Zootaxa 3681 (3): 270–280
www.mapress.com/zootaxa/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

http://dx.doi.org/10.11646/zootaxa.3681.3.6
http://zoobank.org/urn:lsid:zoobank.org:pub:F8E35CEC-5741-474F-B363-E7BC6DA678D3

Ramazzottius thulini (Pilato, 1970) *bona species* and description of *Ramazzottius libycus* sp. nov. (Eutardigrada, Ramazzottidae)

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Abstract

Comparing the type series of *Ramazzottius thulini* with specimens of *Ramazzottius oberhaeuseri*, from the *locus typicus*, we confirmed there are morphological and metric differences between these two species. This evidence convinced us that *R. thulini* should be considered a *bona species*. A new species, *Ramazzottius libycus* **sp. nov.,** is also described. *Ramazzottius libycus* **sp. nov.** differs from *R. oberhaeuseri* in having more slender internal claws I–III and anterior claws IV, and other metric differences relative to all claws. The new species differs from *R. thulini* in details of the cuticular ornamentation, stylet supports inserted on the buccal tube in a slightly more caudal position, internal claws slightly different in shape, and some metric differences relative to all claws. It appears *R. thulini* and *R. libycus* **sp. nov.** are sibling species of *R. oberhaeuseri* and therefore the authors expect that there are other cryptic species included under the *R. oberhaeuseri* name still awaiting to be recognized and described.

Key words: Eutardigrada, *Ramazzottius oberhaeuseri*, *R. thulini*, *R. libycus* **sp. nov.**

Introduction

Since the original description, *Ramazzottius thulini* (Pilato, 1970) has appeared as a problematic species, some authors considered it as a synonym of *Ramazzottius oberhaeuseri* (Doyère, 1840) (e.g. Maucci, 1986), others considered *R. thulini* as a valid species or left the problem of an eventual synonymy unsolved (e.g. Biserov 1997/98).

At present 26 species of the genus *Ramazzottius* are known (Degma *et al*., 2012; some of which have a smooth cuticle, while in others the dorsal and lateral cuticle is completely or partially sculptured. In some species a degree of individual variability may be noted in the extent and visibility of the cuticular ornamentation. Specimens have been ascribed to *Ramazzottius semisculptus* Pilato & Rebecchi, 1992, which exhibit either a faint cuticular ornamentation or a smooth cuticle. Specimens attributed to *R. oberhaeuseri* have generally sculptured cuticle, but also specimens with smooth cuticle have been attributed to that species (Durante Pasa & Maucci, 1979). All the known *Ramazzottius* species lay eggs free and with variously shaped processes but only two (*R. oberhaeuseri* and *R. thulini*) produce eggs with mostly hemispherical and few conical or trunco-conical processes. This character reduced to two the number of comparative species required for us to establish whether *R. thulini* is or is not a *bona species*. However, a specific diagnosis of *R. oberhaeuseri sensu stricto* is a difficult problem to solve because there is no type-series and, as mentioned above, authors attributed to this species both specimens with sculptured and smooth cuticle. Richters (1908) instituted the form *R. o. granulosus* for the specimens with ornamented cuticle, but most authors have not considered it opportune to distinguish between sculptured and smooth cuticle forms.

In the species description, Doyère (1840 as *Macrobiotus oberhaeuseri*) does not specifically mention any cuticular ornamentation. This implies either the cuticle was smooth or that the quality of Doyère's microscope was not good enough to observe sculptured ornamentation. The latter hypothesis is less than plausible since Spallanzani (1776), several years earlier, noted cuticular ornamentation of the first tardigrade examined (which according to some authors (Perty, 1835; Della Valle 1915) could have been *R. oberhaeuseri*).

 Accepted by Sandra J. McInnes: 17 May 2013; published: 21 Jun. 2013

On examining slide mounted specimens attributable to *R. oberhaeuseri* collected in the *locus typicus*, we noted that the dorsal cuticle, from the anterior to the posterior end of the body was ornamented with many polygonal tubercles (less visible in the anterior portion of the body and gradually more visible in the posterior portion). It is possible to hypothesize that the name *oberhaeuseri* has been attributed to more than one species, as had occurred in other tardigrade species (e.g. for the *Paramacrobiotus richtersi* group or the *Macrobiotus hufelandi* group). In this situation, it is not possible to ascertain whether the cuticle of *R. oberhaeuseri sensu stricto* is smooth or ornamented, or whether this is a variable character. However, although type material is not available we do have at hand slides of specimens attributable to *R. oberhaeuseri* and collected in the *locus typicus*, with which we compared *R. thulini* taking into consideration all characters, not just cuticle ornamentation. We also compared examples from a homogeneous Swedish population of smooth cuticle specimens that were attributed to *R. oberhaeuseri* by Durante Pasa & Maucci (1979), which seemed to us to belong to an undetermined species. Finally, we examined new material from Libya and described a new species *R. libycus* **sp. nov.**, a sibling species to *R. thulini* and *R. oberhaeuseri*.

Material and methods

We examined the type series of *R. thulini* (from Apuane Alps, Levigliani), *locus typicus* (Paris) specimens of *R. oberhaeuseri* (kindly sent to us by Lorena Rebecchi and Tiziana Altiero, colleagues at the University of Modena and Reggio Emilia), two specimens from Sweden (Furuvik) and eight specimens from Libya (Barce) (all specimens collected in moss samples). All the examined specimens (mounted in polyvinyl lactophenol) are deposited in the Binda & Pilato collection (Department of Animal Biology "Marcello La Greca", University of Catania, Italy).

Measurements, given in micrometers (µm), and photomicrographs, were made with a Leica Phase Contrast Microscope equipped with a "Canon S40" digital camera and using Adobe Photoshop Elements digital imaging software.

Structures in comparable orientations were measured only if they were not damaged. The body length, excluding the hind legs, was measured from the anterior margin to the end of the body. We calculated the values of the *pt* index relative to many structures; in particular the value of that index relative to the stylet supports insertion point on the buccal tube is very useful, as it proved to be only slightly variable within each species (Pilato, 1981; Pilato *et al*., 1982, Pilato *et al*. 2007). The buccal tube length and the level of the stylet supports insertion on the buccal tube were measured according to Pilato (1981).

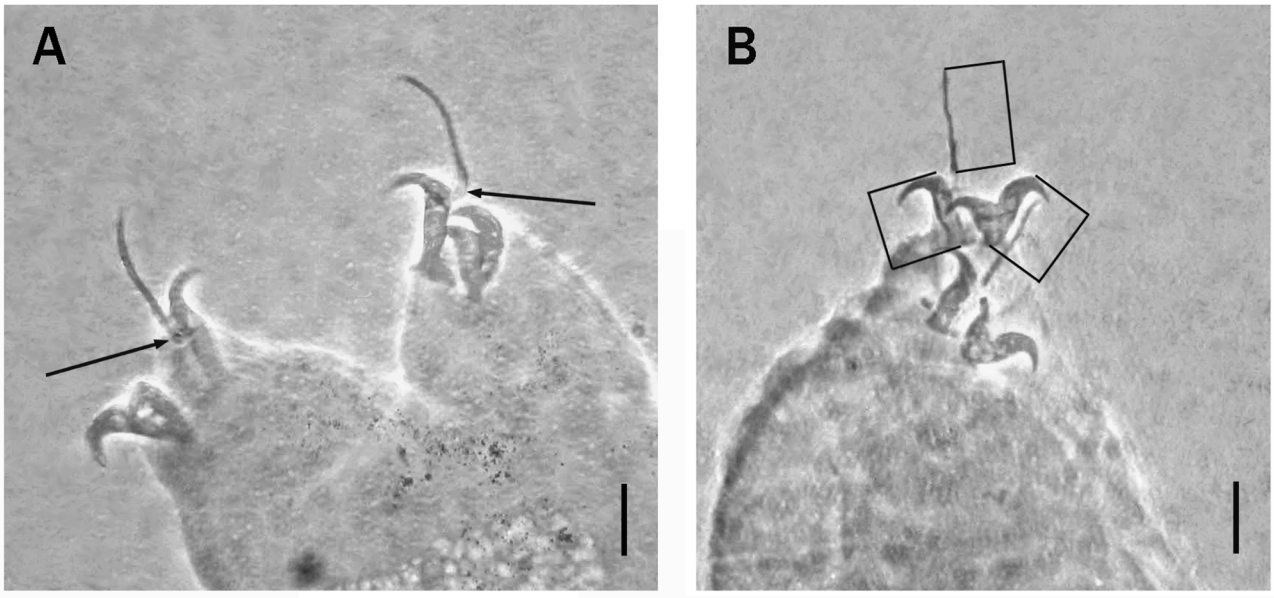

FIGURE 1. A, Arrows indicate the flexible tract by which the main branch is connected to the basal portion + secondary branch in the external claws. B, Measured sections of the claws.

The flexibility of the tract which connects the external claw main branch to the basal portion + secondary branch is a known problem in the genus *Ramazzottius*. This long, flexible main branch can be more or less bent (Fig. 1A) or variably orientated making total claw length appear inconsistent even within the same specimen (Figs. 2B). As a consequence, only claws in exactly the same condition and orientation are comparable; but this is rare. To avoid this difficulty in measuring the external claws, we measured separately only the sclerified portion of the main branch, and the common portion + the secondary branch (as shown in Fig. 1B). We also calculated the ratio of the main branch length with respect to the length of the common portion + the secondary branch, expressed as a percent. It was not clear whether the *HBI* ratio used by Dastych (2006), included the flexible portion of the claw. We are only measuring the sclerified portions of the claw, excluding the flexible section.

Results

A comparison of morphological and metric characters of the holotype and 6 paratypes of *R. thulini* with *locus typicus* (Paris) specimens attributable to *R. oberhaeuseri* (with ornamented cuticle), allowed us to notice that the former can be distinguished from the latter as follows:

Eight transversal bands of reddish pigment but longitudinal uncoloured bands are not present (compared with the five longitudinal bands, where the median is especially remarkable for *R. oberhaeuseri*). Unfortunately because we lacked access to more abundant populations, we were unable to notice any individual variability of this character.

The dorsal and lateral cuticle is ornamented with polygonal tubercles (up to 3 µm in the posterior portion of the body) but, unlike the specimens from the *locus typicus* of *R. oberhaeuseri*, the anterior portion, from the head to the first half segment over the first pair of legs, is smooth. In *R. oberhaeuseri* the cuticular ornamentation is present from the head to the caudal portion of the body but, as in some other species of the genus, it is less visible in anterior section.

The stylet supports in *R. thulini* are inserted on the buccal tube in a slightly more cephalic position (*pt = 57.3–57.*9 in *R. thulini*, *58.3–60.0* in *R. oberhaeuseri*); the *pt* values relative to the internal claws I–III and to the anterior claws IV are slightly higher in *R. thulini* (Table 1).

Differences can be noted in the claw shape. In *R. thulini* the secondary branch of each internal claw has a stouter basal portion; it narrows abruptly (while in *R. oberhaeuseri* it tapers gradually), therefore, its terminal portion is relatively thinner and slightly shorter than in *R. oberhaeuseri* (Figs. 2C–E and 3D, E). No metric difference can be noted relative to the measured portions of the external claws (Table 1).

The eggs of the compared species are similar.

As the individual variability of the characters studied for *Ramazzottius thulini* are low and, though similar, provide sufficient differences from the *locus typicu*s specimens of *R. oberhaeuseri*, we therefore considered it a valid species.

As mentioned above, there is a possibility that the true *R. oberhaeuseri* has smooth cuticle, so we compared *R. thulini* with two unsculptured specimens (from Sweden) and again we noted clear differences for quantitative and qualitative characters. The buccal tube is slightly longer with respect to the body length (Table 1); *R. thulini* has stylet supports inserted on the buccal tube in a slightly more cephalic position (*pt = 57.3 – 57.9* in *R. thulini*, *59.2* in one smooth (though slightly larger) Swedish specimen (Table 1); the internal claws have wider basal extremities and stouter, slightly shorter, main branches (Figs. 2C, D and 5A); in the external claws the *pt* values relative to the sclerified portion of the main branches are lower, and the percent ratio between the main branch length and the length of the basal portion + secondary branch is also clearly lower (Table 1).

The smooth specimens also differ from the *locus typicus* sculptured form of *R. oberhaeuseri*; the buccal tube is slightly shorter with respect to the body length (Table 1); the internal claws have more slender basal extremities and more slender, slightly longer main branches (Figs. 5A and 3B–E); in the external claws the *pt* values relative to the sclerified portion of the main branches are higher, and the percent ratio between the main branch length and the length of the basal portion + secondary branch is also higher (Table 1).

It is evident that the smooth specimens we examined belong to a different species; neither *R. thulini* nor the sculptured *locus typicus* form of *R. oberhaeuseri*. However, these samples came from Sweden not the species *locus typicus*, and we have no eggs; nor did Durante Pasa & Maucci (1979) mention any eggs. We therefore have to consider these specimens belong to a different, undetermined species.

In conclusion, as a result of the above mentioned comparisons, we confirm that *R. thulini* should be considered a *bona species.*

TABLE 1. Length (in µm) and other parameters of selected structures (using, where possible, animals with similar body length), for two specimens of *Ramazzottius thulini*, two *R. oberhaeuseri* from the *locus typicus*, one Swedish *Ramazzottius* sp. (with smooth cuticle), and three *Ramazzottius libycus* **sp. nov**. Due to the variable orientation of the external claws, only the length of the sclerified portion of the main branch and basal portion + secondary branch of each claw have been measured. *Pt* ratios in italics.

Specimens	*R. thulini*		From *locus typicus* of *R. oberhaeuseri*		Swedish *Ramaz-zottius sp.*	*R. libycus* **sp. nov.**		
Slide No.	918	917 holotype	5506	5505	2695	2754 holotype	2754	2753
Body length	234	257	248	274	336	286	289	301
Buccal tube length	28.0	29.9	29.8	31.5	32.2	29.0	29.0	30.2
% buccal tube length/body length	*12.0*	*11.6*	*12.0*	*11.5*	*9.6*	*10.1*	*10.0*	*10.0*
Internal buccal tube width	1.5	1.5	1.2	1.3	1.5	1.5	1.5	1.6
pt	*5.4*	*5.0*	*4.3*	*4.1*	*4.7*	*5.2*	*5.2*	*5.2*
Stylet support insertion point	*57.9*	*57.3*	*58.3*	*60.0*	*59.2*	*60.0*	*59.6*	*61.7*
Placoid row	7.0	7.7	7.4	8.0	7.9	7.6	7.7	7.5
pt	*25.0*	*25.7*	*24.8*	*25.4*	*24.5*	*26.2*	*26.6*	*24.8*
First placoid	3.7	3.8	3.8	4.3	4.6	3.9	3.9	4.0
pt	*13.2*	*12.7*	*12.8*	*13.6*	*14.3*	*13.4*	*13.4*	*13.2*
Second placoid	3.0	3.3	3.1	3.4	3.4	3.1	3.0	3.2
pt	*10.7*	*11.0*	*10.4*	*10.8*	*10.6*	*10.7*	*10.3*	*10.6*
Ext. claw II main branch	8.3	10.4	9.7	?	12.1	?	?	12.8
pt	*29.6*	*34.8*	*32.6*	*?*	*37.6*	*?*	*?*	*42.4*
Basal portion + sec. branch	7.9	9.6	8.9	?	10.2	?	?	12.0
pt	*28.2*	*32.1*	*29.9*	*?*	*31.7*	*?*	*?*	*39.7*
% main branch / basal portion +sec. branch	105.1	108.3	109.0	?	118.6	?	?	106.7
Int. claw II	9.5	9.9	9.1	?	10.9	11.2	11.2	11.6
pt	*33.9*	*33.1*	*30.5*	*?*	*33.9*	*38.6*	*38.6*	*38.4*
Ext. claw III main branch	?	10.6	10.6	12.0	?	11.9	12.4	13.2
pt	*?*	*35.4*	*35.6*	*38.1*	*?*	*41.0*	*42.8*	*43.7*
Basal portion + sec. branch	?	9.6	9.4	10.9	?	11.0	10.9	12.0
pt	*?*	*32.1*	*31.5*	*34.6*	*?*	*37.9*	*37.6*	*39.7*
% main branch / basal portion + sec. branch	*?*	*110.4*	*112.8*	*110.1*	*?*	*108.2*	*113.8*	*110.0*
Int. claw III	?	?	9.3	9.9	?	11.2	11.1	12.2
pt	*?*	*?*	*31.2*	*31.4*	*?*	*38.6*	*38.3*	*40.4*
Post. claw IV main branch	12.2	14.5	13.0	14.5	?	15.3	15.1	15.1
pt	*43.6*	*48.5*	*43.6*	*46.0*	*?*	*52.8*	*52.1*	*50.0*
Basal portion + sec. branch	10.1	11.6	10.2	11.6	?	11.2	11.3	11.5
pt	36.1	38.8	34.2	36.8	?	38.6	39.0	38.1
% main branch / basal port.+ sec. branch	*121.0*	*125.0*	*127.4*	*125.0*	*?*	*136.6*	*133.6*	*131.3*
Ant. claw IV	?	11.2	10.3	11.5	?	12.2	?	12.3
pt	*?*	*37.4*	*34.6*	*36.5*	*?*	*42.1*	*?*	*40.7*

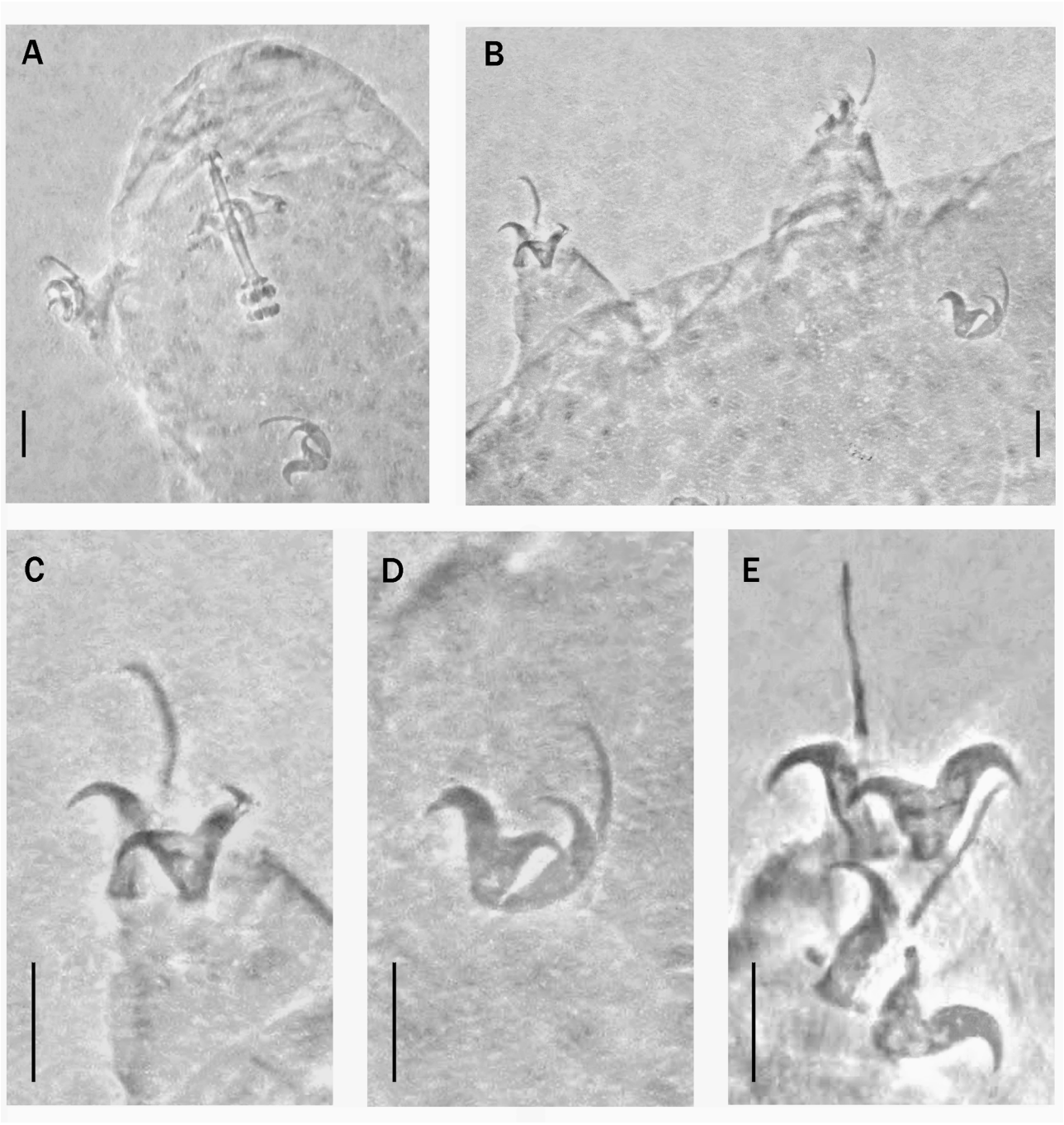

FIGURE 2. A–E, *Ramazzottius thulini* (holotype). A, Buccal-pharyngeal apparatus. B, Claws of the second and third pairs of legs. C, Claws of the second pair of legs. D, Claws of the third pair of legs. E, Claws of the hind legs. Scale bar = 10 μm.

***Ramazzottius libycus* sp. nov**.
(Figs. 5B–D, 6)

Material examined. Libya, Barce (32°29'17"N; 20°50'19"E), in the neighbourhood of the town; moss sample: holotype (slide No. 2754), 7 paratypes (slides No. 2753 and 2754), and three eggs (slides No. 2731 and 2752) collected in 1975 by the colleagues Francesco Furnari and Salvatore Brullo (University of Catania).

Type repository. Holotype and paratypes are deposited in the Binda & Pilato collection (Museum of the Department of Animal Biology "Marcello La Greca", University of Catania, Italy).

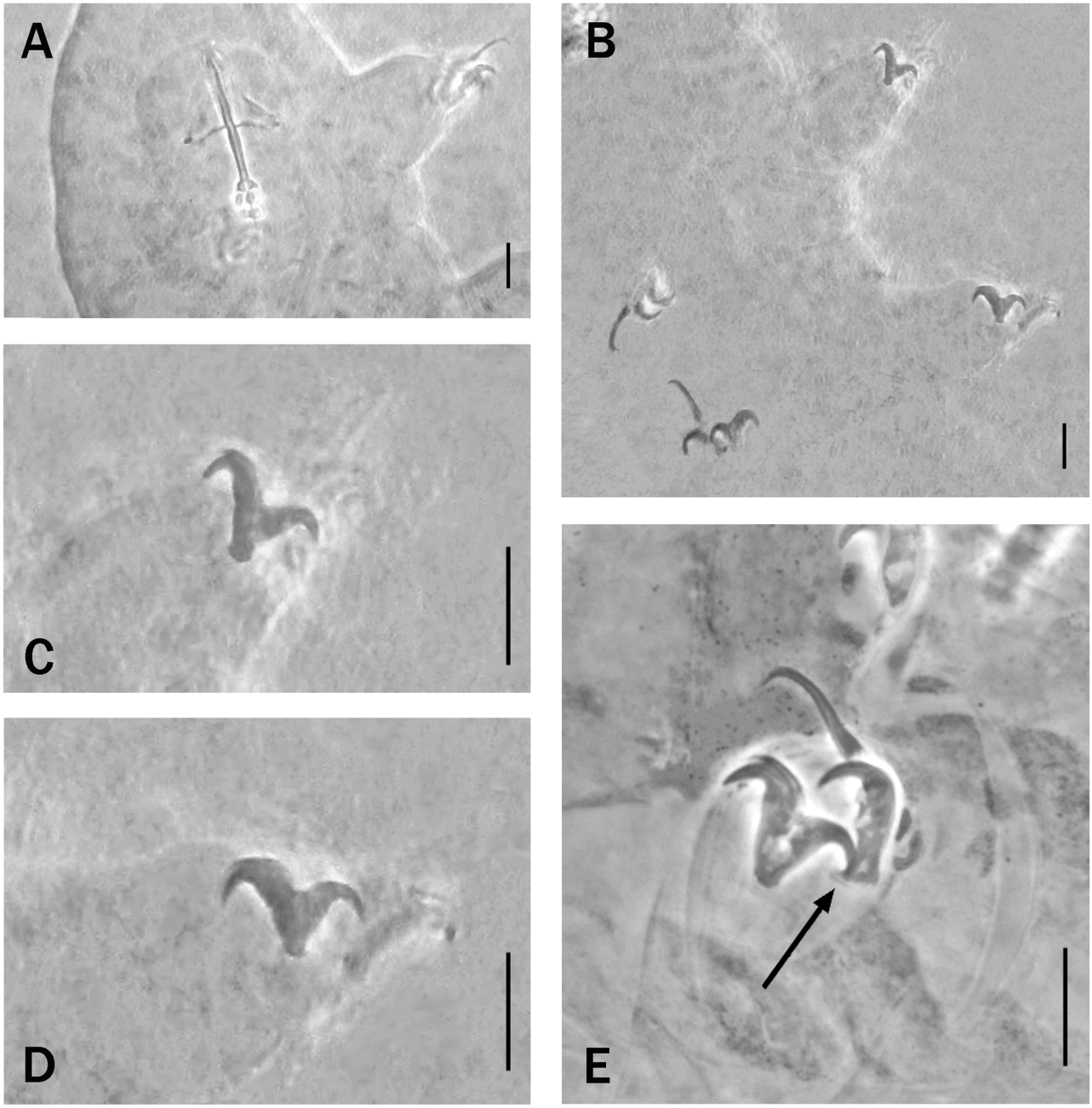

FIGURE 3. A–E, *Ramazzotius* specimens from the *locus typicus* of *R. oberhaeuseri*. A, Buccal-pharyngeal apparatus. B, Claws of the first and second pairs of legs. C, Internal claw of the first pair of legs. D, Internal claw of the third pair of legs. E, Claws of the second pair of legs. Arrow indicates a visible lunule. Scale bar = 10 µm.

Specific diagnosis. Cuticle ornamentation and colour (5 longitudinal and 9 transverse pigmented bands) as usually in *R. oberhaeuseri*; eye spots absent; a pair of elliptical cephalic organs present; buccal-pharyngeal apparatus of *Hypsibius* type (*Ramazzottius* variant), pharyngeal bulb with two macroplacoids; microplacoid and septulum absent; claws of *Ramazzottius* type; small lunules, difficult to see, present. Eggs, laid free, with hemispherical and, very rarely, trunco-conical and conical processes.

Description of the holotype. Body length 286 µm; colour (5 longitudinal and 9 transverse reddish bands separated by unpigmented bands) and cuticular ornamentation as usually in *R. oberhaeuseri*; polygonal tubercles, less visible in the anterior portion of the body and more visible in the posterior portion, are present having diameter up to 2.6 µm (Fig. 5B); eye spots absent; a paired of cephalic, elliptical, organs present as ascertained in most species of the genus; buccal-pharyngeal apparatus of *Hypsibius* type (*Ramazzottius* variant) i.e. with rigid buccal

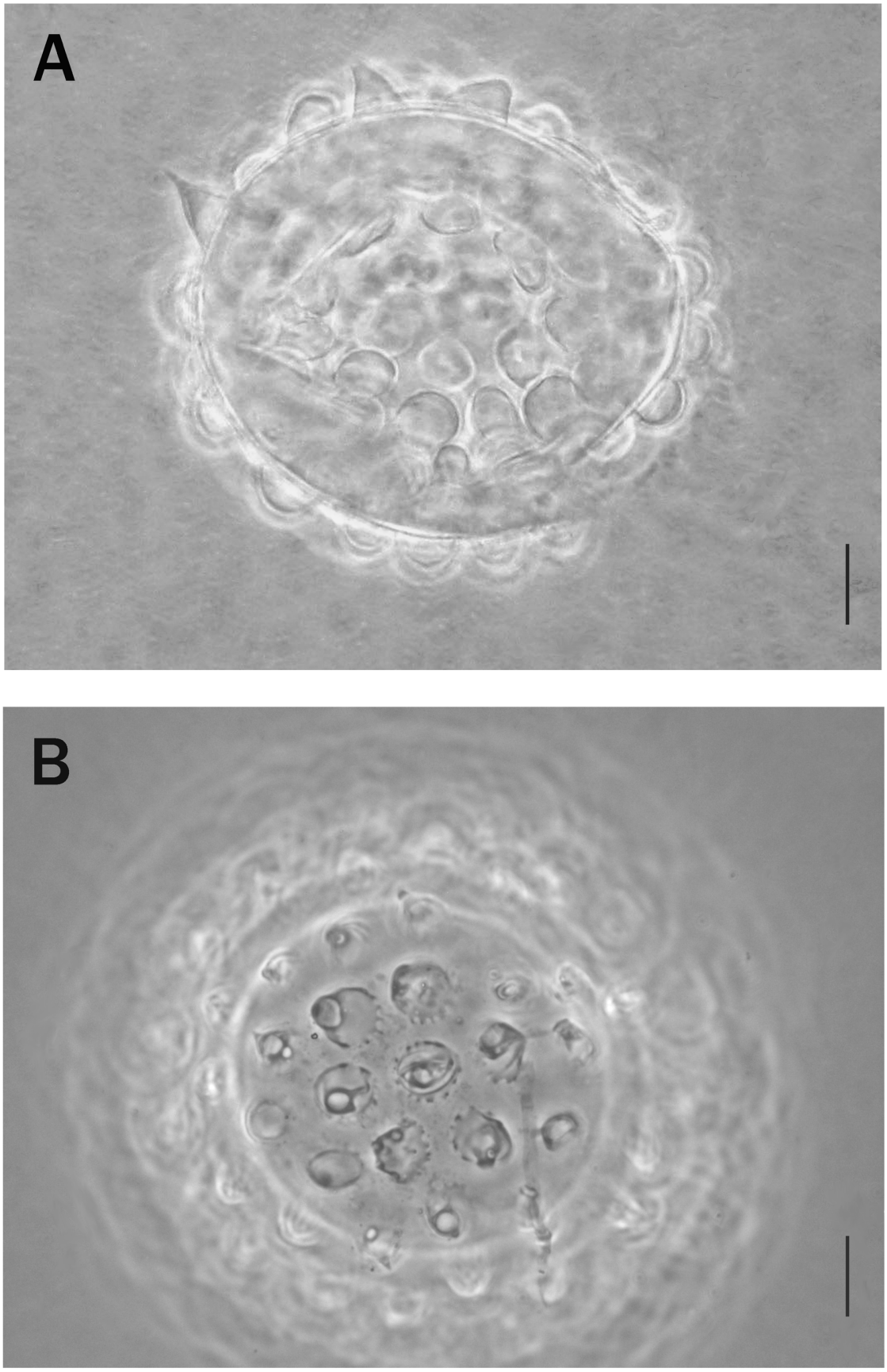

FIGURE 4. A–B, *Ramazzottius* egg from the *locus typicus* of *R. oberhaeuseri*. Scale bar = 10 µm.

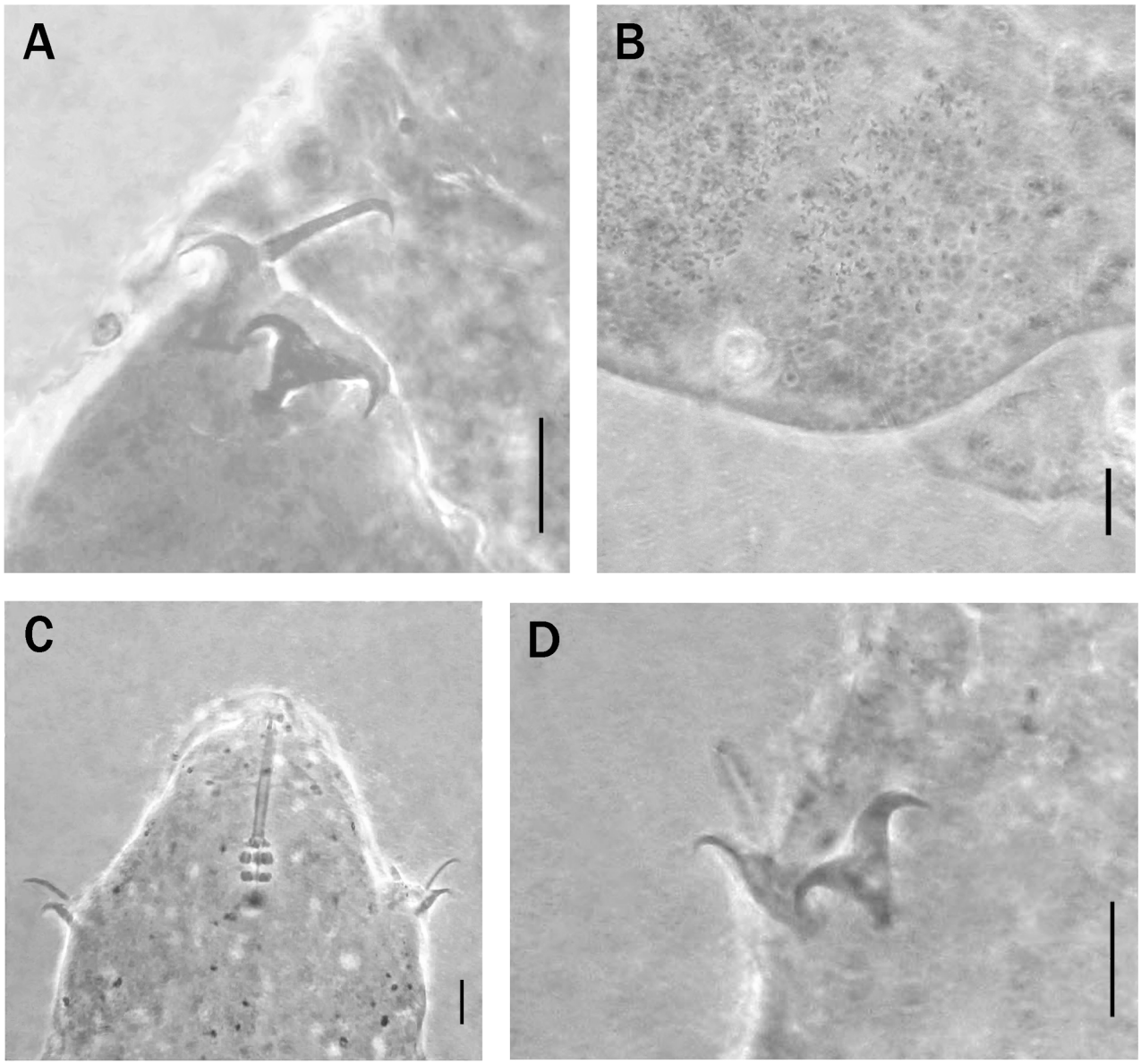

FIGURE 5. A, *Ramazzottius* **sp.** with smooth cuticle from Sweden. Claws of the second pair of legs. B–D, *Ramazzottius libycus* **sp. nov.** B, Cuticle ornamentation (paratype). C, Buccal-pharyngeal apparatus of the holotype. D, Claws of the second pair of legs of the holotype. Scale bar = 10 µm.

tube without ventral lamina, dorsal and ventral apophyses for the insertion of the stylet muscles in shape of blunt hooks, asymmetrical with respect to the frontal plane; peribuccal lamellae and peribuccal papulae absent (Pilato & Binda, 2010). Buccal tube, 29 µm long (with a slightly thicker tube wall immediately caudally to the insertion of the stylet supports) (Fig. 5C), and 1.5 µm wide internally (*pt = 5.2*). Stylet supports inserted on the buccal tube at 60 % of its length (*pt = 60.0*). Pharyngeal bulb with apophyses and two short macroplacoids; microplacoid and septulum absent (Fig. 5C); first macroplacoid 3.9 µm long (*pt = 13.4*), second 3.1 µm long (*pt = 10.7*); entire placoid row 7.6 µm long (*pt* = 26.2).

Claws, of *Ramazzottius* type (Fig. 5C, D and 6A, B), well developed; length of external and internal claws of the third pair of legs 19.1 µm (*pt = 65.9*) and 11.2 µm (*pt = 38.6*), respectively; length of posterior and anterior claws of the hind legs 24.6 µm (*pt = 84.8*) and 12.2 µm (*pt = 42.1*), respectively. Due to the difficulty in measuring the total length of the external claws, we provided the separate lengths of the sclerified portions of the main branches and the basal portion + secondary branch in Table 1. Thin accessory points present on the main branches of all claws. Small, difficult to see, lunules present on all legs (Fig. 6A, B). Other cuticular thickening on the legs, absent.

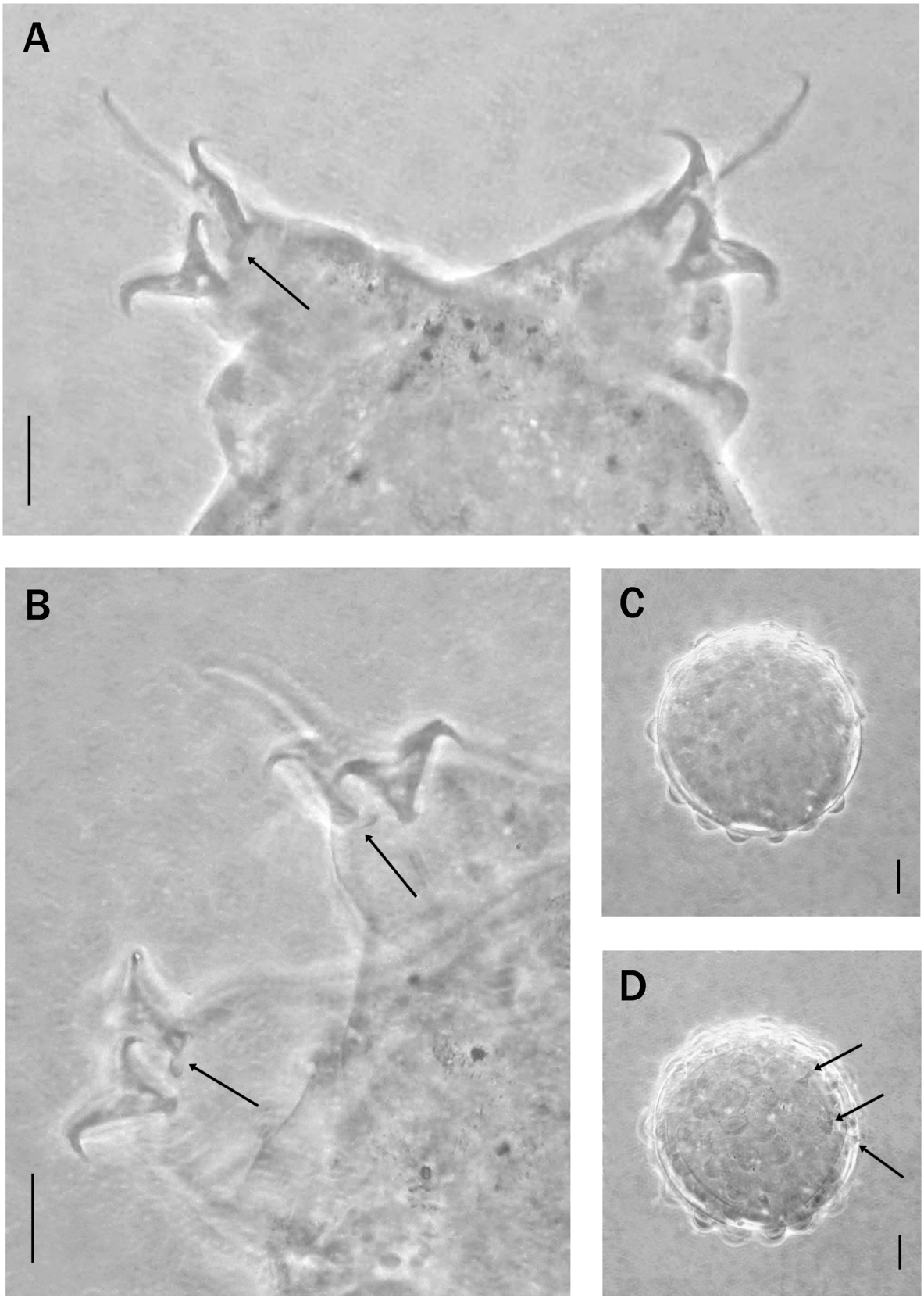

FIGURE 6. A–D, *Ramazzottius libycus* **sp. nov.** A, claws of the hind legs of the holotype. Arrow indicates a visible lunule. B, Claws of the hind legs of a paratype. Arrows indicate visible lunules. C, D, Eggs. Arrows indicate some conical or truncoconical processes. Scale bar = 10 µm.

Eggs laid freely (Fig. 6C,D); we found three eggs but only one is measurable; its the diameter is 60.0 µm excluding and 64.0 µm including processes; there are 16 processes in the circumference and 48 in the hemisphere. Most processes are hemispherical, 3.0–3.4 µm high, with the basal diameter 8.0–8.6 µm. Few processes are trunco-conical or conical in shape (Fig. 6D).

Remarks. Paratypes similar to the holotype in both qualitative and metric characters: Measurements of some structures are given in Table 1.

Etymology. The name refers to the geographic area (Libya) of the *locus typicus* (Barce).

Differential diagnosis. For many characters of the adults and the eggs, *Ramazzottius libycus* **sp. nov**. is very similar to *R. oberhaeuseri* and *R. thulini*. However, it differs from *R. oberhaeuseri* in having slightly shorter buccal tube with respect to the body length (% ratio = 10.0–10.1 in *R. libycus* **sp. nov**.,11.5–12.0 in *R. oberhaeuseri*); more slender internal claws I–III and anterior claws IV (Figs. 5D, 6A, B and 3C–E) with higher values of the *pt* index (Table 1); higher *pt* values of the sclerified portion of both the main branches and basal portion + secondary branch in the external claws; the percent ratio between these portions of the claw is the same in the legs I–III but higher in the hind legs (Table 1). The eggs have a lower number of processes in the hemisphere (48 in *R. libycus* **sp. nov**., 57–60 in *R. oberhaeuseri*), but the number of eggs we examined is too low for us to know the range of individual variability of this character and therefore whether or not this is a significant difference.

R. libycus **sp. nov**., differs from *R. thulini* in having polygonal tubercles also in the anterior extremity of the body, slightly shorter buccal tube with respect to the body length (% ratio = 10.0–10.1 in *R. libycus* **sp. nov**., 11.6–12.0 in *R. thulini*); stylet supports inserted on the buccal tube in a slightly more caudal position (*pt* = 59.6 – 61.7 in *R. libycus* **sp. nov**., 57.3 – 57.9 in *R. thulini*: Table 1); different shape of the internal claws: the main branches of *R. libycus* **sp. nov**. are longer and more slender, and the secondary branches are longer and taper gradually, whereas in *R. thulini* the secondary branches taper more abruptly (Figs. 5D, 6A, B and 2C, D, E); higher *pt* index values relative to internal claws I–III and anterior claws IV (Table 1); in the external claws the *pt* values of both the sclerified portion of the main branches and the basal portion + secondary branches length are higher; in legs I–III the percent ratio between the length of these structures is the same but it is clearly higher in the hind legs (Table 1). The eggs have a lower number of processes in the hemisphere (about 48 in *R. libycus* **sp. nov**., 57–60 in *R. thulini*), but the limited number of eggs we examined for *R. libycus* **sp. nov**. has not provided the range of the individual variability and we cannot say this is a significant difference.

Ramazzottius libycus **sp. nov**. is clearly different from the Swedish smooth specimens, which Durante Pasa & Maucci (1979) attributed to *R. oberhaeuseri*, in having ornamented cuticle; higher *pt* index values relative to the internal claws (Table 1); external claws with larger *pt* values relative to the basal portion + secondary branch (Figs. 5C, 6A, B and 5A; Table 1) and different percent ratio between the main branch and the basal portion + secondary branch (Table 1). We have no eggs produced by the specimens with smooth cuticle for comparison.

Conclusion

There is a degree of uncertainty about the individual variability of the cuticular ornamentation of *R. oberhaeuseri sensu stricto*. However, our comparison of *R. thulini* with both ornamented specimens (*R. oberhaeuseri* from *locus typicus*) and smooth cuticle specimens (that in our opinion belongs to an undetermined *R. oberhaeuseri*-type species), confirmed that *R. thulini* is a distinct species. While very similar to *R. oberhaeuseri*, *R. thulini* must to be considered as a *bona species.* We also described a new species, *R. libycus* **sp. nov.**, from Libya, which is similar to *R. oberhaeuseri* and *R. thulini* in many characters of the adults and eggs. These results lead us to consider *R. thulini* and *R. libycus* **sp. nov.** as sibling species of R. *oberhaeuseri*, and to believe that a group of cryptic species is probably included under the name *R. oberhaeuseri*, which still await to be recognized and described. In this case, the geographic distribution of *R. oberhaeuseri*, today considered as a cosmopolitan species, will require revision in future.

Acknowledgements

We want to thank Lorena Rebecchi and Tiziana Altiero (University of Modena e Reggio Emilia) who furnished

specimens of *R. oberhaeuseri* collected in the *locus typicus*, and W. Maucci (Trieste) who sent us the specimens from Sweden to enrich the Binda & Pilato collection; Francesco Furnari and Salvatore Brullo (University of Catania) who collected moss samples in Libya. We also thank Sandra McInnes who improved our English. The research was supported by the University of Catania (Fondo Ricerca d'Ateneo).

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