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Comparative analysis of the tardigrade feeding apparatus: adaptive convergence and evolutionary pattern of the piercing stylet system

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ABSTRACT

*A thorough analysis of the cuticular parts of tardigrade feeding apparatuses was performed in order to provide a more complete understanding of their evolution and their potential homologies with other animal phyla (*e.g. *Cycloneuralia and Arthropoda). The buccal-pharyngeal apparatuses of eight species belonging to both Eutardigrada and Heterotardigrada were studied using light and scanning electron microscopy. This study supports and completes a previous study on the relationships between form and function in the buccal-pharyngeal apparatus of eutardigrades. The common sclerified structures of the tardigrade buccal-pharyngeal apparatus are: a buccal ring connected to a straight buccal tube, a buccal crown, longitudinal thickenings within the pharynx, and a stylet system composed of piercing stylets within stylet coats, and stylet supports. Specifically, heterotardigrades (Echiniscoidea) have a narrow buccal tube; long piercing stylets, each with a longitudinal groove, that cross one another before exiting the mouth; pharyngeal bars and secondary longitudinal thickenings within the pharynx. In contrast, eutardigrades have stylets which are shorter than the buccal tube; Parachela have pharyngeal apophyses and placoids within the pharynx, while Apochela lack a buccal crown and cuticular thickenings within the pharynx, the buccal tube is very wide, and the short stylets are associated with triangular-shaped stylet supports. In both classes, when the piercing stylet tips emerge from the mouth to pierce food, the buccal tube opening is almost completely obstructed, which may hinder food uptake. In heterotardigrades, the crossing of the piercing stylets may further decrease food uptake, however this disadvantage may have been reduced in echiniscids by the evolution of a long buccal tube and long stylets able to run more parallel to the buccal tube. In contrast, eutardigrades evolved different strategies. In the order Apochela and in several Itaquasconinae (Parachela), the buccal tube is wide, the stylets are short and run parallel to the tube without crossing. In other Parachela, the piercing stylets do not cross one another because they are curved. Further, the development of an anterior bend in the buccal tube (*e.g. in *Doryphoribius *and Macrobiotoidea) may allow the shift of the stylet sheaths to a more ventral position so that a wide portion of the mouth is free during the piercing stylet movements. The possible convergent evolution of several structures of the buccal-pharyngeal apparatus (*e.g. *ventral lamina, pharyngeal tube, wide buccal tube without buccal crown, buccal lamellae) was analysed and discussed.*

Key words: buccal-pharyngeal apparatus, evolution, adaptation, Tardigrada.

INTRODUCTION

Tardigrades, together with Onychophora and Arthropoda, belong to Panarthropoda, as supported by both morphological and molecular studies (Campbell *et al.,* 2011; Nielsen 2012), although some molecular phylogenies have placed tardigrades within Cycloneuralia (Nematoda, Nematomorpha, Kinorhyncha, Loricifera, Priapulida; Dunn *et al*., 2008; Meusemann *et al*., 2010). Several morphological characters are similar in tardigrades and Cycloneuralia, but some of them should be considered homoplastic or plesiomorphic characters (Edgecombe, 2009). As a consequence, further studies are needed to evaluate potential homologies of these characters. A circum-oesophageal brain is considered a synapomorphy of Cycloneuralia. The presence of this feature in tardigrades has been proposed by Hejnol and Schnabel (2005), but Persson *et al.* (2012) do not agree with this interpretation and provide evidence for a dorsal trilobed brain connected by three commissures to a subpharyngeal ganglion, supporting the phylogenetic position of Tardigrada within Panarthropoda. More evidence is needed to determine if the tardigrade subpharyngeal ganglion is really part of the brain forming the circumbuccal ring as it is in Cycloneuralia.

The tardigrade buccal-pharyngeal apparatus shares some characters with the feeding apparatuses of some Cycloneuralia phyla. In particular, a protrusible mouth cone, circumoral ring, a tripartite myoepithelial pharynx with cuticular reinforcements and piercing stylets are shared with Loricifera and Nematoda. According to Eibye-Jacobsen (2001b), the overall structure of the nematode pharynx differs from the tardigrade organ in several aspects and the placoid-like structures in nematodes may well have evolved through adaptive convergence. The placoid-like structures of the loriciferan pharynx are found only in the family Nanaloricidae (Kristensen, 1991), while they are absent in all loriciferan larvae and in the other loriciferan family. Therefore this character should be considered an autapomorphy of the family and thus not homologous to the tardigrade placoids (Eibye-Jacobsen, 2001b). For a more complete understanding of the evolutionary origin and transformation of the tardigrade feeding apparatuses and their potential homologies with other animal phyla, a more thorough analysis of their cuticular parts was needed. Innovative and important comparative studies on the fine structure and organisation of the tardigrade feeding apparatus were performed by several authors using transmission and/or scanning electron microscopy (SEM) (Dewel and Clark 1973; Dewel and Wallis 1973; Schuster *et al*., 1980; Eibye-Jacobsen 1997, 2001a, 2001b; Dewel and Eibye-Jacobsen 2006; Pilato *et al.,* 2006; Guidetti *et al.,* 2012), but the number of analyzed species is still limited. For this reason, the buccal-pharyngeal apparatuses of eight additional species belonging to both tardigrade classes (Eutardigrada and Heterotardigrada) were studied by light and scanning electron microscopy. This study extends, supports and completes a previous study on the relationships between form and function in the buccal-pharyngeal apparatus of eutardigrades (Guidetti *et al*., 2012). In that study the anatomy of some parts of the feeding apparatus of 12 species in 8 genera were reconsidered and new terms were introduced to better define their cuticular organization. The sclerified structures of the tardigrade buccal-pharyngeal apparatus basically consist of a buccal ring articulated with a buccal tube, the latter totally or partially rigid and surrounded anteriorly by a buccal crown formed by crests and laminae for muscle attachments, a stylet system, and longitudinal bars or placoids within the muscular sucking pharynx (Guidetti *et al.,* 2012). The stylet system is formed by two stylets and two stylet supports connecting the caudal end of the stylet (the stylet furca) to the buccal tube. Each stylet consists of a stylet coat (made up of the anterior stylet sheath and the posterior stylet furca) containing a $CaCO_3$ piercing stylet (Guidetti *et al.,* 2012).

The findings reported here illustrate that comparative morphological fine scale analysis improves our understanding of the structure and function of tardigrade anatomy and provides new details for taxonomic and evolutionary studies.

METHODS

The anatomy of the sclerified structures of the buccal-pharyngeal apparatuses of eight species of tardigrades belonging to eight genera in four families (Tab. 1) of both classes were examined by SEM and light microscopy (LM). The species considered were *Ramazzottius* cf. *oberhaeuseri, Diphascon* cf. *patanei, Platicrista angustata* (Murray, 1905)*, Doryphoribius flavus* (Iharos, 1966)*, Thulinius stephaniae* (Pilato, 1974), *Echiniscus blumi* Richters, 1903*, Pseudechiniscus* sp.*,* and *Cornechiniscus lobatus* (Ramazzotti, 1943). In addition, for comparison, a further study on the piercing stylets of *Paramacrobiotus richtersi* (Macrobiotidae) extracted from leaf litter collected in Modena (Italy) was performed.

The buccal-pharyngeal apparatuses of animals mounted in Faure-Berlese fluid were observed by phase contrast (PhC) or differential interference contrast (DIC) with a Leitz DM RB microscope. The buccal-pharyngeal apparatuses were prepared for SEM observations with the sodium hypochlorite (NaClO) extraction method developed by Eibye-Jacobsen (2001a). In the hypochlorite extraction method, the animal body was torn with needles within a drop of diluted NaClO solution. After the tissues around the buccal apparatus had been destroyed, the apparatus was collected with a glass pipette, and transferred onto a coverglass positioned on a stub. Finally, the buccal-pharyngeal apparatus was covered with gold-palladium and analyzed by a SEM XL 40 (Fei Company-Oxford Instruments, Hillsboro, OR, USA) available at the *Centro Interdipartimentale Grandi Strumenti* of the *Università di Modena e Reggio Emilia*.

RESULTS

Descriptions of the apparatuses of the analysed species, focusing mainly on distinctive characters are presented below and summarised in Tab. 2.

Eutardigrada

The buccal-pharyngeal apparatus of *Thulinius stephaniae* exhibits an anterior buccal ring bearing a ring of

Tab. 1. Systematic position of the analysed species, their colonised substrates and sampling sites.

Class	Superfamily	Family	Species	Substrate	GPS coordinates	
Eutardigrada	Hypsibioidea	Ramazzottidae	*Ramazzottius* cf. *oberhaeuseri*	Lichen on tree	44° N 18.788	10° E 47.761
	Hypsibioidea	Hypsibiidae	*Diphascon* cf. *patanei*	Leaf litter	44° N 11.860	10° E 47.923
	Hypsibioidea	Hypsibiidae	*Platicrista angustata*	Moss on rock	44° N 07.688	10° E 35.289
	Isohypsibioidea	Isohypsibiidae	*Thulinius stephaniae*	Freshwater sediment	44° N 35.702	10° E 59.657
	Isohypsibioidea	Isohypsibiidae	*Doryphoribius flavus*	Moss on rock	44° N 12.871	10° E 33.282
Heterotardigrada		Echiniscidae	*Echiniscus blumi*	Moss on rock	46° N 10.133	11° E 00.017
		Echiniscidae	*Pseudechiniscus* sp.	Moss on tree	44° N 30.434	10° E 47.139
		Echiniscidae	*Cornechiniscus lobatus*	Lichen on tree	37° N 51.690	14° E 49.095

Tab. 2. Distinctive characters of the tardigrade genera analysed in this study and in the previous study by Guidetti *et al.* (2012).

Genus	Mouth cone	Buccal ring with	Buccal tube	Ventral lamina	Buccal crown	Oval perforated areas	Terminal buccal tube margin	Pharyngeal apophyses	Macro placoids	Piercing stylets	Branch of furca	Furca condyle	Apophyses on furca branch
Echiniscus°	Present	Round cuticular lamina	Narrow, straight	Absent	Present	Covered by buccal crown	Not enlarged	Absent	Absent	Straight, cross each other	Wide, short	Wide, flat	Absent
Pseudechiniscus°	Present	Round cuticular lamina	Narrow, straight	Absent	Present	Covered by buccal crown	Not enlarged	Absent	Absent	Straight, cross each other	Wide, short	Wide, flat	Absent
Cornechiniscus°	Present	Round cuticular lamina	Narrow, straight, with flexible portion	Absent	Present	Covered by buccal crown	Not enlarged	Absent	Absent	Straight, cross each other	Wide, short	Wide, flat	Absent
Thulinius°	Absent	Ring of fused lamellae	Quite large, straight	Absent	Present	Small, covered by buccal crown	Thick	Long, slender	Three, long, thin	Curved	Long, slender	Round	Absent
Platicrista°	Absent	Round cuticular lamina	Large, straight, with flexible portion	Absent	Absent	Large	Not enlarged	Very small	Two, long, thin, 2nd longer	Straight	Short, arched	Small, pointed	Absent
Diphascon (Diphascon)°	Absent	Round cuticular lamina	Narrow, straight, with flexible portion	Absent	Present	Small, covered by buccal crown	Not enlarged	Bilobed, large margins	Two, quite long, thick	Curved	Mean length	Round	Present
Ramazzottius°	Absent	Round cuticular lamina	Narrow with ventral posterior turn	Absent	Present	Small, covered by buccal crown	Thick	Bilobed, large margins	Two, short, thick	Curved	Mean length	Round	Present
Doryphorybius°	Absent	Round cuticular lamina	Quite large with ventral anterior turn	Present	Present	Small, covered by buccal crown	Thick	Bilobed, large margins	Two-three, short, thick	Curved	Mean length	Round	Present
*Macrobiotus**	Absent	Peribuccal lamellae	Quite large with ventral anterior turn	Present	Present	Small, covered by buccal crown	Thick	Bilobed	Two-three, quite long, thin	Curved	Mean length	Round	Absent
*Paramacrobiotus**	Absent	Peribuccal lamellae	Quite large with ventral anterior turn	Present	Present	Small, covered by buccal crown	Thick	Bilobed	Three, quite long, thin	Curved	Mean length	Round	Absent
*Minibiotus**	Absent	Peribuccal papulae	Narrow with ventral anterior turn	Present	Present	Small, covered by buccal crown	Thick	Large	Three, short, thick	Curved	Mean length	Round	Absent
*Richtersius**	Absent	Ring of fused lamellae	Narrow with anterior and posterior turns	Present	Present	Small, covered by buccal crown	Thick	Bilobed with large margins	Two, short, thick	Curved	Wide, long	Cylindrical or round	Absent
*Adorybiotus**	Absent	Ring of fused lamellae	Narrow with anterior and posterior turns	Present	Present	Small, covered by buccal crown	Thick	Bilobed, large margins	Two, quite long, thick	Curved	Wide, long	Wide, with a point	Absent
*Dactylobiotus**	Absent	Peribuccal lamellae	Quite large with ventral anterior turn	Present	Present	Small, covered by buccal crown	Thick	Bilobed	Two, quite long, thin	Curved	Long, slender	Long, slender	Absent
*Murrayon**	Absent	Peribuccal lamellae	Quite large with ventral anterior turn	Present	Present	Small, covered by buccal crown	Thick	Bilobed, large margins	Two, quite long, thin	Curved	Long, slender	Long, slender	Absent
*Milnesium**	Absent	Peribuccal lamellae	Very large, straight	Absent	Absent	Large	With 3 indentations	Absent	Absent	Straight	Short, triangular	Absent	Absent

*°Distinctive characters of the tardigrade genera analysed in this study; *distinctive characters of the tardigrade genera analysed in the previous study by Guidetti* et al. *(2012).*

fused lamellae on its distal edge (Fig. 1a). The buccal ring of all the other eutardigrade species considered here bears a round cuticular lamina (Fig. 1b).

The buccal tube is large and straight in *Platicrista angustata* (Figs. 2 and 3), quite large and straight in *T. stephaniae* (Fig. 4), narrow and straight in *Diphascon* cf. *patanei* (Fig. 5), and narrow in *Ramazzottius* cf. *oberhaeuseri* (Fig. 6). In the latter species the buccal tube turns ventrally after the insertion of the stylet supports. The buccal tube of *Doryphoribius flavus* is quite large and anteriorly bent, bearing a ventral longitudinal reinforcement (ventral lamina) (Fig. 7). In *T. stephaniae*, the anterior part of the buccal tube bears an inner band of prominent teeth (Figs. 1a and 4b). In *P. angustata* and *D.* cf. *patanei* the buccal tube wall becomes flexible posteriorly (Figs. 2, 3a and 5); this flexible part is generally called the pharyngeal tube. The pharyngeal tube of these two species is characterised by a coiled cuticular wall in which each coil is made up of a cylindrical fibre of about 0.3 μm in diameter. In *P. angustata,* the pharyngeal tube begins immediately after the stylet support insertion on the buccal tube and continues to the end of the buccal tube (Fig. 2). In *D.* cf. *patanei,* the pharyngeal tube begins more posteriorly with respect to the stylet support insertions, at the level of a wide cuticular apophysis in the shape of a drop (commonly called drop-like thickening), and ends within the pharynx with a short, non-coiled and rigid terminal portion (Fig. 5).

In all species, with the exception of *P. angustata,* a buccal crown with prominent laminae for muscle attachments is present on the anterior portion of the buccal tube (Figs. 4-7). The buccal crown has cuticular crests mid-dorsally and mid-ventrally (Figs. 4-7); in lateral view, these crests are the so called *apophyses for the insertion of the stylet muscles*. Their margins differ among the species and are used for taxonomic purposes. In *T. stephaniae*, the dorsal and ventral crests of the buccal crown are flat and wide, and the two lateral rod-shaped thickenings are large and prominent (Fig. 4b). In *R.* cf. *oberhaeuseri* the buccal crown crests are evident; the dorsal crest is posteriorly bifurcated, while the ventral crest has a bulbous ending (Figs. 6b and 6c). Unfortunately, the specimen of *D.* cf. *patanei* examined here did not permit an understanding of the shapes of the crests. In *D. flavus*, the dorsal

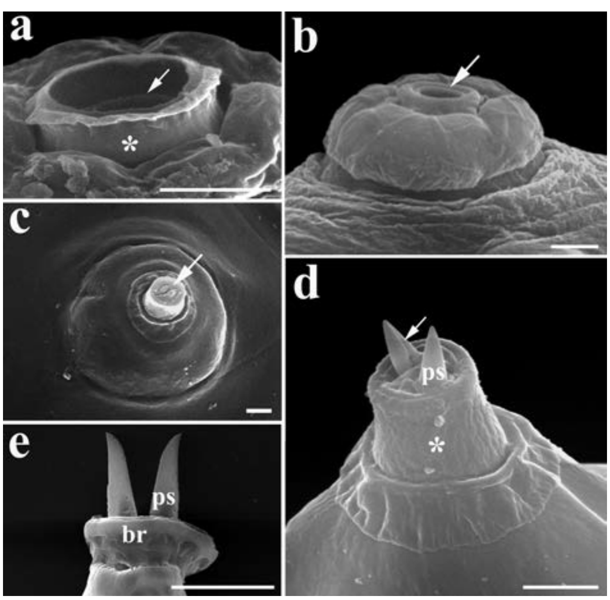

Fig. 1. a) *Thulinius stephaniae* (analysed using scanning electron microscopy). Mouth opening surrounded by fused buccal lamellae (asterisk); band of teeth in the inner surface of the buccal tube (arrow); b) *Ramazzottius* cf. *oberhaeuseri*. Mouth opening surrounded by a circular lamina (arrow); c) *Echiniscus blumi*. Mouth opening (arrow); d) *Echiniscus blumi*. Tips of the piercing stylets out of the mouth opening, showing a longitudinal groove on the internal side (arrow); mouth cone visible (asterisk); e) *Paramacrobiotus richtersi*. The tips of piercing stylets run parallel to each other outside the mouth opening. br=buccal ring; ps=piercing stylet. Scale bars=2 μm.

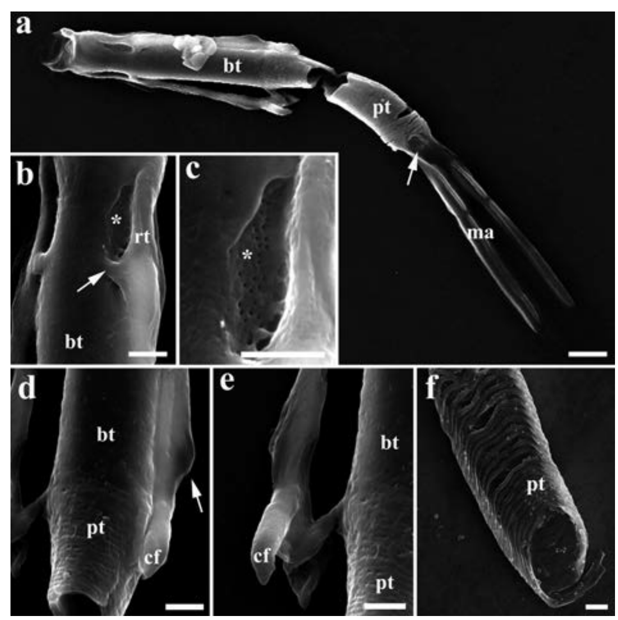

Fig. 2. *Platicrista angustata* (analysed using scanning electron microscopy). a) Buccal-pharyngeal apparatus; b) oval perforated area (asterisk) and cuticular bridges connecting the buccal tube to the stylet sheath (arrow); c) Oval perforated area (asterisk); d) transition area between rigid buccal tube and flexible pharyngeal tube; arrow indicates the stylet elbow; e) stylet furca and stylet support; f) coils of the pharyngeal tube. bt=buccal tube; pt=pharyngeal tube; ma=macroplacoid; rt=rod-shaped thickening; cf=condyle of the furca. Scale bars of a)=10 μm and of b-f)=2 μm.

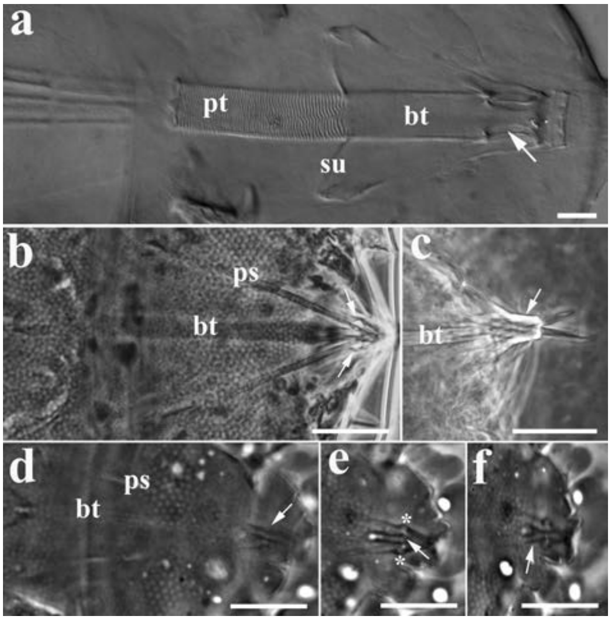

Fig. 3. a) Buccal-pharyngeal apparatus of *Platicrista angustata* with oval perforated areas (arrow). b-f) Buccal-pharyngeal apparatus of *in vivo Echiniscus blumi*: b) buccal-pharyngeal apparatus with the two spherical enlargements located within the buccal crown (arrows); c) Piercing stylets crossing within the buccal crown (arrow); d-f) three successive focuses of the same buccal-pharyngeal apparatus (from ventral to dorsal) showing in d) the left stylet sheath (arrow), in e) the buccal tube opening with the buccal crown (arrow), and two spherical enlargements within the buccal crown (asterisks), and in f) right stylet sheath (arrow). pt=pharyngeal tube; su=stylet support; bt=buccal tube; ps=piercing stylet. a) observed by differential interference contrast, b-f) observed by phase contrast. Scale bars=10 µm.

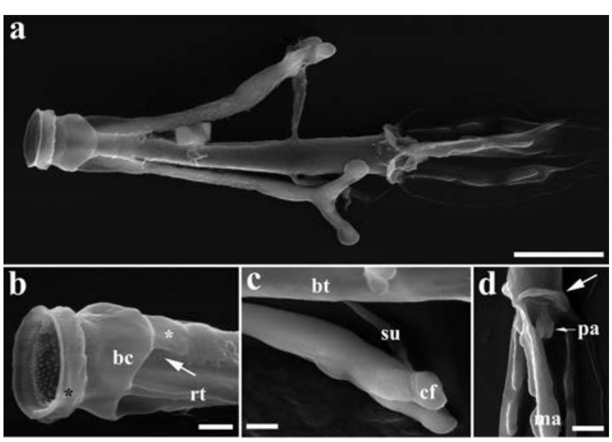

Fig. 4. *Thulinius stephaniae* (analysed using scanning electron microscopy). a) Buccal-pharyngeal apparatus; b) buccal ring with fused buccal lamellae (black asterisk) and buccal crown with dorsal crest (white asterisk). Arrow indicates the oval perforated area; c) condyles of the stylet furca and stylet support; d) posterior end of buccal tube with laminae (arrow) and placoids within the pharynx. bc=buccal crown; rt=rod-shaped thickening; bt=buccal tube; su=stylet support; cf=condyle of the furca; pa=pharyngeal apophyses; ma=macroplacoid. Scale bars of a)=10 µm and of b-d)=2 µm.

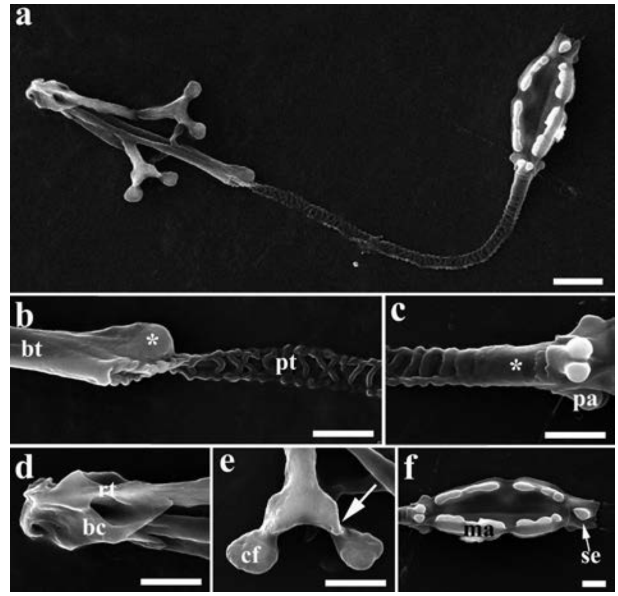

Fig. 5. *Diphascon* cf. *patanei* (analysed using scanning electron microscopy). a) Buccal-pharyngeal apparatus; b) drop-like thickening (asterisk) on the buccal tube at junction with pharyngeal tube; c) end of the pharyngeal tube (asterisk); d) buccal crown; e) stylet furca with apophyses on its branches (arrow); f) apophyses, macroplacoids and septula within the pharynx. bt=buccal tube; pt=pharyngeal tube; pa=pharyngeal apophyses; rt=rod-shaped thickening; bc=buccal crown; cf=condyle of the furca; ma=macroplacoid; se=septulum. Scale bars of a)=10 µm and of b-f)=2 µm.

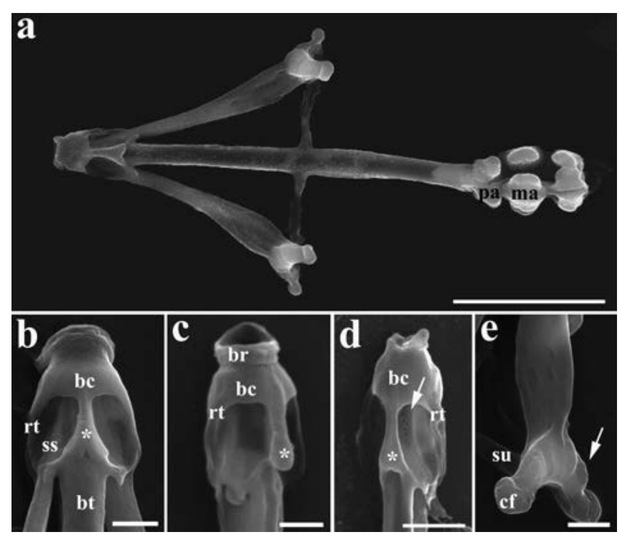

Fig. 6. *Ramazzottius* cf. *oberhaeuseri* (analysed using scanning electron microscopy). a) Buccal-pharyngeal apparatus; b) buccal crown with ventral crest (asterisk); c) buccal crown with dorsal crest (asterisk); d) buccal crown with ventral crest (asterisk), and oval perforated area (arrow); e) stylet furca with apophyses on its branches (arrow). pa=pharyngeal apophyses; ma=macroplacoid; bc=buccal crown; rt=rod-shaped thickening; ss=stylet sheath; bt=buccal tube; br=buccal ring; su=stylet support; cf=condyle of the furca. Scale bars of a)=10 µm and of b-e)=2 µm.

crest is absent and the ventral one is formed by the anterior portion of the ventral lamina (Figs. 7b and 7c). The ventral lamina of *D. flavus* is a crest-shaped structure with its proximal margin fused with the buccal tube (Fig. 7b).

Four oval perforated areas are present on the buccal tube wall of all examined species and are symmetrically oriented on each side of the stylet sheaths, one dorsal and one ventral (Figs. 2-4, 6 and 7). These oval perforated areas are the regions through which the microvillus-like sensory processes of the sensitive buccal sensory organs (also called pharyngeal organs) cross the buccal tube and reach the inner surface of the mouth. The oval perforated areas are particularly evident and wide in *P. angustata*, while in the other species they can be smaller and totally or partially covered by the crests of the buccal crown. In *P. angustata*, cuticular bridges connect the buccal tube to the stylet sheaths at the level of the posterior end of the oval perforated areas (Fig. 2b).

The posterior margin of the buccal tube ends within the pharynx with thicker margins in *R.* cf. *oberhaeuseri*, *D. flavus* and *T. stephaniae;* in the latter species the margins form expanded laminae (Fig. 4d). The buccal tube is in cuticular continuity with the cuticular lining of the pharynx, which is reinforced by apophyses and placoids. Pharyngeal apophyses are large and bilobed in *R.* cf. *oberhaeuseri, D. flavus,* and *D.* cf. *patanei*, small and bilobed in *T. stephaniae* and very small, not visible by LM, in *P. angustata* (Figs. 2-7). There are three lines of macroplacoids, located at 120° to each other, one ventrally and two dorso-laterally. In each line, the macroplacoids are as follows: three, long and thin in *T. stephaniae*; two, quite long, and thick in *D.* cf. *patanei*; two, short, and thick in *R.* cf. *oberhaeuseri* and *D. flavus*; two, very long, and thin in *P. angustata*. *Diphascon* cf. *patanei* has another cuticular pharyngeal thickening, called a septulum. Septula are the same in number as the macroplacoid lines, but each one of them is positioned between two lines of macroplacoids and is aligned with the pharyngeal apophyses (Fig. 5f). The cuticular lining of the pharynx continues with the narrow cylindrical oesophagus.

In all species, the stylet system is made up of the stylet coats containing the piercing stylets, and the stylet sup-

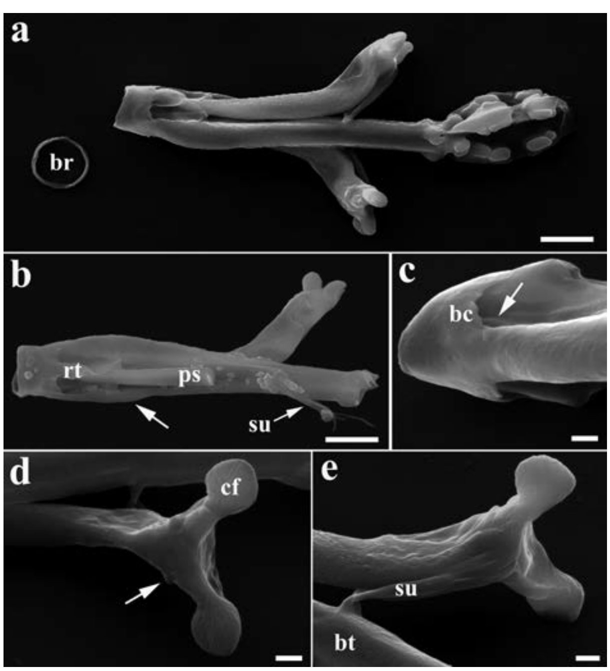

Fig 7. *Doryphoribius flavus* (analysed using scanning electron microscopy). a) Buccal-pharyngeal apparatus (buccal ring separated from buccal tube due to specimen preparation); b) buccal-pharyngeal apparatus in lateral view; c) oval perforated area (arrow); d) stylet furca with apophyses on its branches (arrow); e) stylet support. br=buccal ring; rt=rod-shaped thickening; ps=piercing stylet; su=stylet support; bc=buccal crown; cf=condyle of the furca; bt=buccal tube. Scale bars of a, b)=10 µm and of c-e)=2 µm.

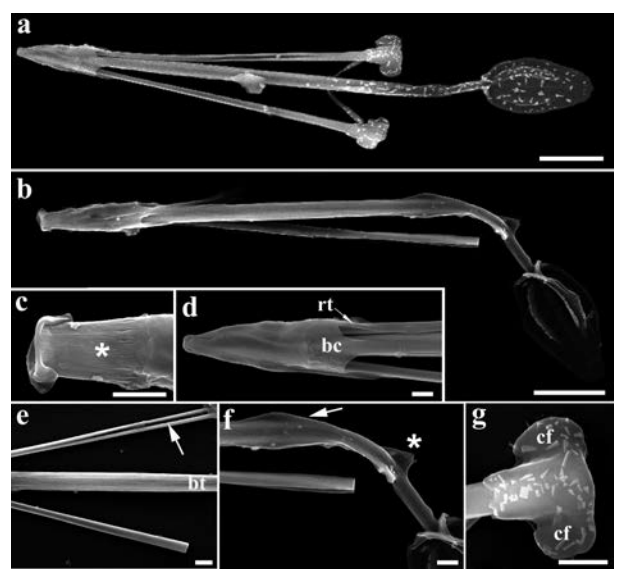

Fig. 8. *Cornechiniscus lobatus* (analysed using scanning electron microscopy). a) Buccal-pharyngeal apparatus; b) buccal-pharyngeal apparatus in lateral view; c) buccal ring (asterisk); d) buccal crown with rod-shaped thickening; e) buccal tube and piercing stylets with longitudinal groove (arrow); f) posterior buccal tube with a longitudinal dorsal crest (arrow) followed by a flexible portion bearing a second dorsal longitudinal crest (asterisk); g) stylet furca. rt=rod-shaped thickening; bc=buccal crown; bt=buccal tube; cf=condyle of the furca. Scale bars of a, b)=10 µm and of c-g)=2 µm.

ports are present on both sides of the buccal tube. The stylet coat is characterised posteriorly by the stylet furca and anteriorly by the stylet sheath. The stylet furcae have two branches with rounded condyles at their extremities. The stylet sheath is a cylindrical structure that opens within the buccal tube and allows egress of the piercing stylet through the mouth opening. It is laterally reinforced by a thin rod-shaped cuticular thickening, which is anteriorly connected to the buccal crown when present (Figs. 2 and 4-7). Each piercing stylet, positioned within each stylet coat, is quite large, curved, and dorso-ventrally compressed in all species, with the exception of *P. angustata* (Figs. 2 and 4-7). In *P. angustata*, the piercing stylets are straight, needle-shaped, and placed within a stylet coat showing a stylet elbow (Fig. 2d). In eutardigrade species the tips of piercing stylets run parallel to each other outside the mouth opening (Fig. 1e). In all examined species, with the exception of *D. flavus,* the piercing stylets run parallel to the buccal tube. In *D. flavus,* the piercing stylets do not run parallel to the buccal tube because the stylet sheaths are located ventro-laterally to the buccal tube, while the stylet furcae are located laterally to the buccal tube (Fig. 7). In this species, the piercing stylet runs within the stylet coat that is positioned in the furrow formed by the buccal tube wall and its ventral thickening (ventral lamina) (Figs. 7a and 7b).

In all species, with the exception of *P. angustata*, the stylet furca has two round, generally laterally compressed condyles; in *T. stephaniae* the condyles have long, slender branches (Fig. 4a), while in *D.* cf. *patanei, R.* cf. *oberhaeuseri* and *D. flavus* two prominent apophyses are present on the furca branches (Figs. 5-7). In *P. angustata* the furca has a peculiar shape: the two branches form a distal arc with a concave margin and the two condyles at the branch extremities are tapered, not enlarged (Figs. 2d and 2e).

In all species examined, the stylet supports are flexible cuticular structures connecting the buccal tube with the stylet furca (Figs. 2-7). The stylet supports are inserted on the buccal tube at about 70% of the length of the rigid portion of the buccal tube in *T. stephaniae, D. flavus,* and *D.* cf. *patanei,* and at about 60% of the buccal tube length in *R.* cf. *oberhaeuseri*. In *P. angustata* they are inserted at the end of the rigid portion of the buccal tube. *Platicrista angustata* has a very short stylet support that can move backwards up to 90°, becoming aligned with the buccal tube (Figs. 2d and 2e). In contrast, in all other species, the stylet support can move backward but never becomes aligned with the buccal tube (Figs. 4-7).

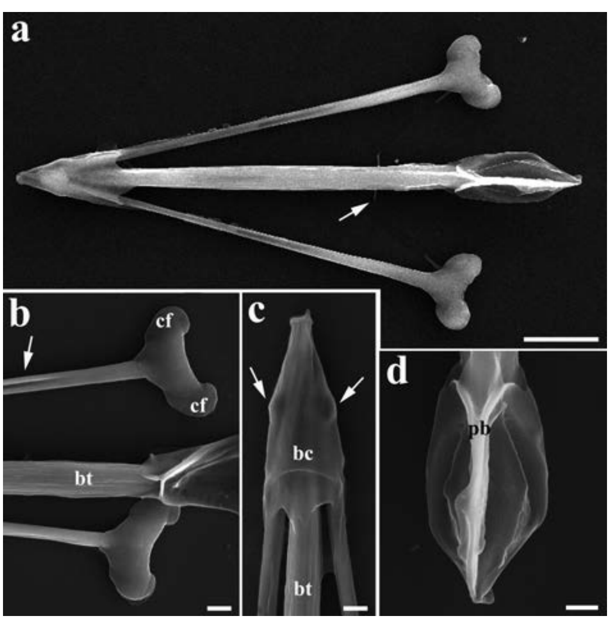

Fig. 9. *Echiniscus blumi* (analysed using scanning electron microscopy). a) Buccal-pharyngeal apparatus; the stylet support (arrow) is very thin; b) piercing stylets with longitudinal groove (arrow) and stylet furcae; c) buccal crown, with two internal spherical enlargements (arrows); d) pharyngeal bars and secondary longitudinal laminar thickenings within the pharynx. cf=condyle of the furca; bt=buccal tube; bc=buccal crown; pb=pharyngeal bars. Scale bars of a)=10 µm and of b-d)=2 µm.

Heterotardigrada

In *Echiniscus blumi*, *Pseudechiniscus* sp. and *Cornechiniscus lobatus*, the mouth opening is very narrow and positioned at the extremity of a cuticular protrusion of the body called the mouth cone (Figs. 1c and 1d). A buccal ring formed by a cuticular ring with a striated surface bearing a cuticular lamina around its distal margin (Figs. 8b and 8c) was detected only in *C. lobatus*. The absence of a cuticular ring in the other heterotardigrade species examined here could be due to loss of the buccal ring as a consequence of the extraction procedures employed (Guidetti *et al.,* 2012).

In all species, the external wall of the anterior portion of the buccal tube is surrounded by the buccal crown: a conical, dorso-ventrally compressed structure and bearing the laminae and crests for the insertion of stylet protractor muscles (Figs. 8d and 9c). The anterior opening of the buccal tube is within this conical structure (Fig. 3e). The buccal tube is narrow and straight with longitudinal striations on its surface. In *E. blumi* and *Pseudechiniscus* sp., the buccal tube is slightly enlarged after the stylet support insertion (Figs. 8-10). In *C. lobatus*, the buccal tube has a longitudinal dorsal crest followed by a narrow, thin, and flexible portion after the stylet support insertion point (Figs. 8b and 8f). This last flexible portion of the buccal tube bears another dorsal longitudinal crest.

In all species, the posterior portion of the buccal tube ends within the pharynx, and it is in cuticular continuity with the cuticle covering the pharyngeal lumen. This covering is characterised by three main longitudinal thickenings (pharyngeal bars), bifurcated at their proximal extremities, and located at 120° to each other, one dorsally and two ventro-laterally (Figs. 8-10). Between each pair of main pharyngeal bars, secondary longitudinal laminar thickenings are present in the inter-radial position (Figs. 8b, 9d and 10b). The cuticular lining of the pharynx continues into the narrow cylindrical oesophagus.

All species have a stylet system made up of stylet coats, piercing stylets and stylet supports on both sides of the buccal tube. The stylet coat is characteried by a long, anterior cylindrical portion corresponding to the stylet sheath. The stylet sheath is laterally reinforced by a rod-shaped cuticular thickening and it is connected to the buccal crown (Fig. 8d). Within the buccal crown, the two stylet sheaths cross each other: the left stylet sheath passes ventrally while the right stylet sheath passes dorsally (Figs. 3b-f). This crossing occurs immediately in front of the buccal tube where there are two symmetrical spherical enlargements located within the buccal crown (Figs. 3b, 3e and 9c). The stylet coat is never in contact with the buccal tube. The stylet furca constitutes the posterior portion of the stylet coat. Each stylet furca bears two large, short and flat branches with condyles at their extremities (Figs. 8g, 9b and 10d). The piercing stylet is a very long, straight needle-like structure, and has a deep longitudinal groove in its internal side (Figs. 8e, 9b and 10a). The two piercing stylets run parallel to the buccal tube but, because of the stylet sheath organisation, they cross each other before exiting the mouth opening. The stylet supports are inserted on the buccal tube at about 80% of the length of the rigid portion of the buccal tube. The distal extremity of the stylet support is fused to the arc between the two condyles of the stylet furca. In *E. blumi* and *Pseudechiniscus* sp., the stylet support is a long, thin cylindrical structure, the middle portion of which often disappears in treated specimens because of its tiny diameter or its chemical composition (Figs. 9a and 10c). In *C. lobatus*, the stylet support is proximally thin and distally enlarged (Fig. 8a).

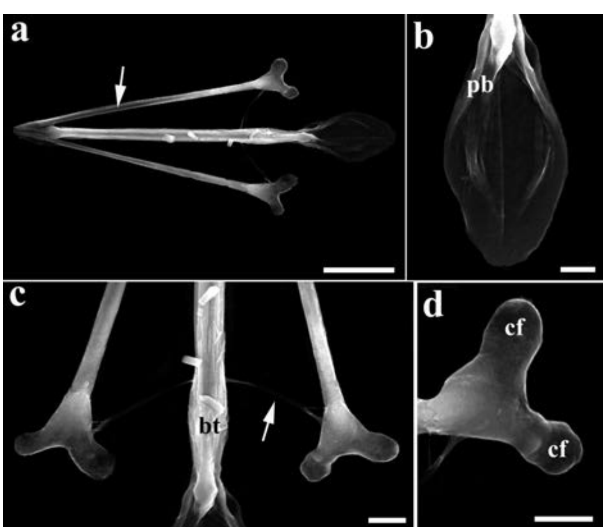

Fig. 10. *Pseudechiniscus* sp. (analysed using scanning electron microscopy). a) Buccal-pharyngeal apparatus with piercing stylets showing longitudinal groove (arrow); b) pharyngeal bars and secondary longitudinal laminar thickenings within the pharynx; c) buccal tube with thin stylet supports (arrow) connected to the style furcae; d) stylet furca.pb=pharyngeal bars; bt=buccal tube; cf=condyle of the furca. Scale bars of a)=10 μm; b-d)=2 μm.

DISCUSSION

Comparative analysis of the buccal-pharyngeal apparatuses in tardigrades

The first authors that truly emphasised the importance of the buccal-pharyngeal apparatus for tardigrade evolution, performing the first comparative study of SEM pictures of the feeding tardigrades structures, were Schuster *et al*. (1980). Based on their results, the data obtained by Guidetti *et al.* (2012), and the comparative analyses presented in this paper, the common sclerified structures of the tardigrade buccal-pharyngeal apparatus are: a buccal ring connected to a straight, rigid buccal tube, a buccal crown with laminae and crests for muscle attachments, a cuticular lining of the pharynx (connected with the buccal tube, and reinforced by longitudinal thickenings positioned at 120° to each other), and a stylet system composed of piercing stylets, enclosed within stylet coats (formed by the stylet sheaths and the stylet furcae), and stylet supports.

According to the results of this and previous studies (Schuster *et al*., 1980; Eibye-Jacobsen, 2001a; Dewel and Eibye-Jacobsen, 2006; Rebecchi *et al.,* 2008), the buccal-pharyngeal apparatuses of the Heterotardigrada Echiniscoidea are characterized by the presence of: i) a narrow, longitudinally striated buccal tube; ii) long stylets with wide stylet furcae; iii) stylets that run parallel to the buccal tube but cross each other before emerging from the mouth opening; iv) two piercing stylets each with a longitudinal groove down the internal side; v) pharyngeal bars in radial positions within the pharynx; vi) secondary longitudinal laminar thickenings in interradial positions (absent in the genus *Echiniscoides*; Eibye-Jacobsen, 2001a). Eibye-Jacobsen (2001b) considered the presence of pharyngeal bars as an apomorphy of the class Heterotardigrada. Eutardigrada differs from the Echiniscoidea by the absence of all the characters listed above for heterotardigrades and by the presence of short stylets that never reach the pharynx, which are therefore shorter than the buccal tube. All the eutardigrades analyzed in this study belong to the order Parachela. This order is characterized by a buccal-pharyngeal apparatus with: i) placoids (cuticular thickenings) in the pharynx; ii) pharyngeal apophyses in radial positions that alternate with rows of a double series of symmetrical cuticular placoids (macroplacoids) in interradial positions. According to Nichols *et al.* (2006), these characters are apomorphies of the order, or according to Eibye-Jacobsen (2001b), even apomorphies of the entire class Eutardigrada (assuming the placoids were lost in Apochela). We stress that *Platicrista* (Pilato, 1987) has pharyngeal apophyses (although they are extremely small) despite what was reported in the original description of the genus. The eutardigrade species belonging to class Apochela are characterized by a buccal-pharyngeal apparatus characterised by the absence of both the reinforcements in the cuticular lining of the pharynx (Eibye-Jacobsen, 2001a, 2001b; Dewel and Eibye-Jacobsen, 2006; Guidetti *et al.,* 2012) and the buccal crown (Guidetti *et al*., 2012), and by the presence of i) a short, wide buccal tube, ii) triangular-shaped stylet supports, and iii) short, thin stylets with stylet elbows (Guidetti *et al.,* 2012).

The heterotardigrade species belonging to Echiniscoidea analyzed so far showed a very uniform and constant shape of the buccal-pharyngeal apparatus (present study; Kristensen, 1987; Eibye-Jaconsen, 2001a; Dewel and Eibye-Jacobsen, 2006; Rebecchi *et al.,* 2008). In contrast, the eutardigrade buccal-pharyngeal apparatus is more heterogeneous and variable among species, and even between species belonging to the same families. For examples, *Doryphoribius flavus* and *Thulinius stephaniae* (present study) belong to the same family, but *Doryphoribius* has a very different buccal-pharyngeal apparatus and is the only genus of the family Isohypsiidae and the superfamily Isohypsibioidea to have a ventral lamina; *Dipascon* cf. *patanei*, *Platicrista angustata* (present study), *Hypsibius dujardini* (Doyère, 1840), and *Borealibius zetlandicus* (Murray, 1907) (Pilato *et al.,* 2006) belong to the same family Hypsibiidae, but they have very different buccal-pharyngeal apparatuses. Similar examples can be recorded among Macrobiotidae, in which the buccal-pharyngeal apparatuses of *Richtersius coronifer* (Richters, 1903) and *Paramacrobiotus richtersi* (Murray, 1911) are very dissimilar (Guidetti *et al.,* 2012). These differences in the degree of diversity of the feeding apparatuses within parachelans (Schuster *et al*., 1980) and between parachelans and echiniscids could be related to the more variable limno-terrestrial habitat colonized by parachelan families (*e.g.* freshwater substrates, lichens, bryophytes, leaf litter, soil, bio-films) that produced wider morphological adaptations to the different food sources as opposed to echiniscids, which are generally restricted only to bryophytes and lichens.

Evolutionary patterns of the piercing stylet system in tardigrades

The stylet system is composed of cuticular structures (*i.e.* stylet coats, stylet supports) and muscular fibres (*i.e.* protractor and retractor stylet muscles) that allow two piercing stylets composed of $CaCO_3$ to be pushed out of the mouth cavity to pierce the body wall or cell wall of a food source to access nutrients (Guidetti *et al.*, 2012). The piercing stylets are moved simultaneously and act symmetrically during their piercing action, so when the tips of the piercing stylets are inserted into the food, the buccal tube opening is almost completely obstructed and the food cannot be sucked into the buccal tube. The food is sucked inward when the piercing stylets are retracted and the myoepithelial muscular pharynx contracts. Therefore the piercing and sucking processes must alternate, which probably reduces food uptake in a given period of time in that sucking cannot take place continually. The fluid pressure existing in most prey (*e.g.* plant cells, nematode body cavity) probably assists the flow of liquid through the tardigrade buccal tube.

In heterotardigrades, the crossing of the piercing stylets before exiting from the mouth (present study; Dewel and Eibye-Jacobsen, 2006) may be disadvantageous because of the strong reduction in the size of the lumen of the buccal tube and the reduction of the penetration force of the piercing stylets due to their oblique trajectories. In echiniscids, this disadvantage may have been reduced by the evolution of a long buccal tube and long stylets. The increased length of these structures allows the piercing stylets to run more parallel to the buccal tube and therefore to exit straighter and more parallel to the mouth, although crossing is still not avoided there (Fig. 11a). Eutardigrades evolved different strategies to avoid problems related to the piercing stylet movements. In *Platicrista* species as well as in some other Itaquasconinae (Hypsibiidae) such as *Astatumen* species, and in the order Apochela (*e.g. Milnesium*; Guidetti *et al*., 2012), the buccal tube is wide or very wide, and the stylets are short and thin. As a consequence the piercing stylets run almost parallel to the buccal tube (Fig. 11b). In these species, the wide mouth opening, the thin stylets, and their parallel running avoid mouth obstruction during the piercing stylet operations [Fig. 12d; see also Fig. 17 in Dewel and Clark (1973) representing the cross-section of a *Milnesium* mouth]. In other Eutardigrada Parachela (*e.g. Ramazzottius* cf. *oberhaeuseri*, *T. stephaniae*; Figs. 4 and 6), the piercing stylets become curved; in this way the stylets do not cross each other when protruded from the mouth (Figs. 1e and 11c). Therefore, obstruction of the mouth opening is reduced and the anterior parts of the piercing stylets exit straight from the mouth, increasing their penetration force. A further development of this evolutionary trend could be represented by the development of an anterior bend in the buccal tube (Fig. 7b). The bend in the buccal tube allows the shift of the stylet sheaths from a lateral position with respect to the buccal tube, as in *T. stephaniae* (Figs. 4a and 12a), to a more ventral position, as occurred in several parachelan species such as in *Doryphoribius* (Fig. 7) and in Macrobiotoidea (Guidetti *et al.,* 2012). This ventral shift of the stylet sheaths is certainly an advantageous condition because it leaves a wide portion of the mouth opening free during the piercing stylet movements [Fig. 12c; see also Fig. 17 in Walz (1978) representing the cross-section of a macrobiotid mouth, and Fig. 65b in Michalczyk and Kaczmarek (2003), in which the stylet sheaths open in ventral position leaving the dorsal portion of the mouth opening free of obstruction]. Ventral stylet sheaths (with respect to the anterior portion of the buccal tube) are always associated with the presence of a ventral reinforcement on the buccal tube (ventral lamina), because it works as guide for the piercing stylet movement (Guidetti *et al*., 2012). In fact, the curved stylets, not aligned with the buccal tube, need a mechanical guide in their movement to reach the mouth opening. A ventral lamina occurs in all species with an evident anterior buccal tube bend such as the species belonging to *Doryphoribius* (present study) and in Macrobiotoidea species. The more the stylets are bent, the longer is the ventral lamina, while when the stylets are straight, the ventral lamina is absent or very short (Guidetti *et al*., 2012).

According to the equation of Hagen-Poiseuille, the length and diameter of the buccal tube strongly also influence the volumetric flow rate and pressure within the buccal tube, together with the sucking power of the pharynx, as also reported by Guidetti *et al*. (2012). Therefore, in addition to the obstructive effect due to the piercing stylet action, other factors are involved in tardigrade feeding strategies, and other selective forces may be involved in the evolution of the stylet system.

Convergent evolution of the structure of the buccal-pharyngeal apparatus in tardigrades

The selective advantage of the organization of the buccal-pharyngeal apparatus reducing the obstruction of the mouth opening has led to the convergent evolution of such organizations in independent lines of eutardigrades. For example, curved piercing stylets and ventral stylet sheaths associated with a ventral lamina may be advantageous for food uptake and have developed in the independent lines

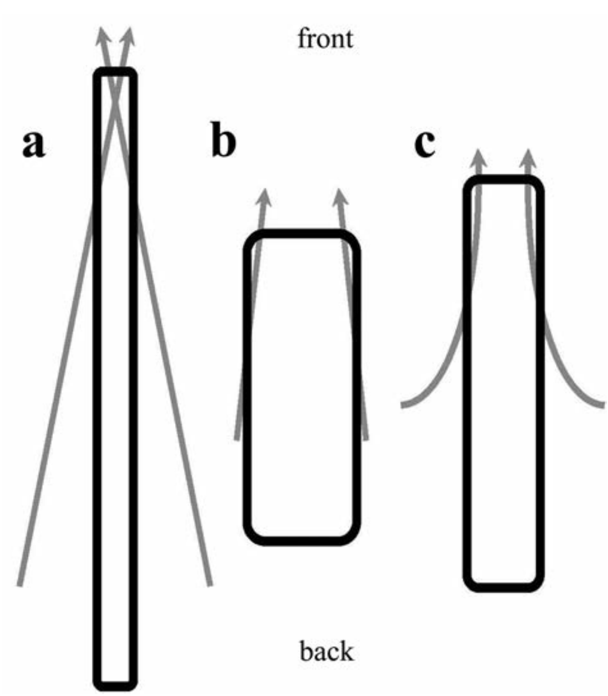

Fig. 11. Schematic relationships between the buccal tube (black) and the stylets (gray) in: a) Echiniscoidea (*e.g. Echiniscus blumi*); b) *Platicrista angustata* and Apochela (*e.g. Milnesium* species); c) Parachela (*e.g. Thulinius stephaniae, Doryphoribius flavus, Paramacrobiotus richtersi*).

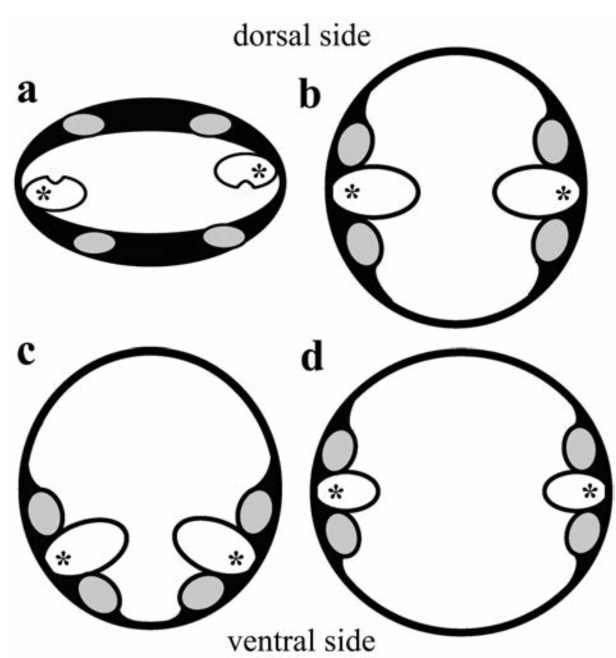

Fig. 12. Schematic representation of the transverse mouth section (at the level of the middle portion of the stylet sheath), representing the relationships between the buccal tube (black), the stylet sheaths (asterisk) and the four buccal sensory organs (gray) in: a) Echiniscoidea (*e.g. Echiniscus blumi*); b) Isohypsibioidea (*e.g. Thulinius stephaniae*); c) *Doryphoribius flavus* and Macrobiotoidea (*e.g. Paramacrobiotus* species); d) *Platicrista angustata* and Apochela (*e.g. Milnesium* species).

of *Doryphoribius* (Isohypsibioidea) and Macrobiotoidea (*e.g. Macrobiotus* and *Paramacrobiotus*). Even though *Doryphoribius* and the genus *Paramacrobiotus* belong to different superfamilies, the general organization of their buccal-pharyngeal apparatus is very similar (present study; Guidetti *et al.,* 2012). The only detectable differences are at the level of the buccal ring (in *Doryphoribius* buccal lamellae are absent, and the buccal armature when present is also different) and of the stylet furca (in *Doryphoribius* apophyses are present on the branches of the furcae). Advantageous anatomical changes may have developed in the buccal-pharyngeal apparatuses as a consequence of convergent evolution in *Milnesium tardigradum* Doyère, 1840 (Apochela) and *Platicrista angustata* (Parachela). Even though they belong to different eutardigrade classes, they share many morphological characters: a wide buccal tube; absence of a buccal crown; wide oval perforated areas; short, thin, and straight stylets with a stylet elbow; small triangular-like stylet furcae with small condyles; short stylet supports that can move backwards, becoming aligned with the buccal tube (present study; Guidetti *et al.,* 2012).

Other characters of the buccal-pharyngeal apparatus shared among eutardigrade species belonging to different evolutionary lines are probably not due to common origin but to convergent evolution. For example, a flexible caudal portion of the buccal tube provided with a spiral thickening such as described here for *Diphascon* cf. *patanei* and *P. angustata* (Hypsibiidae) also developed in several evolutionary lines of eutardigrades, belonging to both Apochela (*i.e. Limmenius*, Milnesiidae) and Parachela (*i.e. Biserovus, Insuetifurca* and *Minilentus*, Macrobiotidae, Macrobiotoidea; *Eohypsibius*, Eohypsibiidae, Eohypsibioidea; *Hebesuncus*, Ramazzottidae, Hypsibioidea) (Guidetti and Pilato, 2003). The selective advantage of this flexible portion may be related to the broader movements of the buccal tube within the body cavity that allow the buccal tube, and the associated piercing stylets, to always be perpendicular to the wall of the food sources during food acquisition. In the eutardigrade genus *Parascon* (Hypsiibidae; Pilato and Binda, 1987) and in the heterotardigrade genera *Cornechiniscus* (present study), *Novechiniscus, Proechiniscus,* and *Mopsechiniscus* (Echiniscidae; Kristensen, 1987; Rebecchi *et al.,* 2008), a flexible posterior portion of the buccal tube is also present, but its flexibility is a consequence of the reduction of the cuticle thickness and not a result of the development of a spiral thickening, as in the previous eutardigrade genera.

CONCLUSIONS

Finally, from an evolutionary point of view, the presence of buccal lamellae on the buccal ring of the buccal-pharyngeal apparatus is another enigmatic character since it is unclear whether or not it developed by convergence in different evolutionary lines. Buccal lamellae can be found in eutardigrades belonging to Parachela (*i.e.* all Macrobiotoidea genera, and the two Isohypsibioidea genera *Thulinius* and *Pseudobiotus*) and in all Apochela genera (*e.g. Milnesium*). The function of these buccal lamellae is still unclear. Only the buccal lamellae of the carnivorous apochelan species are wide enough to be able to close the mouth opening and possibly used to grasp prey. Those of macrobiotoids and isohypsibioids are small compared with their mouth opening and are probably used to increase the adhesion of the mouth opening to the substrate, thus enabling a more efficient sucking action (Guidetti *et al*., 2012).

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