The types of supplements in the family Tobrilidae (Nematoda, Enoplia)

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Summary. The structure of supplementary organs and buccal cavity are the main diagnostic features for identification of Tobrilidae species. Four main supplement types can be distinguished among representatives of this family. Type I supplements are typical for *Tobrilus*, *Lamuania* and *Semitobrilus* and are characterised by their small size and slightly protruding external part. There are two variations of the type I supplement structure: *amabilis* and *gracilis*. Type II is typical for several *Eutobrilus* species (*E. peregrinator*, *E. prodigiosus*, *E. strenuus*, *E. nothus*). These supplements are very similar to the type I supplements but are characterised in having a highly protruding torus with numerous microthorns and a bulbulus situated at the base of the ampoule. Type III is typical for *Eutobrilus* species from the Tobrilini tribe, *i.e.*, *E. graciliformes*, *E. papilicaudatus* and *E. differtus*, and *Mesotobrilus* spp. from the Paratrilobini tribe and is characterised by a well-defined cap and a bulbulus situated at the base of the ampoule. Type IV is observed in the majority of *Eutobrilus*, *Paratrilobus*, *Brevitobrilus* and *Neotobrilus* and is the most complex supplement type with a mobile cap and an apical bulbulus. The origin of nematode supplementary organs from unspecialised setae can be illustrated by the evolution of tobrilid supplements.

Key words: evolution, free-living nematodes, morphology, supplementary organs, Tobrilidae.

Males in the family Tobrilidae (Triplonchida) have numerous and often large midventral precloacal supplementary organs (or supplements). The supplementary apparatus is a row of precloacal supplements acting as an orientation organ that is used for attachment to the female body during copulation. Supplement structure is a convenient object for construction of evolutionary theories for tobrilid taxa.

The supplement structure was usually considered as the main diagnostic feature in the family Tobrilidae. Tsalolikhin (1983) had divided the family Tobrilidae into two subfamilies, Tobrilinae (with simple supplements) and Eutobrilinae (with echinate supplements). Nowadays, the buccal cavity structures are considered as the main features for the tobrilid taxonomy and the value of a supplement structure is decreased, although it still remains the basis for the division of certain genera (Tsalolikhin, 2001). Currently, the taxonomy system of tobrilids proposed by Tsalolikhin (1983) is accepted by the majority of specialists in its original scope (Zullini, 2006) or with minor changes (Andrássy, 2009).

The morphology of tobrilid supplements was described in several papers (Tsalolikhin, 1972, 1981a, 1983, 2006). All tobrilids have supplements except for the genus *Asperotobrilus* Shoshin, 1991 (Shoshin, 1998). A supplement consists of several parts according to terminology proposed by Tsalolikhin (1983) with some additions made by Shoshin & Shoshina (1999), which may be described as follows: each supplement consists of an ampoule, a protruding cap in the centre of the supplement, and a central spinule at the apex of the cap (Fig. 1); a ring fold separates cap from the cuticularised torus (shoulders). A torus and a cap are covered by numerous microthorns; a bulbulus is the probable derivate of a dendritic process typical for all sensory organs of nematodes (Ryss, 1981; Decraemer *et al.*, 2014). At present, two types of supplements are distinguished: a submerged and an echinate supplement. The most highly developed

supplements belong to the echinate type which consists of a central spinule, a cap, a torus covered by microthorns, and an ampoule (Fig. 1). Conversely, submerged supplements consist of a spinule, a smooth cap and an ampoule.

In this paper, we intend to clarify the terminology describing supplement structure. We propose to consider yet another supplement structure, the bulbulus. We also propose to divide supplements into four types: type I a simple submerged supplement; type II a simple convex supplement; type III a complex supplement with a basal bulbulus; type IV a complex supplement with an apical bulbulus (Shoshin & Shoshina, 1999). Scanning electron microscopy images have revealed the new information, which may shed light on the origin and evolution of supplementary apparatus.

MATERIALS AND METHODS

Specimens were collected by E.A. Shoshina from Lake Baikal, Gulf of Finland and the Neva River. Nematodes from the glycerin collection of the Zoological Institute of the Russian Academy of Sciences were also used, specifically materials collected by A.V. Shoshin from Lake Baikal and water bodies of Samara at the Volga River, and materials collected by D.D. Danilin from water bodies of Kamchatka.

Selected specimens were mounted on slides after impregnation in glycerin. Differential interference contrast (DIC) photographs were taken using microscopes Leica TCS SP5, Leica DMI-6000 and Lieca DM 6000 (Research Resource Center for Molecular and Cell Technologies of Saint Petersburg State University).

For scanning electron microscopy (SEM) nematodes were washed from glycerin and dehydrated in a graded ethanol series. Final dehydration was carried out using hexamethyldisilazane or a critical point dryer, Leica EM CPD300. Specimens were covered by gold or platinum by vacuum evaporator Jeol JEE-420D and observed using microscopes Hitachi S-800, S-1000 (Zoological Institute of the Russian Academy of Sciences), Zeiss Merlin (Interdisciplinary Resource Center for Nanotechnology) and Tescan Mira3 (Research Resource Center for Molecular and Cell Technologies of Saint Petersburg State University).

RESULTS

On the basis of morphological characteristics, four types of supplements can be distinguished.

Type I: a simple submerged supplement (Figs 2A-D & 5A). Supplements of this type are small. The exterior part slightly protrudes above the surface of cuticle. Height of torus and central spinule do not exceed the length of ampoule. Bulbulus is located at the base of an ampoule. It is typical for the genera *Tobrilus* Andrássy, 1959 (Tsalolikhin, 1981b, 1983), *Lamuania* Tsalolikhin, 1976 (Shoshin & Shoshina, 2002), *Semitobrilus* Tsalolikhin, 1981 (Tsalolikhin, 1981b). Two groups of species characterised by the type I supplements could be distinguished among *Tobrilus* species.

***Amabilis* group** is characterised by a differentiated supplementary apparatus (Fig. 2A, B). Supplements increase in size towards the cloaca. The distance from the cloaca to the nearest (posteriormost) supplement is smaller than the distances between the rests of the supplements. Supplements are small with a slightly protruding outer part, a rounded ampoule with thin and uneven walls. The number of supplements varies from 6 to 13.

This type of supplement could be found in *T. amabilis* Tsalolikhin, 1974, *T. undophilus* Shoshin, 1988, *T. latens* Tsalolikhin, 1974 and *T. incognitus* Tsalolikhin, 1972. The representatives of the genus *Semitobrilus* have similar supplements.

***Gracilis* group** is characterised by the equally sized supplements. The distance from cloaca to the nearest supplement exceeds the distance between the nearest and the following supplement. All supplements have a flattened ampoule with thick walls and a torus with microthorns. A short central spinule has the slightly swollen base. Torus is separated from the central spinule by a circular fold. The central spinule is asymmetrical and tilted towards the head end (Fig. 2C, D). This type of supplements are found in *T. gracilis* (Bastian, 1865), *T. helveticus* (Hofmänner & Menzel, 1914), *T. wesenbergi* (Micoletzky, 1925), *T. zacopanensis* (Stefanski, 1924), *T. macramphis* Tsalolikhin, 1977, *T. aberrans* (Filipjev, 1928), *T. brevisetosus* (Schneider, 1925), *T. nepalensis* Tsalolikhin, 1983, *T. phantasus* Tsalolikhin, 1983, *T. bekmanae* Tsalolikhin, 1975, *T. tripylis* Gagarin, 1991, *T. parvus* Gagarin, 1991, *T. unisecsus* Gagarin, 1989. It is also found in *Lamuania orientalis* Tsalolikhin, 1976.

Type II: a simple convex supplement characterised by the presence of a highly protruding torus (Figs 2E, F & 5B), equal sizes of torus, central spinule and ampoule depth. Torus is covered with numerous microthorns. Bulbulus is situated at the base of the ampoule. Supplements of this type are immobile and cannot retract.

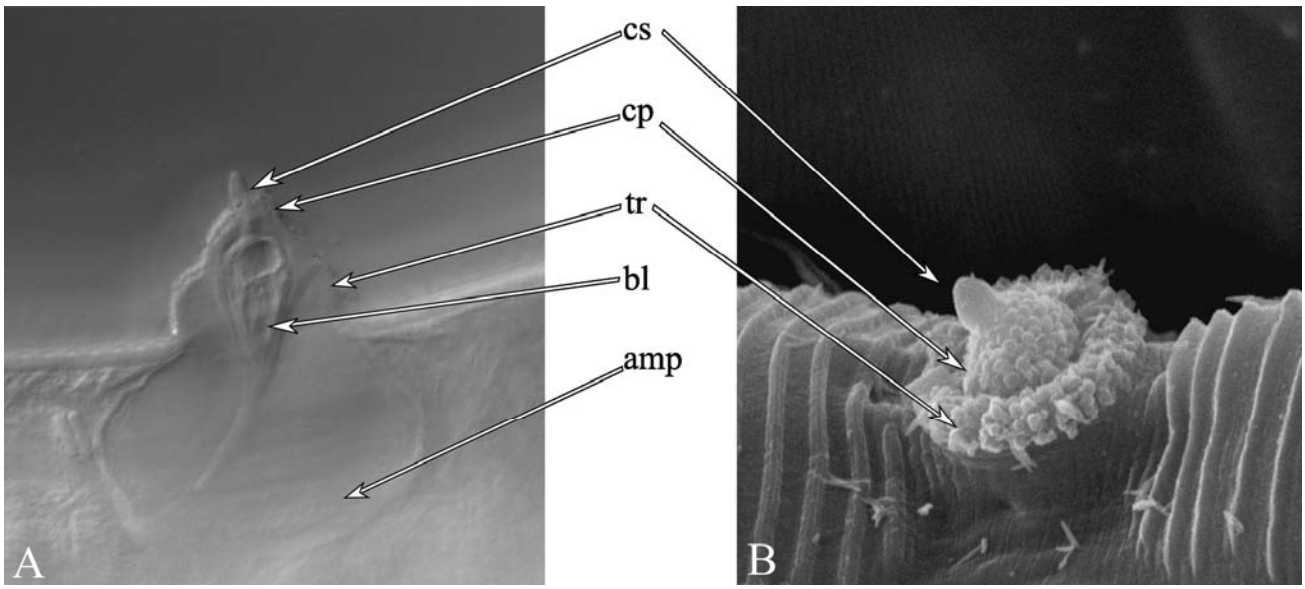

Fig. 1. General morphology of a tobrilid supplement (*Paratrilobus* sp.) A – DIC; B – SEM. Supplements consisting of broad ampoule (amp) submerged into cuticle with central inner bulbulus (bl). Bulbulus continuing to central spinule (cs) and encircled by basal torus (tr) and apical cap (cp).

Type II supplements are found in the genus *Eutobrilus* Tsalolikhin, 1981: *E. peregrinator* Tsalolikhin, 1983, *E. prodigiosus* Shoshin, 1988, *E. strenuus* Gagarin, 1991, *E. nothus* Gagarin, 1989, *Eutobrilus* sp. (Fig. 2E, F).

Type III: a complex supplement with the basal bulbulus and the cap covered with microthorns. The cap is separated from the torus by a ring fold. The bulbulus is at the base of ampoule or slightly elevated but never located in the cap (Figs 2G, H & 5C). The cap is retractable. This type of supplements are found in the genera *Mesotobrilus* Tsalolikhin, 1981 and *Eutobrilus*: *E. graciliformes* (Altherr & Delamare Deboutteville, 1972), *E. papilicaudatus* (Altherr, 1963), *E. differtus* Shoshin, 1988, *E. arcticus* Gagarin, 1991, *E. angarensis* Gagarin, 1991.

Type IV: a complex supplement with the apical bulbulus (Figs 3, 4 & 5D, E). Usually supplements of this type are large and most complex. They are found in the representatives of *Eutobrilus*, *Neotobrilus* Tsalolikhin, 1981, *Brevitobrilus* Tsalolikhin, 1981 and *Epitobrilus* Tsalolikhin, 1981.

There are some differences in type IV supplements structure in subfamilies Tobrilinae and Neotobrilinae.

In the subfamily Tobrilinae, supplements are characterised by the presence of an apical retractable cap (Figs 3A-F & 5D). In the majority of *Eutobrilus* (Fig. 3A, B) and *Paratrilobus* Micoletzky, 1922 (Fig. 3C, D) supplements are of equal size, except for the one closest to the cloacal opening, which can be smaller than the others. Supplements in the genus *Kurikania* Tsalolikhin, 1976 are reduced in size, but have all the features of a complex supplement (Fig. 3E, F).

In the subfamily Neotobrilinae the supplements are characterised by the partial or total reduction of an apical cap. In particular, the supplements of *Neotobrilus* are characterised by the partial reduction of an apical cap. The first three supplements from the cloaca are reduced in size and characterised by the rectangular torus and the absence of a cap (Fig. 4A, F). The next three supplements are gigantic (Figs 4B-E & G) and have lateral pillow-like thickening (Fig. 4B). The lateral parts of the torus enclose the cap and central spinule like a shell. The supplements of *Brevitobrilus* are the most simple with a thin smooth torus and no cap at the base of a central spinule. Nevertheless, these supplements have the bulbulus in the apical position and can be assigned to the type IV.

Ventral crests and 'micropapillae'. The entire surface of the body is thinly annulated, annuli 1.1-1.4 μm wide. In the supplementary field, the annulation character changes sharply and there are clearly visible crests (observed even under an optical microscope). Scanning electron microscopy data showed that these crests are regular, with the crest occurring approximately every third ring. The length of the field of folded cuticle differs from species to species and can slightly exceed the length of supplementary field. For example, in *Neotobrilus* crests are present only between the first three supplements (Fig. 4F, G).

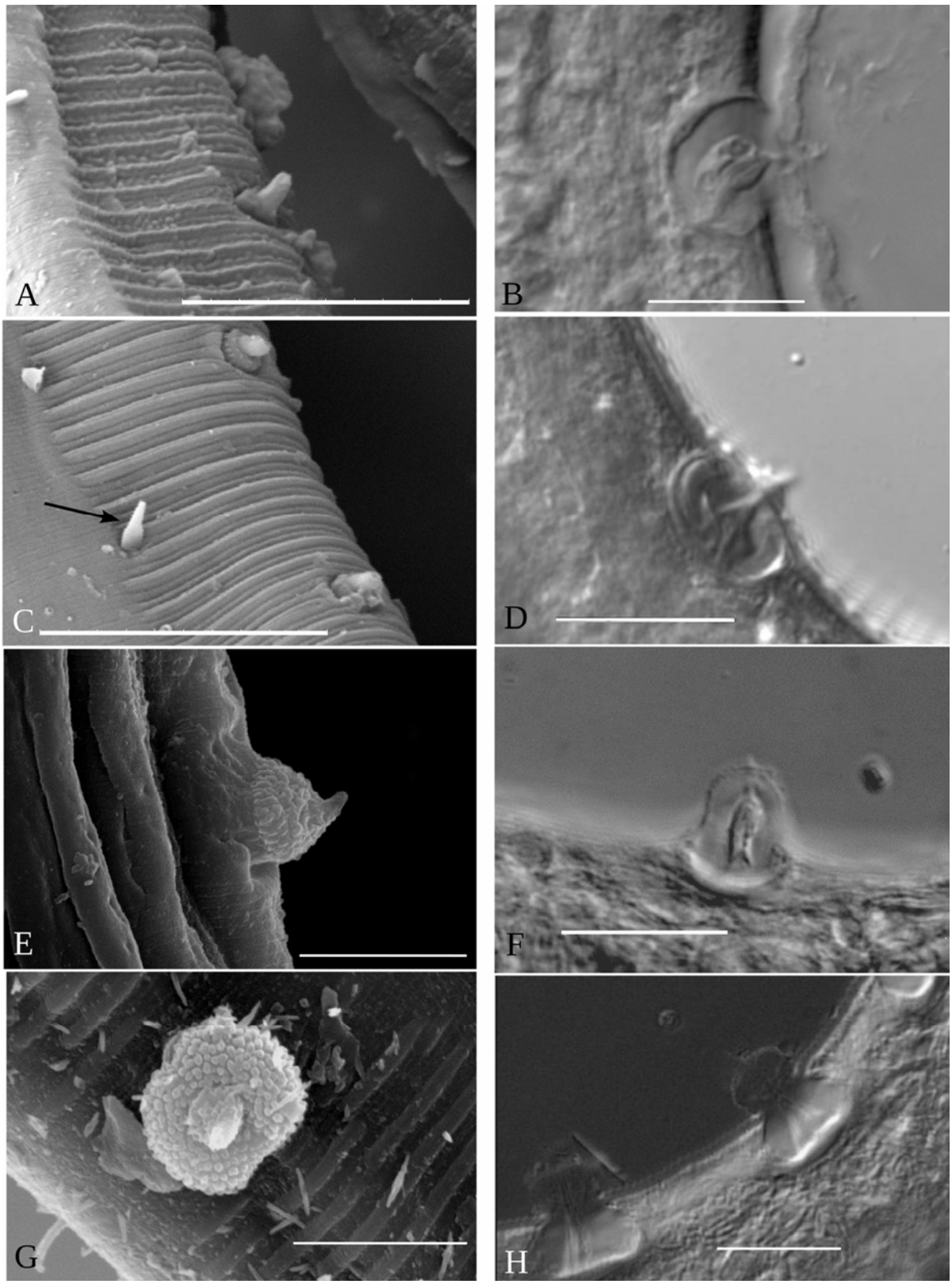

Fig. 2. Different types of supplements. A, C, E, G – SEM; B, D, F, H – DIC. A, B: morphology of supplement type I, *amabilis* group (*Tobrilus amabilis*); C, D: morphology of supplement type I, *gracilis* group (*Tobrilus gracilis*); E, F: morphology of supplement type II (*Eutobrilis* sp.); G, H: morphology of supplement type III (*Mesotobrilus delicatus*). Scale bars: A, C = 10 μm; E, G = 6 μm; B = 5 μm; D = 3 μm; F = 10 μm, H = 7 μm.

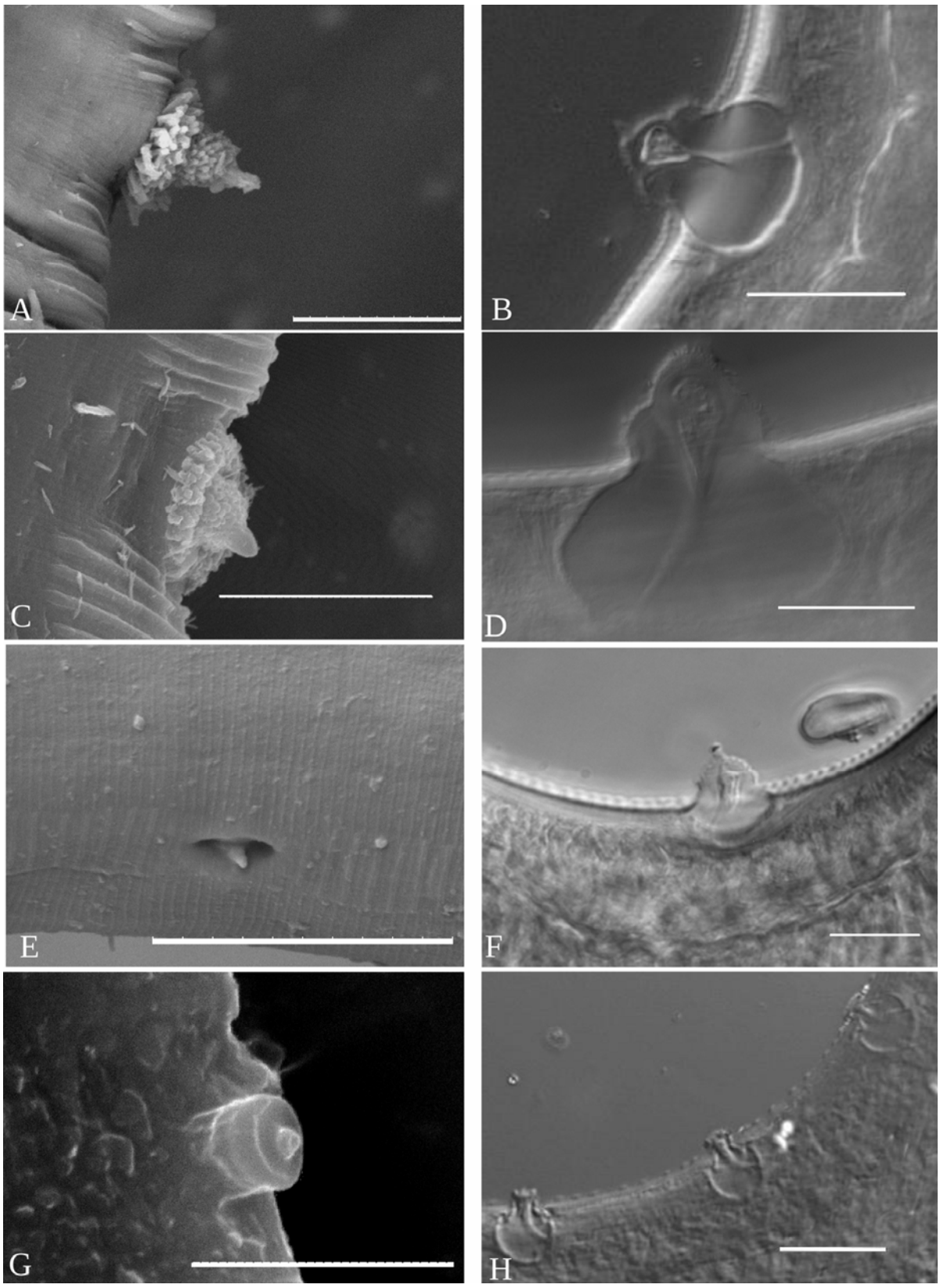

Fig. 3. Different variants of type IV supplements. A, C, E, G – SEM; B, D, F, H – DIC. A, B: *Eutobrilus grandipapillatus*; C, D: *Paratrilobus* sp.; E, F: simplified supplements of *Brevitobrilus* sp.; G, H: modified supplement of *Kurikania tsalolikhini*. Scale bars: A, C = 10 μm; B = 8 μm; D = 5 μm; E = 5 μm; F = 3.5 μm; G = 20 μm; H = 6 μm.

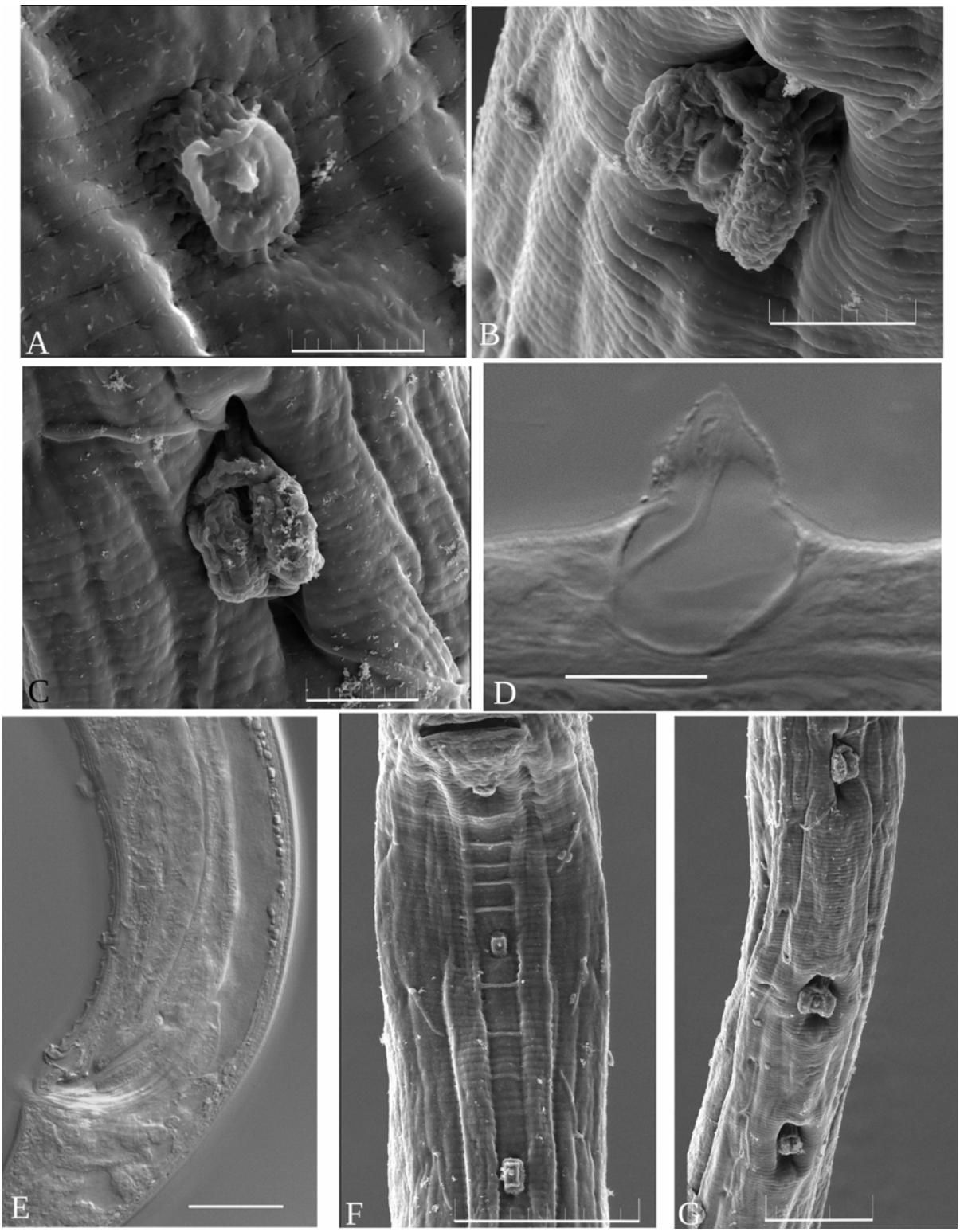

Fig. 4. Type IV supplements with apical bulbulus (*Neotobrilus filipjevi*). A, B, C, F, G – SEM; D, E – DIC. A: second supplement from cloacal opening; B: fifth supplement from cloacal opening; C: sixth supplement from cloacal opening; D: first supplement from cloacal opening; E: precloacal region of the male showing three closest to cloacal opening supplements and ventral crests (lateral view); F: precloacal region showing three closest to cloacal opening supplements and ventral crests (ventral view); G: precloacal region showing three furthest from cloacal opening supplements. Scale bars: A-C = 5 μm; E-G = 5 μm; D = 10 μm.

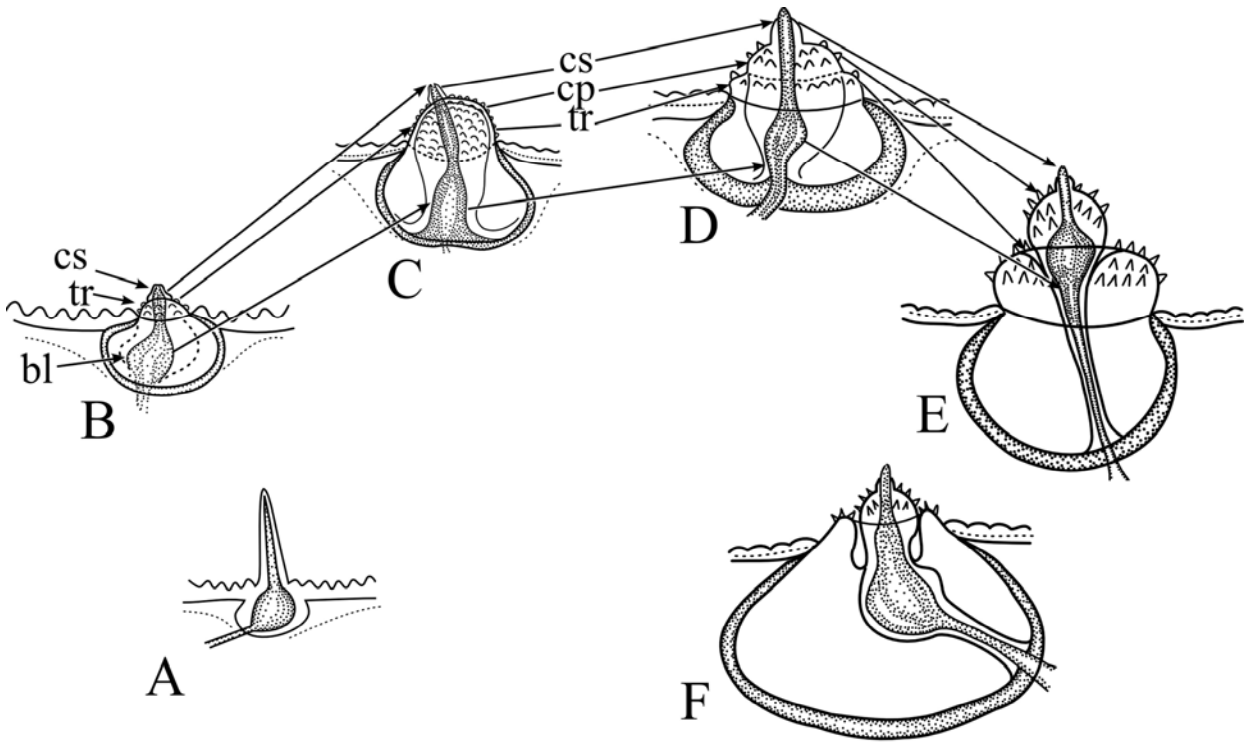

Fig. 5. Schematic representation of the morphology of four types of supplements. A: somatic seta, hypothetical ancestor of supplementary organ; B: type I supplement with basal bulbulus (bl) and short torus (tr); C: type II supplement with basal bulbulus and protruding torus; D: type III supplement with basal bulbulus and cap (cp) separated from torus by ring fold; E: type IV supplement with protracted torus and cap; F: type IV supplement with retracted torus and cap. cs – central spinule, arrows indicate homologous structures (not to scale).

The width of a field of folded cuticle also differs from species to species, and can be equal to the width of supplements (as in *Kurikania tsalolikhini* Shoshin, 1991 (Fig. 3E)) or exceeds it (as in the most of the species studied (Fig. 2A, C)).

Using light microscopy, crests were previously described as 'micropapillae' (Fig. 4E), and the number of micropapillae is frequently used in species identification.

DISCUSSION

According to Malakhov (1986), supplementary sensory organs evolved from unspecialised sensory organs, like a somatic seta or a pore in the cuticle of the nematode's ancestor. An illustration of this can be found when comparing the structure of somatic setae and supplements of *Tobrilis gracilis* (Fig. 2C). Here we may see the similarities: both seta and supplement have a cylindrical basis (stipes) and a sharpened apex. However, in a supplement the stipes is usually shorter and expanded compared to that of a seta and could be surrounded by additional structures such as a ring fold, microthorns *etc*.

The probable sequence of the formation of a supplement could be described as follows (Fig. 5). The ampoule increased in size resulting in expansion of the outer part of a seta. At the next step, a stipes transforms into the torus and an apex transforms into the central spinule. The bulbulus is in the basal position. Thus, the simple submerged supplement appears (type I) (Fig. 5B).

The next evolutionary step is the oligomerisation of the supplementary apparatus (Tsalolikhin, 1977, 1983). This is a process of reducing the number of homological organs while increasing their functions. The size of an ampoule and outer parts of supplements increases, resulting in the appearance of a simple convex supplement (type II) (Fig. 5C).

The appearance of such massive supplements might have affected nematode movement and probably lead to the emergence of supplements that were able to retract. Supplements of type III with a mobile cap and ability to retract slightly could be an illustration of this process (Fig. 5D).

Further development of supplements leads to the modification in which a bulbulus shifts to the cap. Such modification results in the appearance of

supplement able to retract inside the body or protruding back outside, depending on the physiological state of nematode (Holovachov & Shoshin, 2014). In this situation, nothing can limit the supplement size, and supplements of type IV could reach the giant size up to half of the body diameter (Fig. 5E, F).

Supplements with the ability to retract are also found in other groups of nematodes. For example, *Chromadoropsis vivipara* (Schuurmans Stekhoven, 1931) (Desmodorida, Spiriniidae) is characterised by the sucker-shaped supplement that is able to retract into the special pit-like cuticular sheath. As in tobrilids, supplements of *C. vivipara* are devoid of own muscles and their retraction/protraction is determined by the alteration of cuticle tension during the body movements. If the ventral side of the body is bent, supplements protrude due to the cuticle pressure. If the ventral side is convex, the body cuticle stretches and a supplement retracts (Tchesunov, 2006).

The results obtained here are in accordance with molecular phylogeny studies. The primitive Tobrilinae with its simple supplements occupies the basal position and more advanced Neotobrilinae with its well-developed supplements appears to be in higher position on the molecular phylogenetic tree (Antofica, 2012). The position of *Semitobrilus* is still under discussion. Traditionally this genus is considered within Neotobrilinae (Tsalolikhin, 2001; Zullini, 2006; Andrássy, 2007). However, the molecular phylogeny analysis (Antofica, 2012) and our data on the supplement structure do not support the accepted taxonomical position of this group (Zullini, 2006; Andrássy, 2007). Maximum parsimony and Neighbour joining trees put *Semitobrilus* in the clade together with *Eutobrilus* and the sister clade is formed with *Tobrilus* (Antofica, 2012). *Semitobrilus* is characterised by the type I supplements that also supports the close affinity of *Semitobrilus* and *Tobrilus*.

Although the modern system of Tobrilidae is based on the stoma structure (Tsalolikhin, 2001; Holovachov & Shoshin, 2014), it is not supported by the molecular phylogenetic studies. Basing on the multidisciplinary approach, Antofica (2012) assumed that the reproductive system has a major impact on the tobrilid evolution. So, we propose to consider a supplementary apparatus as the main diagnostic feature in the family Tobrilidae.

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A.V. Shoshin, E.A. Shoshina and J.K. Zograf. Типы суплементов у нематод семейства Tobrilidae (Nematoda, Enoplia).

Резюме. Строение стомы и суплементов являются главными диагностическими признаками для определения видов семейства Tobrilidae. Четыре основных типа суплементов можно выделить среди представителей семейства. Первый тип суплементов найден у *Tobrilus*, *Lamuania* и *Semitobrilus* и характеризуется малыми размерами и слегка выступающей наружной частью. Среди суплементов первого типа можно выделить две вариации – *amabilis* вариант и *gracilis* вариант. Суплемент второго типа, найденный у некоторых *Eutobrilus* (*E. peregrinator*, *E. prodigiosus*, *E. strenuus*, *E. nothus*), схож с предыдущим, но отличается сильно выступающими плечиками с многочисленными микрошипами и луковичкой, расположенной базально. Третий тип суплементов, найденный у некоторых *Eutobrilus* (*E. graciliformes*, *E. papilicaudatus*, *E. differtus*) и *Mesotobrilus* spp., характеризуется развитием апикальной шапочки и луковичкой, расположенной в основании ампулы. Четвертый тип суплементов, найденный у большинства *Eutobrilus*, *Paratrilobus*, *Brevitobrilus*, *Neotobrilus*, характеризуется подвижной шапочкой и апикальным расположением луковички. На примере развития суплементов у тобрилид сделано предположение о происхождении суплементарных органов нематод из неспециализированных щетинок.