

## Research Paper

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# Redescription of *Diastolaimus grossus* (Truskova & Eroshenko, 1977) Andrassy, 1984 (Rhabditida, Chambersiellidae) from Europe and comments on its phylogenetic position

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**Abstract**

Two populations of the species *Diastolaimus grossus* have been obtained from bark of trees in Bosnia and Herzegovina and the Czech Republic. The species is described and characterized in detail using morphological techniques (light and electron scanning microscopy) and morphometrical (Gower General Similarity coefficient of morphological characters) and molecular analyses, including phylogenetic analysis of all related and already sequenced species of the family Chambersiellidae. Morphological and molecular analyses, based on 18S and 28S ribosomal DNA sequences, show that the family Chambersiellidae is polyphyletic, being the subfamily Chambersiellinae related with Cephalobomorpha and Tylenchomorpha, and the subfamily Macrolaiminae is located into Panagrolaimomorpha. The genus *Diastolaimus*, previously belonging to Macrolaiminae, is transferred to Chambersiellinae. *Diastolaimus mexicanus* is proposed as a junior synonym of *D. grossus*.

**Introduction**

The genus *Diastolaimus* Rahm, 1928 includes six species (Andrassy, 1984; Cid del Prado, 2012), most of them poorly described. The main morphological diagnostic characters of the genus are the presence of seta-like inner labial papillae, structure and proportion of the cheilostom–gymnostom, didelphic–amphidelphic female genital system, male and female tails with elongate mucro having bifurcate tip, and the morphology of the spicules and the gubernaculum. However, in general, some of these diagnostic characters remain unknown or were described inaccurately.

*Diastolaimus grossus* (Truskova & Eroshenko, 1977) Andrassy, 1984, was originally described as *Fescia grossa* by Truskova & Eroshenko (1977) from Chuguyevsky district, Primorsky Krai (Far Eastern Siberia, Russia) examining four females and two males collected from the branches of *Abies* sp. In the present paper, *D. grossus* is morphologically, morphometrically and molecularly characterized through material found in two forest locations in the Czech Republic and Bosnia and Herzegovina. In addition, new molecular data of *D. grossus* (18S ribosomal DNA (rDNA): OM691517; 28S rDNA: OM691516), *Macrolaimus canadensis* Sanwal, 1960 (18S rDNA: OM691513; 28S rDNA: OM691511, OM691512) and *Macrolaimus ruehmi* Andrassy, 1966 (18S rDNA: OM691506; 28S rDNA: OM691514, OM691515) from the Czech Republic, *Macrolaimus arboreus* Truskova & Eroshenko, 1977 from Iran (28S rDNA: MF996701, MF996702) and *Macrolaimus crucis* Maupas, 1900 (18S rDNA: OM691510; 28S rDNA: OM691509) from Spain are provided.

**Materials and methods****Sampling and nematode extraction**

Specimens of *D. grossus* were extracted from the bark or barked wood samples taken from Náměšť na Hané, Czech Republic (*Salix* sp.) and from Banja Luka, Bosnia and Herzegovina (*Pinus nigra* Arnold). Molecular data of *M. canadensis* and *M. ruehmi* were obtained from samples taken during the monitoring of *Macrolaimus* species (Abolafia *et al.*, 2018).

Nematodes from the collected samples were extracted using a modified Baermann (1917) funnel technique, killed by heat, fixed in 4% formalin, transferred to pure glycerine following De Grisse's (1969) technique for the Czech and Iranian specimens and following Siddiqi's (1964) technique for the Spanish specimens, and mounted on permanent glass slides according to the de Maeseneer & d'Herde (1963) technique, but somewhat modified.

### Light microscopy (LM)

Measurements were taken for specimens mounted on permanent slides. Pictures were taken with a Leica DM2500 (Leica, Wetzlar, Germany) light microscope provided with differential interference contrast optics and a Leica DMC2900 camera. Micrographs were combined using Adobe® Photoshop® CS (Adobe Inc., San José, USA). The terminology used for the morphology of stoma and spicules follows the proposals by De Ley *et al.* (1995) and Abolafia & Peña-Santiago (2017), respectively.

### Scanning electron microscopy (SEM)

Specimens preserved in glycerine were selected for observation under SEM according to Abolafia (2015). The nematodes were hydrated in distilled water, dehydrated in a graded ethanol-acetone series, critical-point dried, coated with gold and females observed with a Zeiss Merlin microscope (5 kV) (Zeiss, Oberkochen, Germany) and males with a Hitachi SU 8010 (Hitachi, Tokyo, Japan).

### DNA amplification and sequencing

#### Czech specimens

DNA amplification was performed by heat gently inactivated specimens identified by LM. DNA was extracted as follows: single specimens were homogenized in 50 µl of nematode lysis buffer (10 mM Tris-hydrochloride (Tris-HCl), pH 8.8; 1 mM ethylenediamine-tetraacetic acid (EDTA); 1% Triton X-100 (v/v); 100 µg ml<sup>-1</sup> proteinase K) in a 1.5 mL Eppendorf tube using a micropipette. Sample incubation at 55°C for 1 h and subsequently at 95°C for 10 min followed. The resulting DNA extract was used as a template for polymerase chain reaction (PCR). Amplification was performed using GoTaq® G2 Flexi DNA Polymerase (Promega, Madison, Wisconsin, USA). The primers used for amplification of the region of 18S ribosomal RNA (rRNA) gene were the forward primers 988 F (5'-CTCAAAGATTAAGC CATGC-3') and 1813F (5'-CTGCGTGAGAGGTGAAAT-3') and the reverse primers 1912R (5'-TTTACGGTCAGAACT AGGG-3') and 2646R (5'-GCTACCTTGTTACGACTTTT-3') (Holterman *et al.*, 2006). The primers used for amplification of the D2–D3 region of 28S rRNA gene were forward primer D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and the reverse primer D3B (5'-TCGGAAGGAACCAGCTACTA-3') (synthesized by Integrated DNA Technologies) targeting the D2–D3 region of 28S rRNA gene (Nunn, 1992; De Ley *et al.*, 1999). The total volume of the reaction mix was 25 µl containing 1× Colorless GoTaq® Flexi Buffer (Promega, Madison, Wisconsin, USA); 1.25 mM magnesium chloride (MgCl<sub>2</sub>); 250 nM deoxyribose nucleoside triphosphates; 600 nM of each primer; 1.25 U of GoTaq® G2 Flexi DNA Polymerase (Promega, Madison, Wisconsin, USA) and 2 µl of undiluted DNA extract. The reaction was performed in the XP thermal cycler (Bioer Technology, Hangzhou, China) and consisted of 3 min at 94°C initial denaturation

followed by 40 cycles of 30 s at 94°C, 1 min at 55°C and 2 min at 72°C, with a final elongation step of 7 min at 72°C. Ten microlitres of the PCR product were run on 1.5% Tris-Borate-EDTA (TBE)-buffered agarose gel to check the amplification. The size of PCR product was approximately 800 bp. The remaining 15 µl of PCR product was submitted for sequencing to the Centre of Region Haná for Biotechnological and Agricultural Research, Institute of Experimental Botany (Olomouc, Czech Republic). Obtained DNA sequences were analysed using the Geneious Bioinformatics software platform (Biomatters, Auckland, New Zealand).

#### Spanish specimens

Nematode DNA was extracted from single fresh individuals using the proteinase K protocol and PCR assays as described by Castillo *et al.* (2003), but somewhat modified (Archidona-Yuste *et al.*, 2016). Specimens were cut into small pieces using a sterilized dental anaesthesia needle on a clean slide with 18 ml of Tris-EDTA buffer (10 mM Tris-HCl + 0.5 mM EDTA; pH 9.0), transferred to a microtube, adding 2 µl proteinase K (700 µg ml<sup>-1</sup>) (Roche, Basel, Switzerland), and stored at –80°C within 15 min (for several days). The microtubes were incubated at 65°C (1 h), then at 95°C (15 min). For DNA amplification, 3 µl of the extracted DNA was transferred to a microtube containing the following: 0.6 µl of each primer (10 mM), 3 µl Master Mix Taq DNA Polymerase (5× Hot FirePol Blend Master Mix, Solis BioDyne, Tartu, Estonia) and double-distilled water (ddH<sub>2</sub>O) to a final volume of 20 µl. The primers used for amplification of the region of 18S rRNA gene were the forward primer 988F (5'-CTCAAAG ATTAAGCCATGC-3') and the reverse primer 1912R (5'-TTT ACGGTCAGAACTAGGG-3') (Holterman *et al.*, 2006). The primers used for amplification of the D2–D3 region of 28S rRNA gene were the forward primer D2A (5'-ACAAGTACCGTGA GGGAAAGTTG-3') and the reverse primer D3B (5'-TCGGA AGGAACCAGCTACTA-3'). PCR cycle conditions were as follows: one cycle of 94°C for 15 min, followed by 35 cycles of 94°C for 45 s + annealing temperature of 55°C for 45 s + 72°C for 45 s, and finally one cycle of 72°C for 5 min. After DNA amplification, 5 µl of product was loaded on a 1% agarose gel in 0.5% Tris-acetate-EDTA (40 mM Tris, 20 mM glacial acetic acid and 2 mM EDTA; pH = 8) to verify the amplification using an electrophoresis system (Labnet Gel XL Ultra V–2, Progen Scientific, London, UK). The bands were stained with RedSafe (Intron Biotechnolog, Seongnam, South Korea) (20,000×) previously added to the agarose gel solution. The sequencing reactions of the PCR products were performed at Sistemas Genómicos (Paterna, Valencia, Spain) according to the Sanger *et al.* (1977) method.

#### Iranian specimens

DNA was extracted from nematodes using the method according to Holovachov *et al.* (2009). Specimens were picked using a fine-tipped needle and transferred to a 1.5 ml microtube containing 25 µl of ddH<sub>2</sub>O. The tube containing the nematode was crushed by a sterile needle. Following this procedure, 20 µl of Chelex 100 and 2 µl proteinase K (20 mg/ml) were added to the nematode substrate and the homogenate incubated at 56°C for 2 h and then at 95°C for 10 min. The supernatant was then extracted from the tube and stored at –20°C. The primers used for amplification of the D2–D3 region of 28S rRNA gene were the D2A (5'-ACAA GTACCGTGAGGGAAAGTTG-3') and the D3B (5'-TCGG AAGGAACCAGCTACTA-3') primers (Nunn, 1992; De Ley *et al.*, 1999). Subsequently, PCR was conducted with

8 µl of the PCR product of the nematode specimens, to which 2.5 µl of PCR buffer, 0.5 µl of DNTP, 1 µl of MgCl<sub>2</sub>, 0.3 µl of Taq-polymerase (CinnaGen, Iran), 1 µl of each primer listed above (10 pmol µl<sup>-1</sup>) and finally ddH<sub>2</sub>O were added, comprising a final volume of 25 µl. The amplification was carried out using an Eppendorf Mastercycler gradient (Eppendorf, Hamburg, Germany), with the following programme: initial denaturation for 3 min at 94°C, 37 cycles of denaturation for 45 s at 94°C, extension for 45 s at 56°C and annealing for 1 min at 72°C, and finally an extension cycle of 6 min at 72°C followed by a holding temperature of 4°C. After DNA amplification, 5 µl of product was loaded on a 2% agarose gel in TBE buffer (40 mM Tris, 40 mM boric acid and 1 mM EDTA) for evaluation of the DNA bands. The bands were stained with 50 mM ethidium bromide and visualized and photographed on an ultraviolet transilluminator. The PCR product was purified for sequencing by the Macrogen Corporation (Republic of Korea).

### Phylogenetic analyses

For phylogenetic relationships, analyses were based on 18S rDNA and 28S rDNA fragments. Other segments of 18S rRNA and 28S rRNA gene sequences available in GenBank were aligned using ClustalX alignment in the program MEGA7 (Kumar *et al.*, 2016). The sequence dataset was analysed with Bayesian inference using MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The analysis under the GTR + G model was initiated with a random starting tree and run with the Markov chain Monte Carlo for 10<sup>6</sup> generations. *Teratocephalus lirellus* (AF036607 for the 18S phylogenetic tree and AB477073 for the 28S phylogenetic tree) was used as the outgroup. The phylogenetic trees were visualized and saved with the program FigTree 1.4.3 (Rambaut, 2014).

### Gower General Similarity coefficient of morphological characters

To examine the similarity of particular taxa of the family Chambersiellidae, a cluster analysis was implemented. The analysis was based on morphological diagnostic characteristics of particular populations, which were obtained within the present study or adopted from previously published studies: M\_a-Ru: *Macrolaimus arboreus* (Russia; Truskova & Eroshenko, 1977); M\_a-Ke: *Macrolaimus arboreus* (Iran; Shokoohi *et al.*, 2018); M\_ca-Ge: *Macrolaimus canadensis* (Germany; Fuchs (1938) as *Macrolaimus crucis*); M\_ca-US/M\_ca-Ca: *Macrolaimus canadensis* (USA/Canada; Sanwal, 1960); M\_ca-CZ/M\_ca-Co: *Macrolaimus canadensis* (Czech Republic/France; Abolafia *et al.*, 2018); M\_cu-Al: *Macrolaimus crucis* (Algeria; Maupas, 1900); M\_cu-Sp: *Macrolaimus crucis* (Spain; Abolafia & Peña-Santiago, 2014); M\_ri-Sa: *Macrolaimus richteri* Swart & Heyns, 1992 (South Africa); M\_h-US: *Macrolaimus hamatus* Thorne, 1937 (USA); M\_n-Ba: *Macrolaimus natator* Timm, 1960 (Pakistan); M\_ru-Ge: *Macrolaimus ruehmi* (Germany; Rühm (1956) as *M. crucis*); M\_ru-CZ/M\_ru-BiH: *Macrolaimus ruehmi* (Czech Republic/Bosnia and Herzegovina; Abolafia *et al.*, 2018); M\_so-It: *Macrolaimus somniorum* Andrassy, 1984 (Italy); M\_ta-US: *Macrolaimus taurus* Thorne, 1937 (USA); D\_c-US: *Diastolaimus croca* (Massey, 1963) Andrassy, 1984 (USA; as *Santafea croca* Massey, 1963); D\_d-US: *Diastolaimus damalis* Andrassy, 1984 (USA; as *Santafea damalis* Massey, 1966); D\_g-Ru: *Diastolaimus grossus* Andrassy, 1984 (Russia; as *Fescia grossa* Truskova & Eroshenko, 1977); D\_g-CZ/D\_g-BiH: *Diastolaimus grossus* (Czech Republic/Bosnia and Herzegovina; present paper); D\_m-Me: *Diastolaimus mexicanus* Cid del

Prado, 2012 (Mexico); D\_p-Br: *Diastolaimus papillatus* Rahm, 1928 (Brazil); Co\_f-Ru: *Cornilaimus furcillus* Truskova & Eroshenko, 1977 (Russia); G\_b-US: *Geraldus bakeri* (Sanwal, 1957) Sanwal, 1971 (USA; as *Chambersiella bakeri* Sanwal, 1957); G\_g-Ec: *Geraldus galapagoensis* Cid del Prado, 2012 (Ecuador); Ch\_r-US: *Chambersiella rodens* Cobb, 1920 (USA). Both, quantitative (such as morphometric) traits as well as qualitative traits were used for the analysis. For the purposes of quantitative traits, mean values for given population were used. Analysis was carried out separately for males and females because of many sex-associated morphological traits. Prior to the cluster analysis, Gower's dissimilarity coefficients were computed using the function 'daisy' in package 'cluster' in R 3.4.1 software, which was appropriate for the mixed-data (numeric/categorical) dataset that we had (Gower, 1971). Subsequently, a matrix of Gower's dissimilarity coefficients was entered into hierarchical cluster analysis computed in R package 'cluster' using function 'agnes'. Ward's (1963) clustering method was used because it exhibited higher agglomerative coefficients (0.9) than other available clustering methods.

## Results

### *Diastolaimus grossus* (Truskova & Eroshenko, 1977) Andrassy, 1984

#### Material from the Czech Republic

Nine females and three males in acceptable conditions. For morphometrics, see table 1.

#### Description

**Adult** (figs 1–3). Moderately slender nematodes of small size, 0.94–1.38 mm long. Body cylindrical, tapering towards both extremities, but more so towards the posterior end because the tail is conical. Habitus slightly curved ventrad after fixation, to an open 'C' shape. Cuticle *ca.* 1 µm thick, with very fine annuli, *c.* 1 µm wide, frequently appearing divided somewhere in its outline, hardly perceptible under LM but readily visible under SEM. Lateral fields 2–4 µm wide, consisting of two lines (three incisions) without transverse striation or any kind of areolation, starting at level of anterior part of corpus, and ending at tail tip. Lip region convex, continuous with the adjacent body, with the oral region strongly elevated or protruding. SEM observations: lips partially fused, but their inner (perioral) region, hemispheroid, forming six curved, triangular liplets covering the oral aperture; anterior sensilla arranged in three circles: six thin seta-like inner labial sensilla located at base of each liplet, six elongate-conoid outer labial sensilla and four papilliform cephalic sensilla. Amphidial apertures small, oval, located *c.* 15 annuli posteriorly to lips. Stoma 0.9–1.1 times longer than wide and 1.0 times as long as lip region diameter, consciously sclerotized, subdivided into cheilo-, gymno- and stegostom; cheilostom *c.* 50% of the stoma length, having cheilorhabdia consciously sclerotized, convergent (curved) and thinner anteriorly; gymnostom short, approximately one half of the cheilostom length, with arched gymnorhabdia arranged in three rows (examined under the LM); stegostom one half of the cheilostom length, with shallow funnel-shaped lumen, enveloped by the anterior end of pharynx, lacking visible rhabdia; dorsal pharyngeal gland opening at base of stegostom. Pharynx cephaloboid: pharyngeal corpus cylindrical, 1.3–1.6 times longer than isthmus, with procorpus and metacarpus similar in width; isthmus narrower than, and clearly delimited from corpus; basal bulb nearly pyriform, with strongly developed valvular apparatus located at its posterior half. Cardia

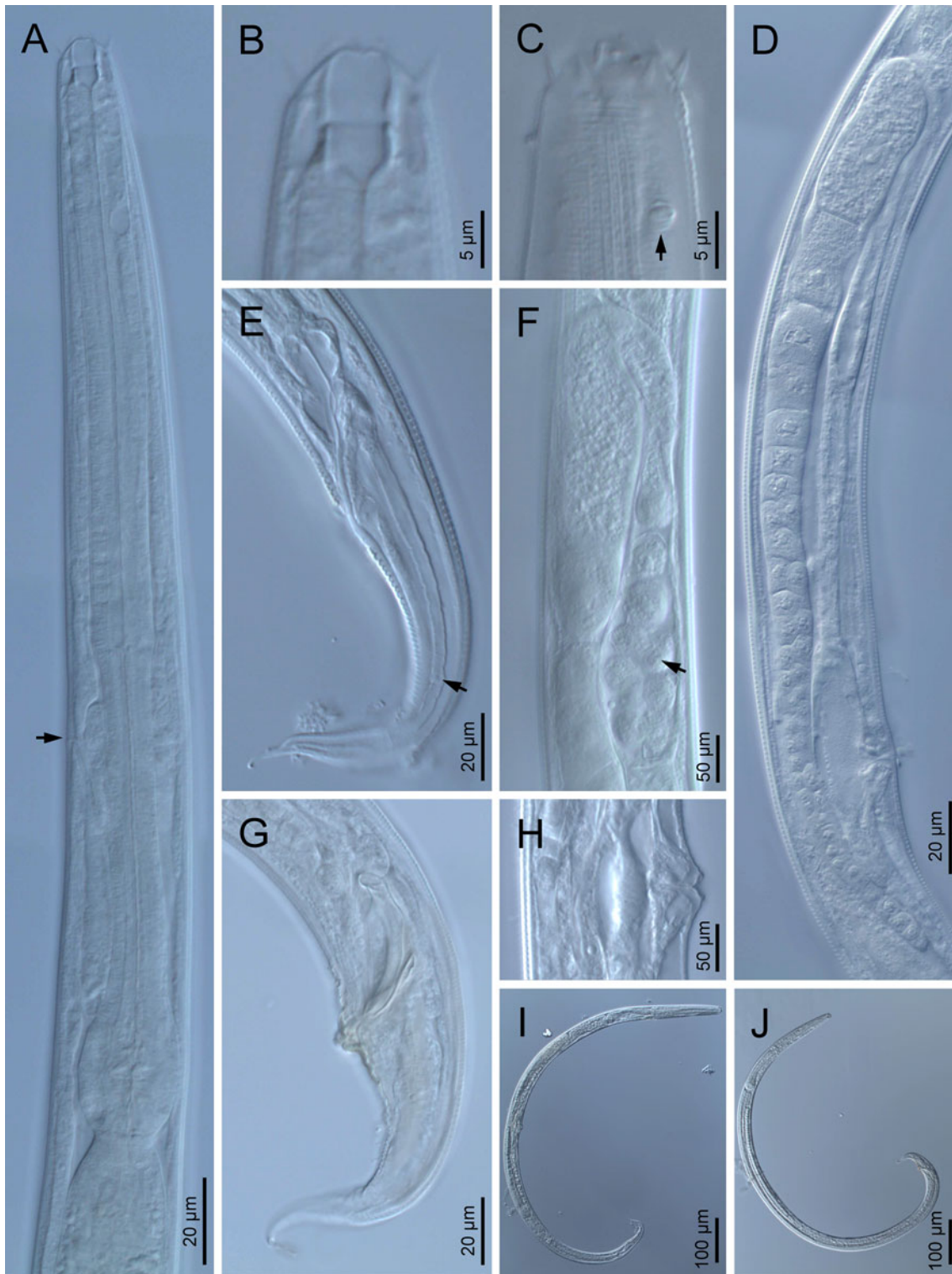
**Table 1.** Morphometrics of *D. grossus* (Truskova & Eroshenko, 1977) Andrassy, 1984. Measurements in  $\mu\text{m}$  and in the form: mean  $\pm$  standard deviation (range) where appropriate.

| Country                       | Czech Republic |                            | Bosnia and Herzegovina     |
|-------------------------------|----------------|----------------------------|----------------------------|
|                               | <i>n</i>       | 9 females                  | 3 males                    |
| Body length                   |                | 1142 $\pm$ 112 (991–1384)  | 1116 $\pm$ 188 (949–1320)  |
| <i>a</i>                      |                | 33.1 $\pm$ 3.3 (30.5–39.0) | 35.8 $\pm$ 3.1 (32.7–38.8) |
| <i>b</i>                      |                | 4.4 $\pm$ 0.2 (4.2–4.7)    | 5.5 $\pm$ 1.5 (4.3–7.1)    |
| <i>c</i>                      |                | 13.1 $\pm$ 0.4 (12.6–13.6) | 14.6 $\pm$ 2.0 (12.8–16.7) |
| <i>c'</i>                     |                | 4.3 $\pm$ 0.4 (3.8–4.8)    | 2.9 $\pm$ 0.2 (2.7–3.2)    |
| <i>V</i>                      |                | 51.6 $\pm$ 0.5 (51–52)     | –                          |
| Lip region width              |                | 13.8 $\pm$ 1.3 (12–16)     | 12.8 $\pm$ 1.7 (12–15)     |
| Stoma length                  |                | 11.2 $\pm$ 1.6 (9–14)      | 10.3 $\pm$ 1.2 (9–11)      |
| Stoma width                   |                | 6.5 $\pm$ 1.6 (4–10)       | 5.9 $\pm$ 0.5 (6–7)        |
| Pharyngeal corpus length      |                | 123.9 $\pm$ 3.4 (118–126)  | 96.0 $\pm$ 26.9 (80–127)   |
| Isthmus length                |                | 82.2 $\pm$ 7.2 (73–91)     | 76.3 $\pm$ 8.8 (69–86)     |
| Bulbus length                 |                | 26.4 $\pm$ 2.6 (24–31)     | 25.7 $\pm$ 0.6 (25–26)     |
| Pharynx length                |                | 229.4 $\pm$ 8.7 (215–241)  | 197.9 $\pm$ 35.7 (174–239) |
| Nerve ring – anterior end     |                | 151.4 $\pm$ 1.9 (148–153)  | 136.7 $\pm$ 9.1 (128–146)  |
| Excretory pore – anterior end |                | 153.0 $\pm$ 10.6 (130–172) | 127.5 $\pm$ 29.0 (107–148) |
| Deirid – anterior end         |                | 151.8 $\pm$ 2.8 (149–155)  | 125.0 $\pm$ 28.3 (105–145) |
| Neck length                   |                | 242.7 $\pm$ 11.8 (223–255) | 208.2 $\pm$ 35.8 (185–249) |
| Body diameter at neck base    |                | 31.1 $\pm$ 1.6 (28–33)     | 28.4 $\pm$ 3.6 (24–31)     |
| Body diameter at midbody      |                | 33.5 $\pm$ 1.9 (29–35)     | 31.0 $\pm$ 2.6 (29–34)     |
| Lateral field width           |                | 3.0 $\pm$ 0.5 (2–4)        | 2.6 ( <i>n</i> = 1)        |
| Anterior ovary/testis length  |                | 280.2 $\pm$ 38.4 (209–322) | 717.3 $\pm$ 153 (578–881)  |
| Anterior oviduct length       |                | 179.0 $\pm$ 33.7 (146–231) | –                          |
| Anterior uterus length        |                | 31.5 $\pm$ 2.5 (29–36)     | –                          |
| Posterior ovary length        |                | 240.4 $\pm$ 38.4 (201–313) | –                          |
| Posterior oviduct length      |                | 166.8 $\pm$ 17.8 (140–199) | –                          |
| Posterior uterus length       |                | 30.5 $\pm$ 3.3 (25–35)     | –                          |
| Vagina length                 |                | 11.5 $\pm$ 2.0 (9–14)      | –                          |
| Vulva – anterior end          |                | 590.4 $\pm$ 62.9 (519–738) | –                          |
| Rectum length                 |                | 36.2 $\pm$ 3.2 (30–40)     | –                          |
| Anal body diameter            |                | 19.6 $\pm$ 1.3 (18–22)     | 26.3 $\pm$ 1.4 (25–28)     |
| Tail length                   |                | 79.9 $\pm$ 6.6 (68–90)     | 76.4 $\pm$ 2.3 (74–79)     |
| Phasmid – anus distance       |                | 32.3 $\pm$ 4.2 (25–36)     | 41.4 $\pm$ 2.7 (39–43)     |
| Tail mucro length             |                | 8.4 $\pm$ 0.4 (8–9)        | 16.5 $\pm$ 5.4 (11–22)     |
| Spicules length               |                | –                          | 36.3 $\pm$ 2.9 (33–39)     |
| Gubernaculum length           |                | –                          | 13.8 $\pm$ 1.3 (13–15)     |

*a* = body length/body diameter; *b* = body length/pharynx length; *c* = body length/tail length; *c'* = tail length/anal body diameter; *V* = (distance from anterior region to vulva/body length)  $\times$  100.

short and subcylindrical 8.3  $\mu\text{m}$  long in average. Nerve ring at 61–66% of neck length, encircling pharynx at level of anterior half of isthmus. Secretory–excretory gland cell located ventrally to isthmus, its excretory pore opening posterior to nerve ring, at 61–63% of neck length, at level of isthmus and nerve ring. Deirids located posterior to nerve ring, inside the lateral field, at 60–63% of neck length close and posterior to excretory pore.

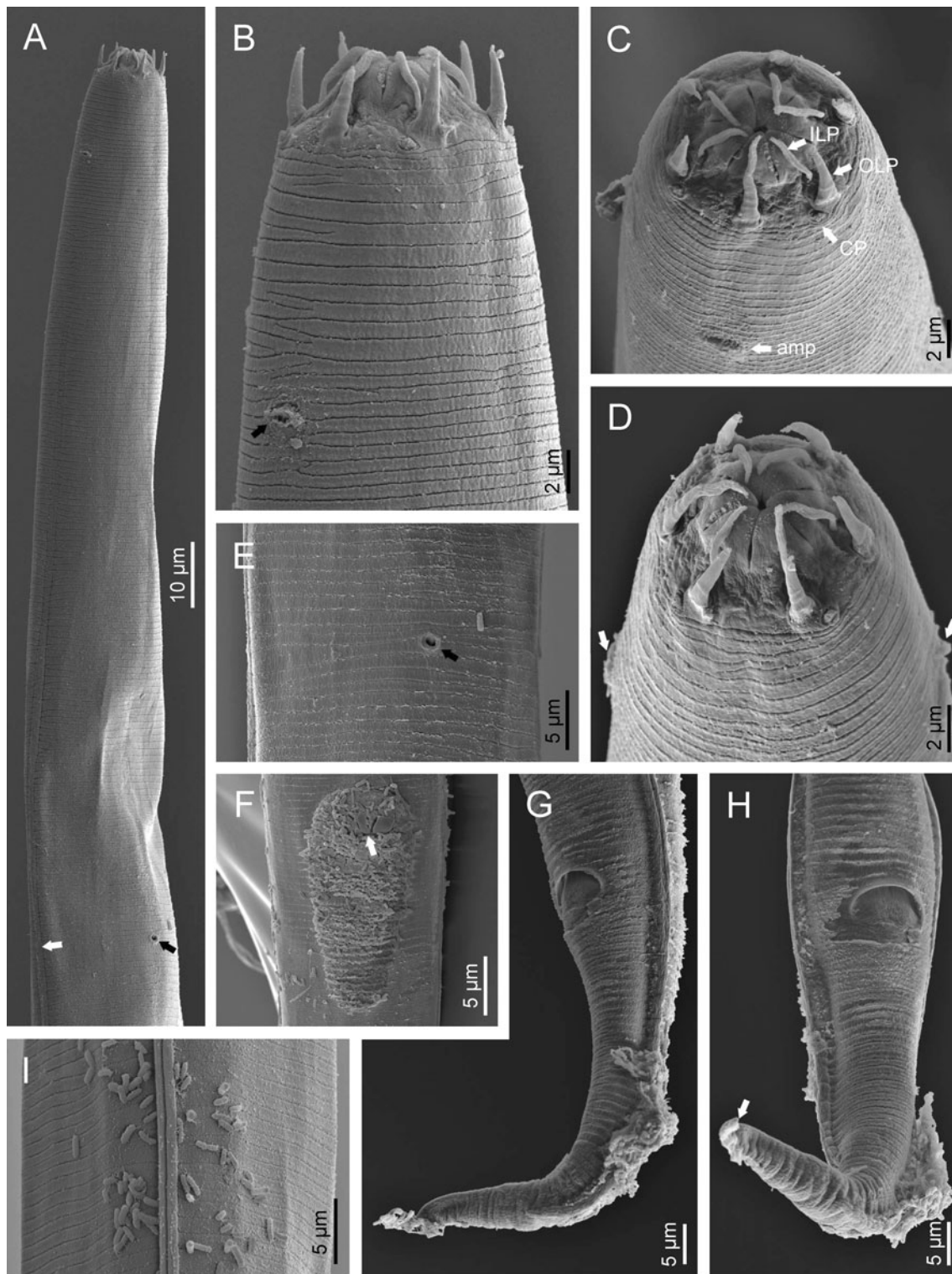
*Female.* Reproductive system didelphic-amphidelphic. Both ovaries reflexed near to oviduct junction, very long, surpassing the level of the vulva; anterior ovary is always longer than posterior one (1.1–1.2 times longer) and can be reflexed two times; oocytes first in two rows, primarily in short ovary reflection at vagina, then in only one row. Oviduct narrow and short with distinct narrowing spermatheca containing big spherical sperms. Uteri located



**Fig. 1.** *Diastolaimus grossus* (Truskova & Eroshenko, 1977) Andrassy, 1984 (LM): (a) Neck (arrow pointing the excretory pore); (b, c) anterior end at stoma and amphid (arrow) levels, respectively; (d) female reproductive system; (e) female posterior end (arrow pointing the phasmid); (f) spermatheca (arrow pointing the sperm); (g) male posterior end; (h) vagina; (i) entire female; (j) entire male.

on right-hand side of intestine; tubular, with thick walls and two times the corresponding body diameter long. Uterine eggs absent in the specimens examined. Vagina straight, occupying one-third (c. 31–40%) of body diameter. Vulva a transverse slit, with distinctly protruded lips that forms a vulval cone; advulval cuticle lacking

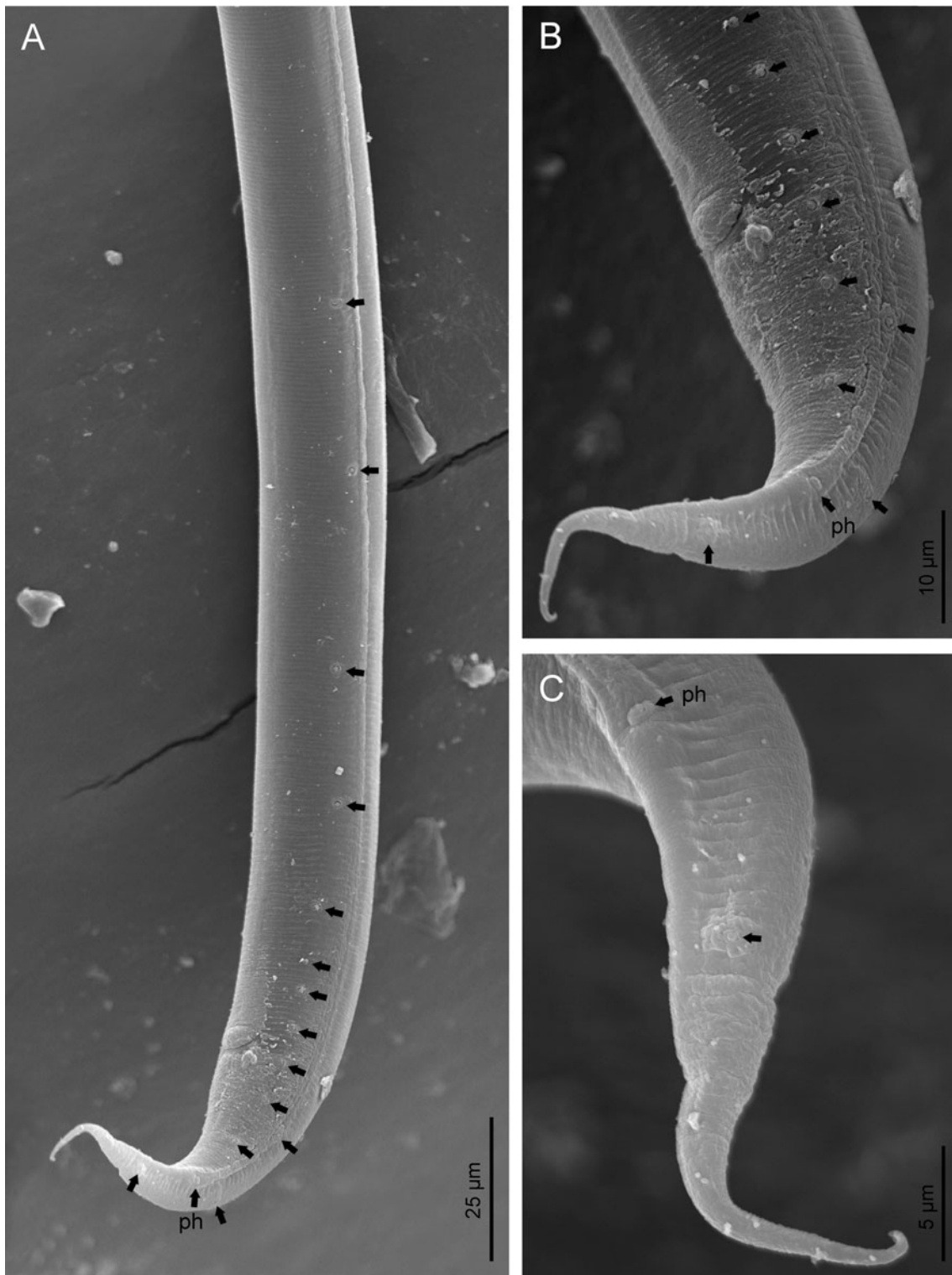
differentiations. Rectum 1.6–1.7 times longer than anal body diameter, its anterior half swollen forming strong sphincter; anal lips not prominent. Tail conical, slightly ventrad curved, tapering very gradually and ending in a filiform forked terminus 8–9 μm long. Phasmids are located at 43–44% of tail length from anus.



**Fig. 2.** *Diastolaimus grossus* (Truskova & Eroshenko, 1977) Andrassy, 1984 (SEM, female): (a) anterior end (black arrow pointing the excretory pore, white arrow pointing the deirid); (b–d) lip region in subventral, fronto-lateral and fronto-ventral views, respectively (arrows pointing the amphids); (e) excretory pore (arrow); (f) vulva (arrow); (g, h) posterior end (arrows pointing forked tail tip); (i) lateral field.

*Male.* Reproductive system monorchic, with testis reflexed ventrad, on right-hand side of intestine. Spermatocytes first in two rows, then in only one row. Spicules paired and symmetrical, 5.7–6.0 times longer than wide, curved ventrad with rounded manubrium, cylindrical calamus, ventrad curved lamina with

reduced dorsal hump and a well-developed ventral velum. Gubernaculum long, with thin manubrium and corpus with low central cuneus and well-developed triangular lateral cura. Fourteen pairs of genital papillae are present, eight subventral precloacal pairs and six postcloacal pairs arranged as follows:



**Fig. 3.** *Diastolaimus grossus* (Truskova & Eroshenko, 1977) Andrásy, 1984 (SEM, male): (a, b) posterior end in ventro-lateral view (arrows pointing genital papillae); (c) tail end in lateral view (arrow pointing last genital papillae). ph, phasmid.

two subventral pairs located posterior to the level of cloacal aperture, one lateral pair located at lateral field, one subventral pair, one subdorsal pair at the posterior half of tail next to the phasmid and one lateral at the end of tail (six annuli before beginning of filiform part of tail end). Tail conoid and tapering gradually and curved ventrad, ending in a short terminally forked

filiform part. Phasmids located at anterior half of tail, at 48% from anus.

*Gower General Similarity coefficient of morphological characters.* The analyses were based on morphometric and morphological characters including all available data of the redescribed populations in this article and taxa of the family Chambersiellidae

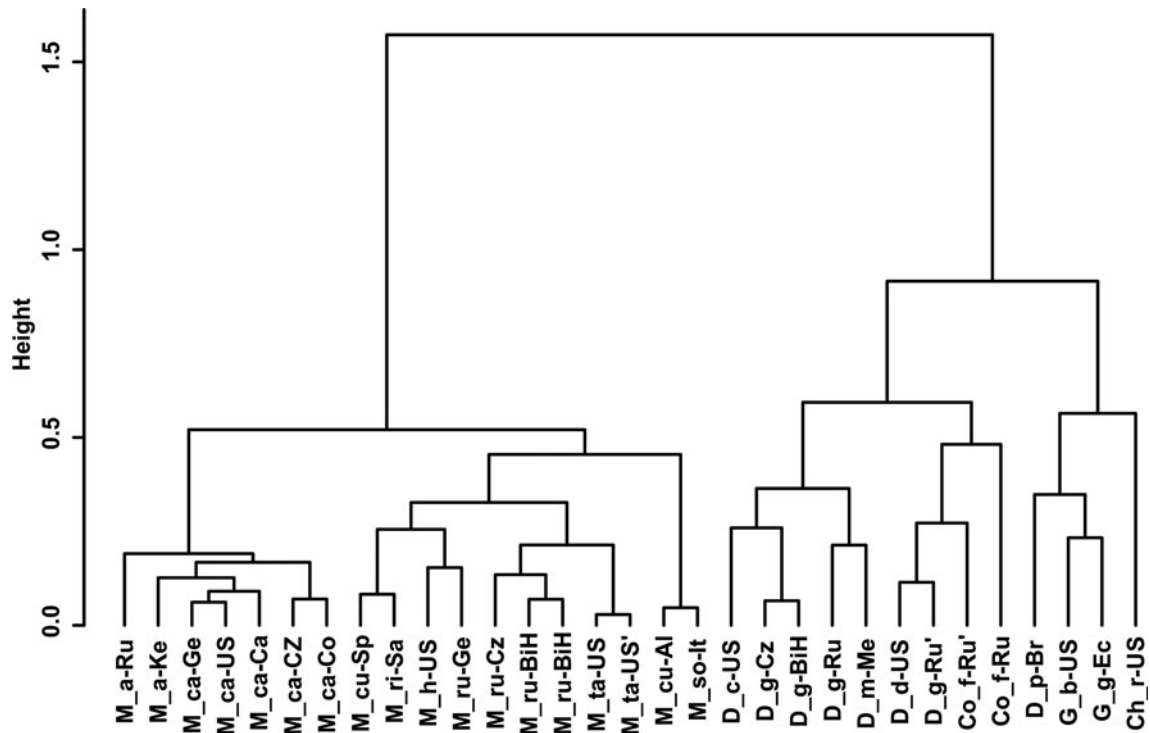


Fig. 4. Dendrogram from hierarchical clustering based on morphological characteristics of published members of the family Chambersiellidae (females).

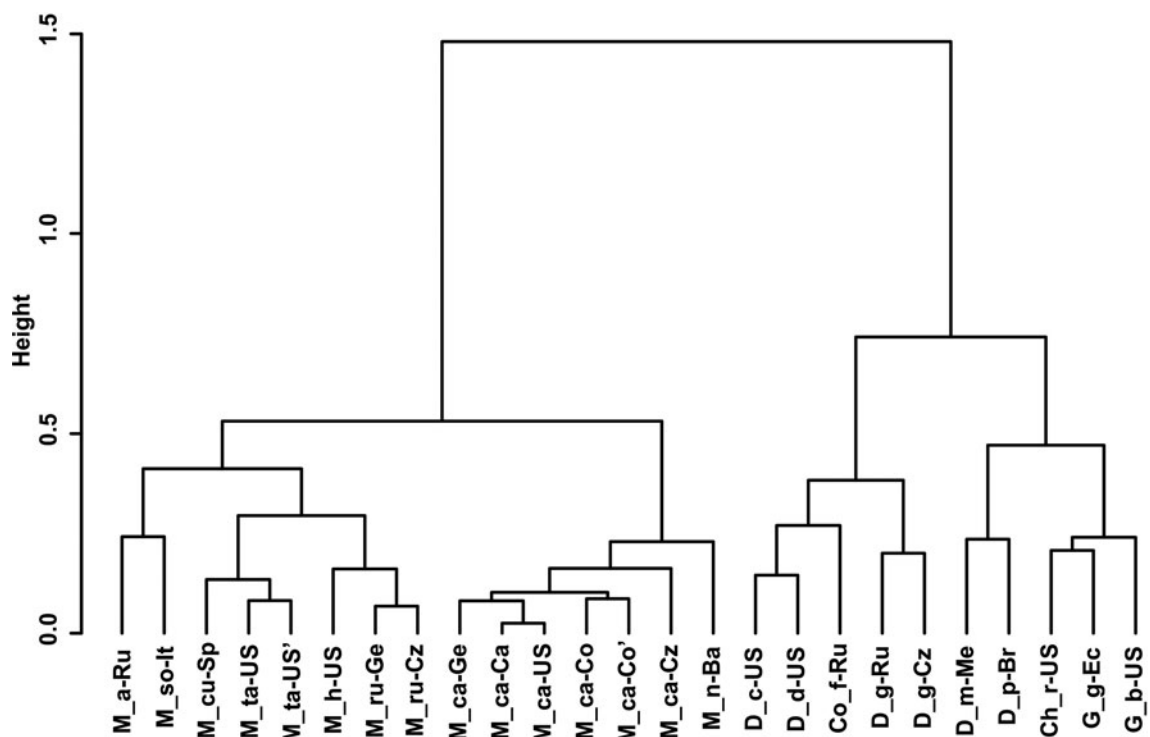


Fig. 5. Dendrogram from hierarchical clustering based on morphological characteristics of published members of the family Chambersiellidae (males).

previously published (see [figs 4 and 5](#)). Analyses of taxa of the family Chambersiellidae showed the identical formation of two main clusters, both with two subclusters for males and females. Main clusters clearly separate the genus *Macrolaimus* from the rest of the genera for Chambersiellidae. The second cluster involves the

genera *Chambersiella*, *Cornilaimus*, *Diastolaimus* and *Geraldus*. Within subcluster A of cluster II, all species of the *Diastolaimus* genus are grouped, except *D. papillatus* in females and *D. papillatus* and *D. mexicanus* in males. *Diastolaimus mexicanus* is grouped together with *D. grossus* (Russian population) in females in



subcluster A of cluster I. *Cornillaimus furcillus* is grouped closely with *D. damalis* in cluster I, subcluster II both for males and females. Males of *D. grossus* of Czech and Russian populations are grouped together in males and separated from other members of subcluster A of cluster I. Females of different populations of *D. grossus* are all grouped in cluster I, subcluster A. The Czech population of *D. grossus* is grouped with Bosnian and Russian populations with *D. mexicanus*.

#### Other material examined

The material studied from Bosnia and Herzegovina (two females) is similar to the specimens from the Czech Republic, only showing slight differences in some morphometrical characters caused by their incomplete maturity based on not fully developed and functional vulva (see table 1).

This is the first record of the genus and species for the Czech Republic and for Bosnia and Herzegovina.

#### Molecular characterization

After sequencing the D2–D3 region of the 28S rRNA and 18S rRNA genes of *D. grossus* PCR product, partial sequences of 745 bp (OM691516) and 1684 bp (OM691517) were obtained, respectively.

Sequence similarity of *D. grossus* (OM691516) compared to *Geraldus* sp. (GU062821) was 85.3% (745 bp). However, the sequence similarity of *D. grossus* (OM691516) and *Geraldus* sp. (GU062821) with member of the subfamily Macrolaiminae Sanwal, 1971 *M. canadensis* (OM691512) was 66.4% (727 bp) and 67.5% (719 bp), respectively. There are no available sequences of any species of the genus *Chambersiella*. The BLAST homology search showed 100% similarity of the 18S rRNA gene sequence with *F. grossa* (KC242218), and close molecular similarity with the sequences of *Acrobeles* species *A. complexus* 88.82% (KU180671) and *A. maximus* 88.89% (EU306344). The similarity of *D. grossus* (OM691517) with members of the subfamily Macrolaiminae Sanwal, 1971 was as follows: *M. canadensis* (OM691513), 72.2% (1709 bp); *M. ruehmi* (OM691506), 78.0% (1682 bp); and *M. crucis* (OM691510), 75.3% (827 bp).

Two obtained 28S sequences of *M. ruehmi* (672 bp and 602 bp, respectively) were compared. The sequence similarity was 98.3%. Sequences of *M. ruehmi* (OM691514 and OM691515) were further compared to the *M. arboreus* sequence (MF996702) with 81.6% (614 bp) and 81.6% (599 bp) similarity, respectively, and to the *M. crucis* sequence (OM691509) with 72.2 (455 bp) and 72.8% (453 bp) similarity, respectively. Sequence similarity of *M. ruehmi* (OM691514) and *M. canadensis* (OM691512) was found to be 84.3%. A similar picture was revealed in comparison of the obtained 18S sequences (1682 bp) of *M. ruehmi* (OM691506) with the *M. canadensis* sequence (OM691513), at 94.3% (1709 bp), and with the *M. crucis* sequence (OM691510), which was also 94.3% (827 bp).

#### Remarks

*Diastolaimus grossus* differs from the original description of the holotype in some morphometrical characters (longer tail expressed by the ratio of tail length to body length (*c*) 12.6–14.7 vs. 19, vulva is located more anteriorly 51–54 vs. 59). From the male allotype in longer tail (*c* = 12.8–16.7 vs. 15–20 and longer spicule 33–39 vs. 30 µm). The main difference is in the number of mail tail papillae. Only six postcloacal papillae are present on the original description and possibly on precloacal vs. eight precloacal and six postcloacal pairs of papillae. The number of pairs of postcloacal papillae is coincident; however, their arrangement is different.

## Discussion

### About the genus *Diastolaimus* Rahm, 1928

The genus *Diastolaimus* was proposed by Rahm (1928) for a new species, *D. papillatus* Rahm, 1928, with reference to the monotypic genus *Chambersiella* Cobb, 1920. Later, Andrassy (1984) synonymized the genus *Diastolaimus* Rahm, 1928 with the genera *Fescia* Truskova & Eroshenko, 1977 and *Santafea* Massey, 1963, all of which had identical morphological characters.

The genus contains five valid species. *Diastolaimus mexicanus*, described by Cid del Prado (2012) from Mexico, has morphology and morphometry identical to *D. grossus*. Thus, *D. mexicanus* is proposed as a new junior synonym of *D. grossus*.

#### List of species

*Diastolaimus aculeatus* (Daday, 1905) Andrassy, 1984

Syn. *Cephalobus aculeatus* Daday, 1905

Syn. *Macrolaimus aculeatus* (Daday, 1905) Thorne, 1937

*Diastolaimus croca* (Massey, 1963) Andrassy, 1984

Syn. *Santafea croca* Massey, 1963

*Diastolaimus damalis* (Massey, 1966) Andrassy, 1984

Syn. *Santafea damalis* Massey, 1966

*Diastolaimus grossus* (Truskova & Eroshenko, 1977) Andrassy, 1984

Syn. *Fescia grossa* Truskova & Eroshenko, 1977

Syn. *D. mexicanus* Cid del Prado, 2012 n. syn.

*Diastolaimus papillatus* Rahm, 1928

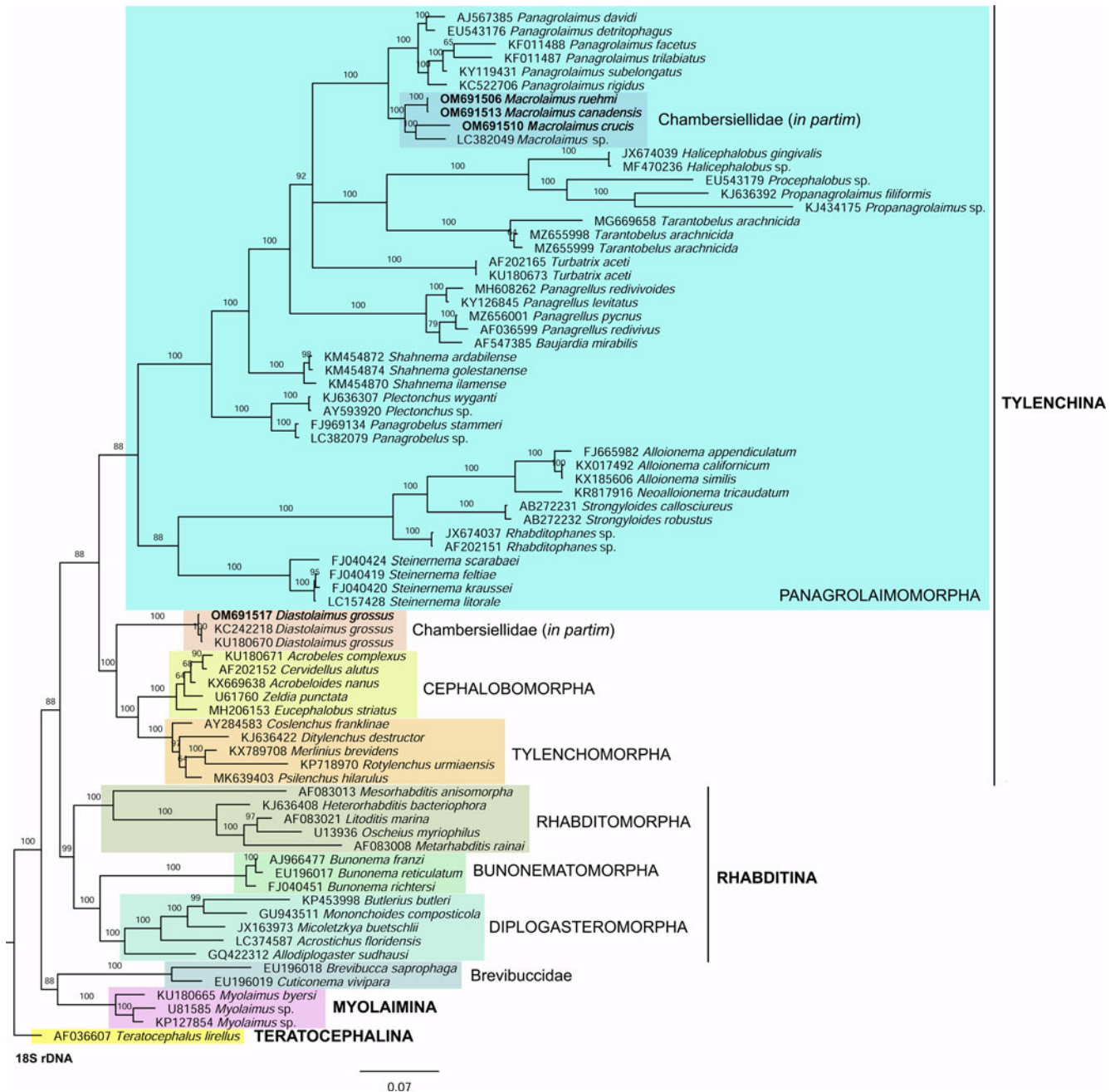
Syn. *Chambersiella papilata* (Rahm, 1928) Sanwal, 1960

#### Key to species identification

- 1a Body more than 1.9 mm . . . . . 2  
 1b Body less than 1.9 mm . . . . . 3  
 2a Spicules with not swollen manubrium; spicules c. 2.5 times the gubernaculum length . . . . . *D. aculeatus*  
 2b Spicules with swollen manubrium; spicules twice the gubernaculum length . . . . . *D. papillatus*  
 3a Cephalic papillae longer than the anterior part of gymnostom; stoma narrow . . . . . *D. damalis*  
 3b Cephalic papillae shorter than the anterior part of the gymnostom; stoma wide . . . . . 4  
 4a Posterior part of gymnostom very short; spicules 52 µm long with manubrium ventrad bent . . . . . *D. croca*  
 4b Posterior part of gymnostom slightly shorter than the anterior part; spicules 30–40 µm long with manubrium not ventrad bent . . . . . *D. grossus*

#### Phylogenetic position of the genus *Diastolaimus* and its relatives

In the phylogenetic study of Nadler *et al.* (2006), *Fescia* (junior synonym of *Diastolaimus*) and *Macrolaimus* were always monophyletic with strong support (100% by maximum parsimony (MP) bootstrap) and in the MP consensus tree were more closely related to members of the clade including Cephalobomorpha and Tylenchomorpha. However, their position among sampled nematodes varied according to tree inference method. De Ley & Blaxter (2004) considered the structure of sensory organs of the members of the family Chambersiellidae as plesiomorphic compared to Cephalobomorpha or Panagrolaimomorpha. Holovachov *et al.*



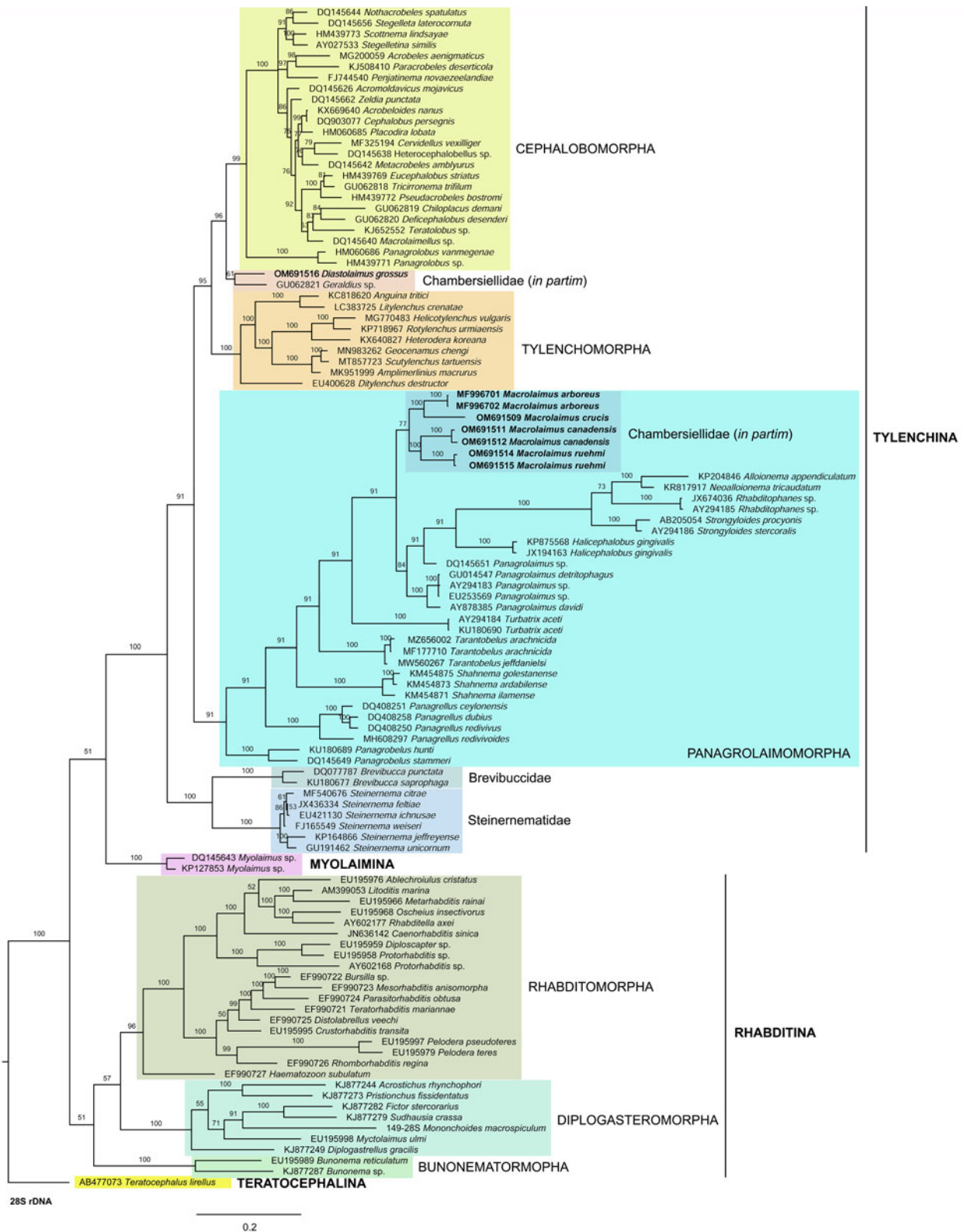
**Fig. 6.** The Bayesian tree inferred from rhabditid species based on sequences of the 18S rDNA region. Bayesian posterior probabilities (%) are given for each clade. Scale bar shows the number of substitutions per site.

(2015) considered that their large 18S + 28S dataset appears to contain insufficient phylogenetic signal for unambiguously resolving the relationship of *F. grossa*. In five out of eight analyses, it was sister taxon to Cephalobomorpha + Tylenchomorpha, but with varying support values. However, for the purposes of the analyses of the unpartitioned secondary structure alignment, *Fescia* was a part of the Panagrolaimomorpha clade (Holovachov et al., 2015).

Phylogenetic analysis based on 18S rDNA (fig. 6) and 28S rDNA (fig. 7) fragments revealed a clear polyphyly of the family Chambersiellidae as well as the subfamily Macrolaiminae Sanwal, 1971, which positively corresponds with the results of Grow–Ward analysis. All the *Macrolaimus* species, which represent a more evolved species with a less

complicated structure of sensory organs (Abolafia et al., 2018; fig. 8), appear as monophyletic taxon in the infraorder Panagrolaimomorpha. On the other hand, the genus *Diastolaimus* (Macrolaiminae cf. Andrassy, 1984, 2005) appears located together with the genus *Geraldus* Sanwal, 1971 (Chambersiellinae). According to this, the genus *Diastolaimus* is transferred to the subfamily Chambersiellinae, which represent taxa including ancestral plesiomorphic characters appearing as a sister taxon of Cephalomorpha, while the subfamily Macrolaiminae, with the only genus *Macrolaimus*, is located in the family Panagrolaimidae.

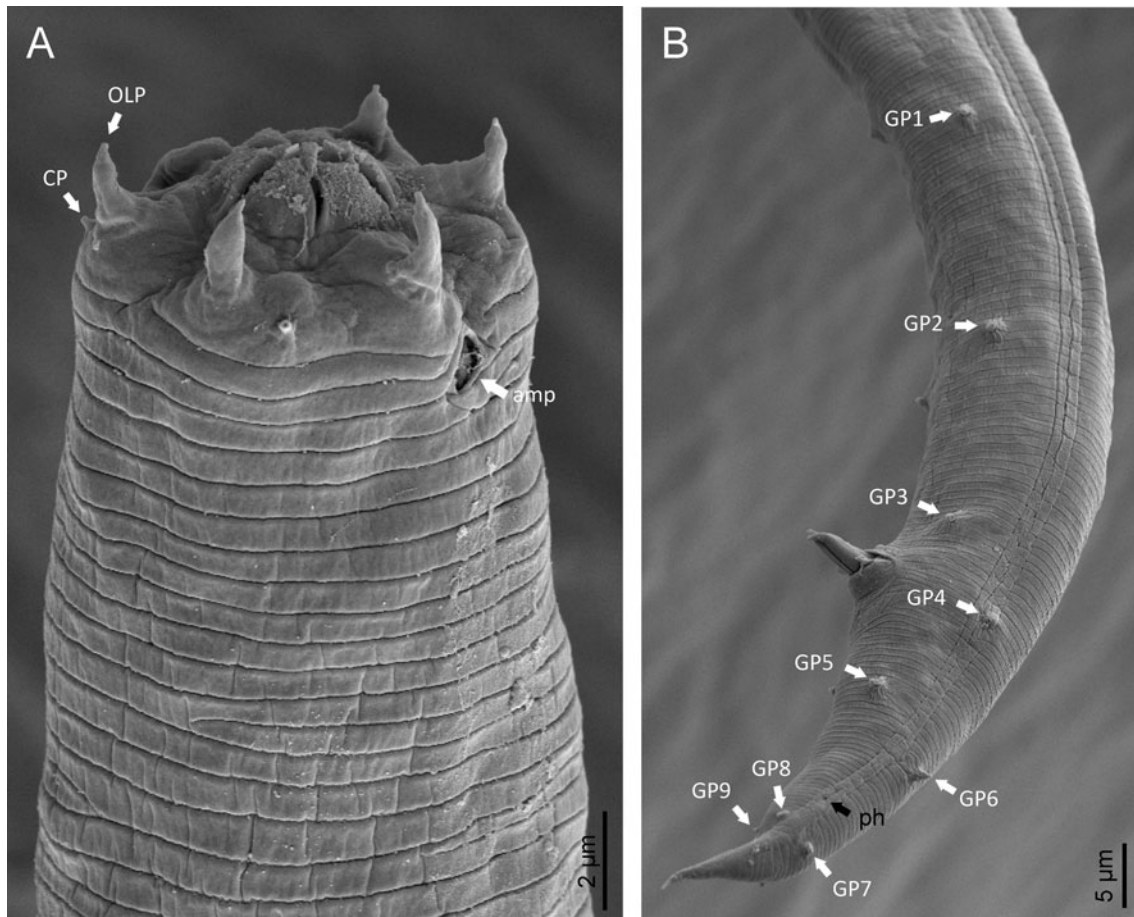
Abolafia et al. (2018) and Nadler et al. (2006) connected the number of preloacal papillae and number of reproductive



**Fig. 7.** The Bayesian tree inferred from rhabditid species based on sequences of the 28S rDNA region. Bayesian posterior probabilities (%) are given for each clade. Scale bar shows the number of substitutions per site.

branches with the phylogenetic position. Species with a didelphic female reproductive system represent an ancestral (plesiomorphic) character, whereas species with a monodelphic

reproductive system represent a derived (apomorphic) character (Chitwood & Chitwood, 1950; Lorenzen, 1978, 1981). In the 28S phylogenetic tree (fig. 7), *D. grossus* (X645-28S) is placed in



**Fig. 8.** Lip region (a) and male posterior end (b) of *Macrolaimus crucis* Maupas, 1900 from coastal sand dune in Alicante (Spain).

one clade with *Geraldius* sp. (GU062821). Both genera share ancestral characters including high number of papillae and didelphic reproductive system in females. The only species of the genus *Geraldius* Sanwal, 1971, *G. bakeri*, bears seven pairs and eight pairs of postcloacal papillae. Other related species *C. rodens* also has a high number of precloacal papillae – six pairs and eight pairs of postcloacal, despite monodelphic female reproductive system. *Diastolaimus croca* has 13 pairs of caudal papillae, four pairs subventral, one pair lateral preanal, one pair postanal and lateral, four pairs postanal subventral and three pairs postanal and subdorsal. Based on the Gower–Ward method (figs 4 and 5), the key grouping morphological character besides number of caudal papillae is also the level of complexity of labial structures. All the genera with higher level of complexity of labial structures are grouped together, regardless of the number of reproductive branches in females. We can assume that the different/lower number of precloacal papillae in the original description of *D. grossus* provided by Truskova & Eroshenko (1977) is not the difference between species, but just that the precloacal papillae was not originally observed.

The latest described species *D. mexicanus* (Cid del Prado, 2012) is very similar to *D. grossus*, especially with respect to females (see fig. 3). Males are slightly different in some morphometrical characters: number of pairs of genital papillae (13 in *D. mexicanus* vs. 14 in *D. grossus*), more posteriorly located excretory pore (158 vs. 128 µm; however, an illustration in Cid del Prado (2012) shows that its position is similar), phasmid–cloaca distance

(18 vs. 41 µm). However, with respect to the number of genital papillae, SEM illustrations in the original description are not clear and perhaps some of the genital papillae were not observed in *D. mexicanus*. The excretory pore is in the same location in both species according to the illustration of *D. mexicanus*. With respect to the phasmid–cloaca distance, at least some measurements of the male phasmid position are incorrect in *D. mexicanus* since the description states 18 µm while the drawing gives the measurement as 28 µm (= 40%) – this range is too large and perhaps the phasmids were confused with genital papillae in some measurements. According to this, we proposed *D. mexicanus* as a junior synonym of *D. grossus*.

#### **Comments on the distribution and ecology of *D. grossus* and its relatives**

To date, *D. grossus* is known only from four countries: Russia (Truskova & Eroshenko, 1977), Ukraine (Nadler et al., 2006), the Czech Republic and Bosnia and Herzegovina (present study). All the members of the subfamily Chambersiellinae can be considered as epiphytic nematodes living on the bark of various tree species and can survive rapid and long dehydration (Cobb, 1920; Rahm, 1928). *Diastolaimus grossus* was originally extracted from the bark of fir tree twigs. Bosnian specimens were also extracted from the bark of a coniferous tree (*P. nigra*); only the Czech population was found on the bark of a broadleaf tree (*Salix* sp.). With respect to other members of the genus

*Diastolaimus*, almost all of the species are associated with tree surface and are mainly distributed in the western hemisphere: *D. croca* and *D. damalis* in association with white fir, juniper and pine tree in North America (Massey, 1963, 1966); *D. papillatus* in South America (Brazil) in moss on orange leaves (Rahm, 1928). Daday (1905) did not note any details about sampling site and associations for *D. aculeatus* except that the location lake was close to Estia Postillon. *Chambersiella rodens* was found in many parts of the eastern US in association with various trees (Cobb, 1920) and *Geraldus backeri* from the bark of the oak tree in Ontario, Canada (Sanwal, 1957). Except for *D. grossus*, the second European member of the subfamily Chambersiellinae is *Cornilaimus furcillus*, which was found in Far East Russia in association with the plant *Cornus* (*Chamaepericlymenum canadensis* L.

Members of the subfamily Macrolaiminae are living both in soil and on the bark of various trees as epiphytic organisms. *Macrolaimus crucis* was found three times in different kinds of soil (Abolafia & Peña-Santiago, 2014); *M. richteri* was extracted from dry sandy soil in rocky desert; *M. arboreus* was found originally on the bark of fir in Russia, and secondary in soil in the rhizosphere of a pine tree in Iran. Based on all known findings of species, *M. ruehmi* generally prefers general broadleaf trees, but it was, however, found repeatedly in one location to be associated with *Pinus sylvestris* (Abolafia et al., 2018). *Macrolaimus canadensis* has not been found to be associated with any broadleaf tree yet (Fuchs, 1938; Massey, 1974; Abolafia et al., 2018). *Macrolaimus canadensis* and *M. ruehmi* seem to be obligatory epiphytic organisms associated with trees.

Members of the subfamily Chambersiellinae can be considered to be less evolved in comparison with Macrolaiminae, especially their way of life and ecological niche (epiphytic/tree association) can be perceived as an ancestral biological character. Soil association probably came later, and can be facultative to a certain degree. The occurrence of the same nematode species in a different niche is well known and spread among different taxonomic groups of nematodes, such as: *Pristionchus* (Sudhaus & Fürst von Lieven, 2003), *Halicephalobus* (Taufel, 1966), *Bursaphelenchus* (Čermák et al., 2014), *Prionchulus* (Andrássy, 2009). This suggests the facultative transition between bark and soil environment can be beneficial for the species survival and improve its competitive strategies.

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**Conflicts of interest.** None.

**Ethical standards.** All procedures contributing to this study comply with the ethical standards of the relevant national and institutional guides on the care and use of animals.

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