitudinal muscles, *vm* ventral longitudinal muscles. **a** DIC optics, **b**, **c** CLSM. *Scale bars* **a**, **b** 50 μm, **d** 25 μm
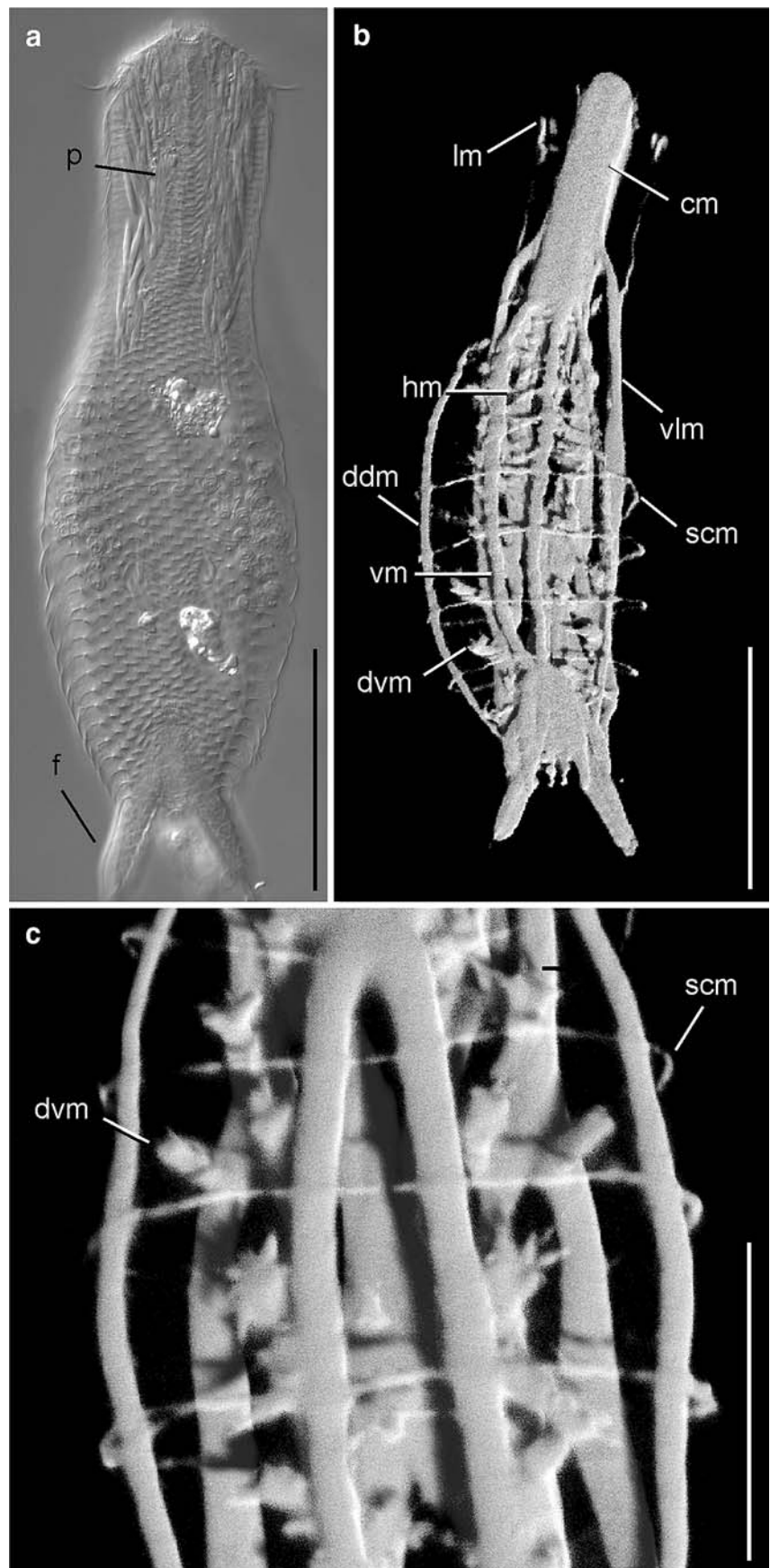
Table 1 Summary statistics for 16 morphometric variables mean ± 1SD range in two populations of *Xenotrichula intermedia* (measurements in μm; *N* = 15 for each collection)

Variable	Italy	Kuwait
	Mean ± 1SD (range)	Mean ± 1SD (range)
Total body length	201.8 ± 3.4 (200.2–204.0)	198.2 ± 1.8 (192.6–201.9)
Pharynx length	55.7 ± 4.6 (54.9–57.7)	53.5 ± 1.2 (47.2–59.3)
Furca length	35.3 ± 3.4 (33.0–37.1)	33.90 ± 1.8 (30.6–35.0)
Adhesive tube length	13.1 ± 1.4 (11.3–14.9)	13.5 ± 1.4 (11.5–15.4)
Pharynx anterior diameter	9.7 ± 1.1 (8.2–11.0)	8.3 ± 1.0 (6.4–9.4)
Pharynx middle diameter	7.6 ± 1.2 (6.0–8.2)	7.6 ± 0.9 (5.8–9.2)
Pharynx posterior diameter	10.0 ± 1.2 (8.6–10.6)	9.3 ± 0.7 (7.5–10.6)
Head width	37.9 ± 2.7 (35.3–41.4)	35.5 ± 2.4 (30.6–36.0)
Neck width	29.7 ± 3.4 (27.3–33.0)	29.1 ± 2.4 (23.4–32.7)
Trunk width	50.3 ± 2.6 (48.4–51.1)	49.6 ± 1.1 (45.2–52.3)
Base furca width	23.9 ± 0.6 (22.2–24.8)	22.3 ± 1.0 (21.5–23.0)
Anus distance from indentation between furcal branches	23.1 ± 1.6 (19.8–27.5)	22.3 ± 3.0 (19.6–24.0)
Mouth diameter	4.3 ± 0.6 (3.9–4.5)	4.3 ± 0.1 (3.3–4.9)
No. of dorsal columns of scales[a]	19.6 ± 0.8 (19.0–21.0)	15.6 ± 0.8 (15.0–17.0)
No. of scales in dorsal median column[a]	51.0 ± 2.1 (50.0–53.0)	48.0 ± 1.2 (45.0–50.0)
No. of scales covering inner margin furca	5.0 ± 0.0 (5.0–5.0)	5.0 ± 0.0 (5.0–5.0)

[a] Differences between populations statistically significant at *P* < 0.05 (*t*-test)

detailed information on the muscles system of the Mediterranean populations of *X. intermedia* see Leasi and Todaro (2008).

Muscular bands were present in circular-, longitudinal-, helicoidal- and dorsoventral arrangements.

Circular muscles

Complete splanchnic circular muscles (cm; 1 μm wide) surrounded the pharynx from the mouth to the pharyngeo-intestinal junction (U20). Along the intestinal tract, the circular fibres (1 cm; 0.7 μm wide) were incomplete and opened onto the dorsal- and ventral sides (Fig. 1c, d). Somatic circular muscles were not present.

Longitudinal muscles

Longitudinal muscles were present in splanchnic and somatic positions. In the splanchnic position, three pairs of muscular bands were present: one pair in the ventral position (vm; 2–2.5 μm wide), and two others in the ventromedial- (vmm; 1.5 μm wide) and dorsal positions (dm; 2 μm wide). Each band inserted into the edge of the mouth, extended to the anal region (U70) and ended in the furca (Fig. 1c, d, e). 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 began from each of the dorsal longitudinal muscles (U30) and joined (U80) with the musculature of the furca.

Helicoidal muscles

Two pairs of thin muscles (hm; 0.5–0.8 μm wide), helicoidally arranged in opposite directions, with an angle of 50–55°, extended from approximately the anterior one-third of the pharynx (U10) to the first-third of the intestine (ca U40; Fig. 1c). Helicoidal muscles surrounded the ventral, ventromedial, and dorsal longitudinal bands.

Dorsoventral muscles

Seven pairs of partial dorsoventral muscles (dvm; a maximum of 3 μm) were positioned lateral to the intestine, in the somatic position, from U33 to U66 (Fig. 1c, d, e). Each muscle, on each of its 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 dorsal, ventromedial, or ventrolateral longitudinal muscle.
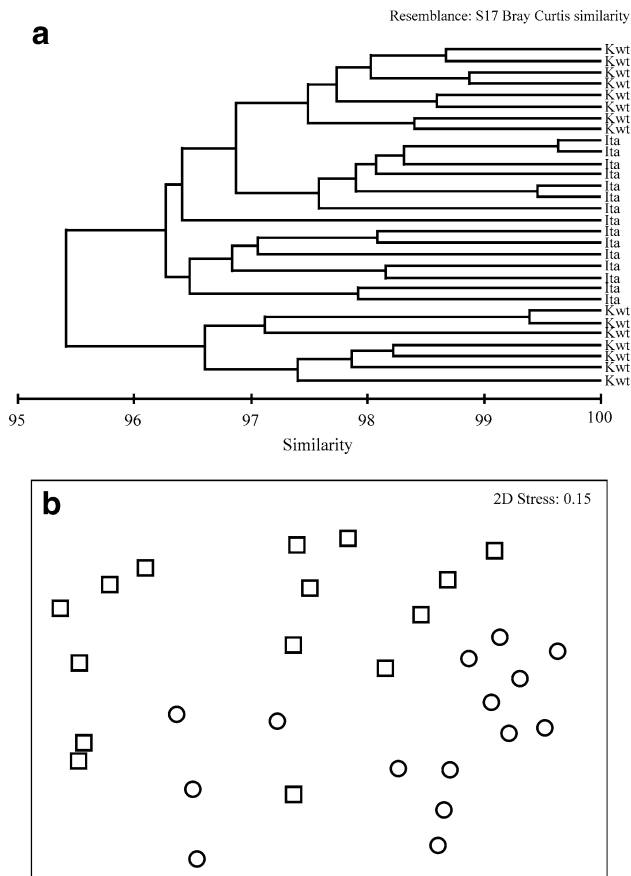
Fig. 3 Outcome of two multivariate analyses. **a** Dendrogram obtained from a group average hierarchical cluster analysis on 16 morphometric traits of 30 adult *Xenotrichula intermedia* spp from Italy (ITA) and Kuwait (KWT). Notice the overall high similarity among specimens. **b** Ordination of 30 adult *Xenotrichula intermedia* spp from Italy (*circles*) and Kuwait (*squares*) by MDS analysis based on Bray-Curtis similarity of 16 morphometric traits. Notice the inability of the analysis to discriminate clearly between the two populations

Other muscle arrangements

Posterior of the anal region, the dorsal longitudinal muscles extended two branches that inserted into the endocuticle at the base of the furca (0.5 μm wide U75–U95; Fig. 1c).

Muscular system of *X. intermedia* (Arabian population)

All the observed specimens showed identical muscular architecture. Muscular bands were present in circular-, longitudinal-, helicoidal- and dorsoventral arrangements.

Circular muscles

Complete circular muscles were present in splanchnic- and somatic positions. In the splanchnic position, circular fibres (cm; 25–30 rings, 1 μm wide) surrounded the pharynx from U10 to U30, that is, from a position slightly posterior to the

mouth to the pharyngeo-intestinal junction (Fig. 2b). Furthermore, in the somatic position, complete circular muscles (scm; 4 rings, 0.5 μm wide) surrounded all the body muscle from about half the intestine, U60, to the anus, U85 (Fig. 2b, c).

Longitudinal muscles

Longitudinal muscles were present in the splanchnic position, extending close to the digestive tube, and in the somatic position, laterally to the gut. In the splanchnic position, three pairs of muscular bands were present: one pair in the ventral position (vm; 2.5 μm wide), the two additional pairs in the ventromedial- (3 μm wide) and dorsal positions (2.5 μm wide), respectively. Each band, inserted at the mouth rim, extended to the anal region (U80), and terminated in the furca (Fig. 2b). Laterally, three additional pairs of longitudinal muscles were present in ventrolateral- (vlm; 3.5 μm wide), lateral- (lm; 1 μm wide) and dorsodermal (ddm; 2 μm wide) arrangements. The thicker ventrolateral muscles inserted anteriorly at the mouth rim while posteriorly merged with all the furcal musculature. The lateral longitudinal muscles inserted anteriorly on the endocuticle side by side of the mouth, extended along the lateral body wall towards the posterior end where they merged with the musculature of the furca at U90. A pair of longitudinal muscular fibres in the dorsodermal position began from each dorsal longitudinal muscle (U35), and joined (U85) the musculature of the furca, flowing close to the dorsal body wall (Fig. 2b).

Helicoidal muscles

Two pairs of thin fibres (hm; 1 μm wide), helicoidally arranged in opposite directions at an angle of 50–55°, ran from about two-third of the pharynx (U30) to half the length of the intestine (about U50). Helicoidal muscles surrounded the longitudinal ventral-, ventromedial- and dorsal bands. At least nine crosses were present on both the ventral and dorsal sides of the digestive tract (two crosses along the pharynx and seven along the intestine; Fig. 2b).

Dorsoventral muscles

Ten pairs of partial dorsoventral muscles (dvm; about 3 μm wide) flanked the intestine in the splanchnic position from U33 to U76. Each muscle, at its each extremity, spread out into two to four branches (each 0.5–0.7 μm wide). Each branch inserted into the basal lamina of the cuticle or connected to the near dorsal-, ventral- or ventromedial longitudinal bands. Three additional pairs of dorsoventral bands were present close to the posterior end of the pharynx (U25–U29; 0.5 μm wide). Additional five thin

dorsoventral-orientated muscles (0.5 μm wide) ran on the internal side of the furcal branches from U78 to U98.

Other muscle arrangements

Posterior to the anal region, the dorsal longitudinal muscles extended two branches that inserted into the endocuticle at the base of the furca (0.5–0.7 μm wide U80–U95; Fig. 2b).

Discussion

Comparison of muscular system between the two populations

This survey of the *X. intermedia* muscular system with CLSM revealed both qualitative and quantitative differences between the two populations.

Circular muscles

The two populations showed complete circular muscles along the entire length of the pharynx, as in all gastrotrichs investigated to date. However, differences were present along the intestinal tract: in the splanchnic position, Italian specimens only possessed circular muscles, which were incomplete, while in the somatic position, Kuwaiti populations only showed complete circular muscles encircling all the body muscles.

Longitudinal muscles

The gastrotrichs of both populations showed longitudinal muscles in the splanchnic- (dorsal, ventral, and ventromedial) and somatic positions (ventrolateral, lateral, and dorsodermal). No significant differences were observed.

Helicoidal muscles

All worms collected from the Italian sea possessed helicoidal muscles that stretched from one-third length of the pharynx to one-third length of intestine (U10–U30); in contrast, the Arabian population showed the same muscles beginning at two-third length of the pharynx and ending more posteriorly, at half the length of the intestine (U30–U50).

Dorsoventral muscles

All of the animals examined possessed dorsoventral muscles that were partially inserted into the basal lamina of the endocuticle (dorsally and ventrally), and partially connected with the closer longitudinal muscles; however,

animals from Italy possessed seven pairs of dorsoventral muscles laterally, in a somatic position, while Arabian specimens possessed ten pairs of dorsoventral fibres in a splanchnic position, along the intestine tract.

Overall comparisons

The external morphology as well as the pharyngeal traits of the examined gastrotrichs appeared very similar in both populations and fell well within the range of measurements reported in the literature for *X. intermedia* (Levi 1950; Gerlach 1953; Rao and Ganapati 1968; Luporini et al. 1973; Todaro et al. 1996). The average value of each character in the two investigated populations did not show statistically significant differences, except for the number of dorsal columns of scales and the number of scales in the dorsal median column; the Arabian population had few scales.

Furthermore, multivariate analyses showing an overall high similarity between the populations (>95%), were unable to clearly distinguish the two groups (Fig. 3). Consequently, from a traditional taxonomic assessment based on these traits alone, as the two populations did not appear different, the species can be considered as cosmopolitan in distribution (Sterrer 1973).

In contrast with the external morphology, the muscular system of the two populations surveyed with CLSM revealed obvious dissimilarities. With the exception of the longitudinal muscles, differences were present in all the other muscular arrangements, such as the circular-, dorsoventral-, and helicoidal organizations. These differences, in particular those concerning the circular- and dorsoventral musculatures of the trunk, have at least two important implications; one directly bound to the scope of the present paper (i.e. identification of cryptic species) and the other, more general, relates to the current hypotheses concerning the evolution of the musculature in Gastrotricha.

Muscles and identification of cryptic species

The muscular system plays a crucial role in several aspects of a gastrotrich's life, e.g. in locomotion, feeding behaviour, defecation, mating, oviposition, sperm transfer. These activities require high coordination among the muscular elements involved; in turn, coordination cannot be divorced from the type and organization of the muscular bands. Consequently, from an evolutionary point of view, it is expected that in taxa that are phylogenetically closely related, the muscular system should have a similar arrangement; possibly, the closer the taxa are the more similar the muscular systems should be. In fact, the different traits of the muscular systems of several species belonging to a wide taxonomic spectrum have been found useful in reconstructing the phylogeny of Paucitubulatina (Leasi and Todaro

2008), while the muscular systems of specimens of *X. intermedia* from another Mediterranean location (viz., Marina di Pisa, Tyrrhenian Sea) are identical to those of the specimens from the Adriatic Sea (F. Leasi and M. A. Todaro, unpublished data). Therefore, we believe that the differences in muscle arrangements between the Italian- and the Arabian populations of *X. intermedia* indicate that the two groups are separate species. A description of the Arabian population as a new species falls beyond the scope of the present paper and will be presented in a forthcoming article.

The musculature of *X. intermedia* from the Atlantic coast of the USA and that of the Mediterranean population originally appeared slightly different (Table 2); these discrepancies were attributed to technical artefacts (Leasi and Todaro 2008). However, these differences may be real and therefore indicative of a different taxonomic status of the two populations. In this regard, it is worth remarking that a high genetic divergence is known to exist between populations from the East coast of the USA and populations from the Adriatic Sea (Todaro et al. 1996). Indeed, a survey of the muscular system of *X. intermedia* from the Atlantic coast of USA using confocal microscopy could clarify whether these gastrotrichs belong to a different cryptic species. Likewise, it would be intriguing to analyze the musculature of specimens of *X. intermedia* from the Atlantic coast of France, investigated by Ruppert (1979), and whose main morphometric traits appear statistically different from those of all the other geographic regions (in Todaro et al. 1996).

Evolution of selected muscular bands

Paucitubulatina contains the only gastrotrichs that possess dorsoventral muscles, which show an ample variety of arrangements over the wide subordinal taxonomic spectrum. Dorsoventral muscles are thought to be derivatives of circular muscles (Hochberg and Litvaitis 2003; Leasi et al. 2006). It has been suggested that they originate from the dorsal- and ventral openings of the complete circular muscles in conjunction with a gradual reduction and their progressive ventral- and dorsal insertion into the cuticle. Such an evolutionary process took place early along the Chaetonotida branch and has proceeded at different rates and extends, with respect to the splanchnic- and somatic positions, into the different Paucitubulatina lineages (Leasi and Todaro 2008).

The presence in the Kuwaiti specimens of complete circular muscles in a somatic position along with the presence of dorsoventral muscles in a splanchnic position seems in contrast with the aforementioned hypothesis regarding the evolution of these muscular bands.

A phylogenetic analysis, based on a matrix of 16 muscular- and bio-ecological characteristics of 12 gastrotrich taxa, indicated the complete circular muscles to be highly plesiomorphic in nature (Leasi and Todaro 2008) and none of the Paucitubulatina investigated so far—13 species, in 11 genera and 4 families—possess them (Hochberg and Litvaitis 2003; Kieneke et al. 2008; Leasi and Todaro 2008), except the Arabian species. Although the existence of taxa with circular muscles could fit in the evolutionary framework highlighted by Leasi and Todaro (2008), the occurrence of such muscles in a species of Xenotrichulidae (a derived taxon) is rather unexpected, particularly because in the specimens from Kuwait circular muscles occur in a somatic position.

According to Leasi and Todaro (2008) somatic circular muscles, and the putative first evolutionary steps (e.g. incomplete circular muscles) of the process towards the formation of dorsoventral muscles should have been present in the very basal taxa, e.g. phylogenetically positioned prior to the branching off that separated the Muselliferidae from the clade formed by Xenotrichulidae and Chaetonotidae. This appears clear while considering that *Musellifer delamarei*

Table 2 Muscular arrangements of *Xenotrichula intermedia* in the three populations (Mediterranean, Arabian and Western Atlantic)

Muscular arrangements	Italy	Kuwait	USA[a]
Somatic			
Circular	n.a.	Complete	Incomplete
Dorsoventral	Partially	n.a.	n.a.
Longitudinal	Lateral-ventrolateral-dorsodermal	Lateral-ventrolateral-dorsodermal	Lateral-ventrolateral-dorsodermal
Splanchnic			
Circular[b]	Incomplete	n.a.	Incomplete
Dorsoventral	n.a.	Partially	n.a.
Helicoidal	U10–U40	U30–U50	?
Longitudinal	Ventral-ventromedial-dorsodermal	Ventral-ventromedial-dorsodermal	Ventral-ventromedial-dorsodermal

n.a. not applicable, *?* unknown

[a] Data from Hochberg and Litvaitis (2003)

[b] Complete circular muscles along the pharynx are been excluded because present in all gastrotrichs

(Muselliferidae), the most basal taxon within Paucitubula-tina, bears incomplete circular muscles in a splanchnic position and incomplete dorsoventral muscles in a somatic position (cf. Fig. 7, Leasi and Todaro 2008), a conditions quite far from the one that theoretically can be considered the most plesiomorphic (i.e. somatic circular and splanchnic circular).

Leasi et al. (2006) suggested the dorsoventral muscles to be functionally related to the interstitial life and have implications for sperm transfer; this hypothesis found support in a later research work (Leasi and Todaro 2008). It is, therefore, possible that the peculiarity of the muscular system of the *Xenotrichula* specimens from Kuwait is linked to their lifestyle and/or reproductive modality. While there do not appear to be obvious differences in the ecology of these specimens compared to their nearest siblings (i.e. xenotrichulids of interstitial, littoral zones), we discern a possible difference in reproductive characteristics. For example, we noticed that most of the adults (>80%) in the male stage had their external body surface covered by coiled cellular elements that appeared similar to the sperm cells seen inside their bodies (Fig. 4). As we are unaware of a similar circumstance occurring in any other xenotrichulid—surely it does not occur in *X. intermedia* from the areas we have studied it (i.e. Mediterranean sea, Virginia, Gulf of Mexico, Somalia)—the presence of spermatozoa in such a large number on the external body surface may be related to differences in sperm transfer modality in the Arabian species (e.g. direct fertilization-by injection—in *X. intermedia* vs. indirect fertilization in *Xenotrichula* from Kuwait), the details of which remain unclear.

Although molecular analysis will eventually be the main avenue to detect potential differences within this complex of sibling species, especially within small-sized taxa with putatively wide geographic ranges, the molecular approach still has many limits. For instance, a possible pit-fall of molecular genetics, especially when focusing on single genes, is that in the absence of other evidence, in most cases it is difficult to indicate clear cut boundaries among species based solely on the degree of molecular divergence (Maltagliati et al. 2000). In contrast, our research shows that genuine cryptic species may be recognized using diagnostic markers of morphological traits that are usually not taken into account during routine taxonomic species assessments. In particular, the survey of fluorescently labelled muscular fibres allowed us to discriminate clearly between specimens belonging to either the Arabian- or the Mediterranean population. Conversely, fluorescent-labelled markers appear to be also extremely useful to identify species when external morphological features vary and such variation is not due to genetic phenomena such as introgressive hybridization (Hummon 1975). For instance, a survey of a fluorescently labelled nervous system under a confocal microscope allowed Rothe and Schmidt-Rhaesa (2008) to recognize a specimen of Gastrotricha as belonging to *Turbanella cornuta* Remane, 1925 when external morphological traits could not be used to differentiate between the co-occurring *T. cornuta* and *T. hyalina* Schultze, 1853.

Owing to the often simple preparation of the samples, the relatively low number of specimens required for the analysis, and the increasing popularity of confocal microscopy, we would recommend this technique to explore the

Fig. 4 *Xenotrichula intermedia* specimens from Arabian population showing coiled sperm-like cells on the body, **a** dorsal side, **b** ventral side. DIC micrographs. *Scale bars* 50 μm
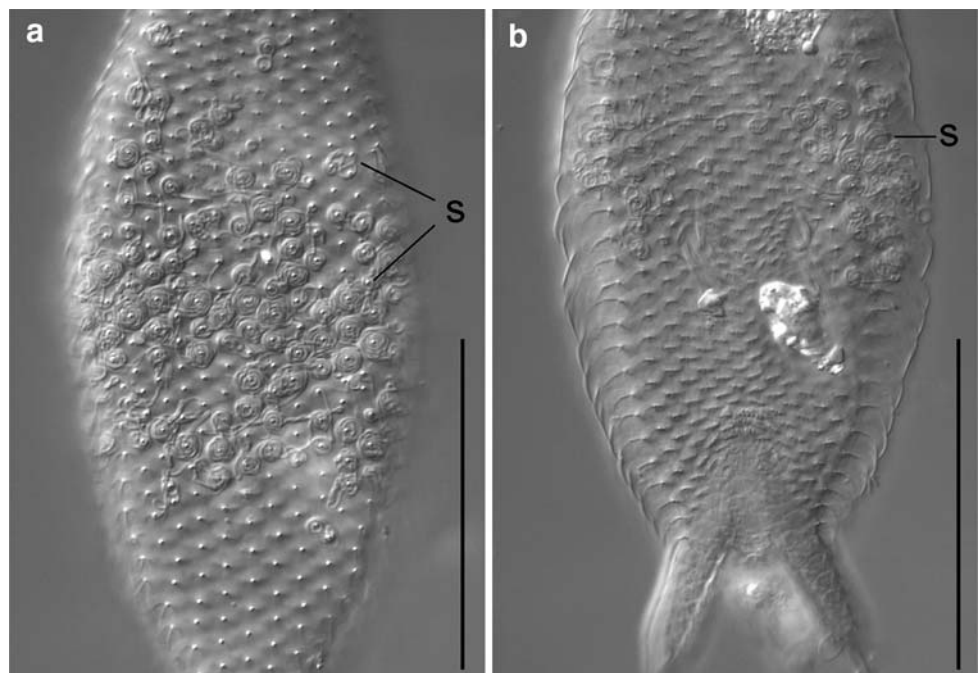
potential occurrence of cryptic species prior to turning to molecular methods.

References

Baker JM, Giribet G (2007) A molecular phylogenetic approach to the phylum Cycliophora provides further evidence for cryptic speciation in *Symbion americanus*. Zool Scr 36:353–359. doi:10.1111/j.1463-6409.2006.00288.x

Baker JM, Funch P, Giribet G (2007) Cryptic speciation in the recently discovered American cycliophoran *Symbion americanus*; genetic structure and population expansion. Mar Biol (Berl) 151:2183–2193. doi:10.1007/s00227-007-0654-8

Casu M, Curini-Galletti M (2004) Sibling species in interstitial flatworms: a case study using *Monocelis lineata* (Proseriata: Monocelididae). Mar Biol (Berl) 145:669–679

Casu M, Curini-Galletti M (2006) Genetic evidence for the existence of cryptic species in the mesopsammic flatworm *Pseudomonocelis ophiocephala* (Rhabditophora: Proseriata). Biol J Linn Soc Lond 87:553–576. doi:10.1111/j.1095-8312.2006.00588.x

Dunn CW, Hejnol A, Matus DQ, Pang K, Browne WE, Smith SA, Seaver E, Rouse GW, Obst M, Edgecombe GD, Sorensen MV, Haddock SHD, Schmidt-Rhaesa A, Okusu A, Kristensen RM, Wheeler WC, Martindale MQ, Giribet G (2008) Broad phylogenomic sampling improves resolution of the animal tree of life. Nature 452:745–749. doi:10.1038/nature06614

Evans WA (1994) Morphological variability in warm-temperate and subtropical populations of *Macrodasys* (Gastrotricha: Macrodasyida: Macrodasyidae) with description of seven new species. Proc Biol Soc Wash 107:239–255

Fenchel T, Finlay BJ (2004) The ubiquity of small species: patterns of local and global diversity. Bioscience 54:777–784. doi:10.1641/0006-3568(2004)054[0777:TUOSSP]2.0.CO;2

Gerlach SA (1953) Gastrotrichen aus dem Kuestengrundwasser des Mittelmeeres. Zool Anz 150:203–211

Gerlach SA (1977) Means of meiofauna dispersal. In: Sterrer W, Ax P (eds) The meiofauna species in time and space, vol 61. Mikrofauna Meeresboden, pp 89–103

Giere O (1993) Meiobenthology, the microscopic fauna in aquatic sediments. Springer, Berlin

Gomez S, Fleeger JW, Rocha OA, Foltz D (2004) Four new species of *Cletocamptus* Schmankewitsch, 1875, closely related to *Cletocamptus deitersi* (Richard, 1897) (Copepoda: Harpacticoida). J Nat Hist 38:2669–2732. doi:10.1080/0022293031000156240

Hochberg R, Gurbuz OA (2007) Functional morphology of somatic muscles and anterolateral setae in *Filinia novaezealandiae* Shiel and Sanoamuang, 1993 (Rotifera). Zool Anz 246:11–22. doi:10.1016/j.jcz.2006.10.002

Hochberg R, Litvaitis MK (2001a) A muscular double helix in Gastrotricha. Zool Anz 240:61–68. doi:10.1078/0044-5231-00006

Hochberg R, Litvaitis MK (2001b) The musculature of *Draculiciteria tessellata*: implications for the evolution of dorsoventral muscles in Gastrotricha. Hydrobiologia 452:155–161. doi:10.1023/A:1011940430507

Hochberg R, Litvaitis MK (2003) Organisation of muscles in Chaetonotida Paucitubulatina. Meiofauna Mar 12:47–58

Hummon WD (1975) Introgressive hybridization between two intertidal species of *Tetranchyrderma* (Gastrotricha, Thaumastodermatidae) with the description of a new species. Mikrofauna Meeresboden 61:113–136

Hummon WD (1994) Trans and Cis-Atlantic distribution of three marine heterotardigrades. Trans Am Microsc Soc 113:333–342. doi:10.2307/3226627

Hummon WD (2007) Global database for marine gastrotricha. http://hummon-nas.biosci.ohiou.edu/

Hummon WD, Todaro MA (2007) A new species of Xenotrichulidae (Gastrotricha) from southern and southeastern USA. Cah Biol Mar 48:297–302

Huys R (1992) The amphiatlantic distribution of Leptastacus macronyx (T. Scott, 1892) (Copepoda: Harpacticoida): a paradigm of taxonomy confusion; and a cladistic approach to the classification of the Leptasticidae Lang, 1948. Mede K Acad Wet Lett Sch Kunst Belg 54:21–196

Kieneke A, Arbizu PM, Riemann O (2008) Body musculature of *Stylochaeta scirtetica* Brunson, 1950 and *Dasydytes* (*Setodytes*) *tongiorgii* (Balsamo, 1982) (Gastrotricha : Dasydytidae): a functional approach. Zool Anz 247:147–158. doi:10.1016/j.jcz.2007.11.001

Leasi F, Ricci C (2008) Musculature in two bdelloid rotifers: *Macrotrachela quadricornifera* and *Adineta ricciae*. Organisation and locomotion in a functional and evolutionary perspective. Proceedings of III Congress of Italian Society for Evolutionary Biology, Italy

Leasi F, Todaro MA (2008) The Muscular system of *Musellifer delamarei* (Reanud-Mornant, 1968) and other chaetonotidans with implication for the phylogeny and systematisation of the Paucitubulatina (Gastrotricha). Biol J Linn Soc Lond 94:379–398. doi:10.1111/j.1095-8312.2008.00974.x

Leasi F, Rothe BH, Schmidt-Rhaesa A, Todaro MA (2006) The musculature of three species of gastrotrichs surveyed with confocal laser scanning microscopy (CLSM). Acta Zool 87:171–180. doi:10.1111/j.1463-6395.2006.00230.x

Levi C (1950) Contribution a l'étude des gastrotriches de la region de Roscoff. Arch Zool Exp Gen 87:31–42

Luporini P, Magagnini G, Tongiorgi P (1973) Chaetonotoid gastrotrichs of the Tuscan Coast. Boll Zool 40:31–40

Maltagliati F, Peru AP, Casu M, Rossi F, Lardicci C, Curini Galletti M, Castelli A (2000) Is *Syllis gracilis* Grube (Polychaeta: Syllidae) a species complex? An allozyme perspective. Mar Biol (Berl) 136:871–879. doi:10.1007/s002270000288

Priyalakshmi G, Menon NR, Todaro MA (2007) A new species of *Pseudostomella* (Gastrotricha:Macrodasyida:Thaumastodermatidae) from a sandy beach of Kerala, India. Zootaxa 1616:61–68

Rao GC, Ganapati PN (1968) Some new interstitial gastrotrichs from the beach sands of Waltair coast. Proc Indiana Acad Sci 67B:35–53

Rocha-Olivarez A, Fleeger JW, Foltz DW (2001) Decoupling of molecular and morphological evolution in deep lineages of a meiobenthic harpacticoid copepod. Mol Biol Evol 18:1088–1102

Rothe BH, Schmidt-Rhaesa A (2008) Variation in the nervous system in three species of the genus *Turbanella* (Gastrotricha, Macrodasyida) Meiofauna Mar 16:175-184

Ruchel J, Müller MCM (2007) F-actin framework in *Spirorbis* cf. *spirorbis* (Annelida: Serpulidae): phalloidin staining investigated and reconstructed by CLSM. Inv Biol 126:173–182. doi:10.1111/j.1744-7410.2007.00087.x

Ruppert EE (1979) Morphology and systematics of the Xenotrichulidae (Gastrotricha, Chaetonotida). Mikrofauna Meeresboden 76:1–56

Ruppert EE (1991) Gastrotricha (Aschelminthes). In: Harrison F, Ruppert EE (eds) Microscopic anatomy of invertebrates, vol 4. Wiley-Liss Press, Washington DC, pp 41–109

Sterrer W (1973) Plate tectonics as a mechanism for dispersal and speciation in interstitial sand fauna. Neth J Sea Res 7:200–222. doi:10.1016/0077-7579(73)90045-8

Suatoni E, Vicario S, Rice S, Snell T, Caccone A (2006) An analysis of species boundaries and biogeographic patterns in a cryptic species complex: the rotifer—*Brachionus plicatilis*. Mol Phylogenet Evol 41:86–98

Todaro MA, Hummon WD (2008) An overview and a dichotomous key to genera of the phylum Gastrotricha. Meiofauna Mar 16:3–20

Todaro MA, Rocha CEF (2004) Diversity and distribution of marine Gastrotricha along the northern beaches of the state of Sao Paulo (Brazil), with description of a new species of *Macrodasys* (Macrodasyida, Macrodasyidae). J Nat Hist 38:1605–1634. doi:10.1080/0022293031000156169

Todaro MA, Fleeger JW, Hu YP, Hrincevich AW, Foltz DW (1996) Are meiofauna species cosmopolitan? Morphological and molecular analysis of *Xenotrichula intermedia* (Gastrotricha: Chaetonotida). Mar Biol (Berl) 125:735–742. doi:10.1007/BF00349256

Todaro MA, Guidi L, Leasi F, Tongiorgi P (2006a) Morphology of *Xenodasys* (Gastrotricha): the first species from the Mediterranean Sea and the establishment of *Chordodasiopsis* gen. nov. and Xenodasyidae fam. nov. J Mar Biol Assoc UK 86:1005–1015. doi:10.1017/S0025315406013981

Todaro MA, Telford MJ, Lockyer AE, Littlewood DTJ (2006b) Interrelationships of the Gastrotricha and their place among the Metazoa inferred from 18S rRNA genes. Zool Scr 35:251–259. doi:10.1111/j.1463-6409.2006.00228.x

Todaro MA, Faraj MN, Al-Kady S, Dal Zotto M (2007) Marine Gastrotricha from the state of Kuwait (Arabian Gulf). Proceedings of XIII international meiofauna conference, Brazil

Todaro MA, Dal Zotto M, Maiorova AS, Adrianov AV (2009) A new species of *Aspidiophorus* (Gastrotricha, Chaetonotida) from the Russian Far East with a key to marine species of the genus. Mar Biol Res (in press). doi:10.1007/s10152-002-0114-2

Westheide W, Schmidt H (2003) Cosmopolitan versus cryptic meiofaunal polychaete species. An approach to a molecular taxonomy. Helgol Mar Res 57:1–6

Westheide W, Hass-Cordes E, Krabusch M, Müller MC (2003) *Ctenodrilus serratus* (Polychaeta: Ctenodrilidae) is a truly amphi-Atlantic meiofauna species—evidence from molecular data. Mar Biol (Berl) 142:637–642

Zelinka C (1889) Die Gastrotrichen. Eine monographische Darstellung ihrer Anatomie, Biologie und Systematic. Z Wiss Zool Abt A 49:209–384

Zrzavý J (2003) Gastrotricha and metazoan phylogeny. Zool Scr 32:61–81. doi:10.1046/j.1463-6409.2003.00104.x