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# A novel endosymbiont-containing trypanosomatid *Phytomonas borealis* sp. n. from the predatory bug *Picromerus bidens* (Heteroptera: Pentatomidae)

Anna I. Ganyukova[1], Alexander O. Frolov[1], Marina N. Malysheva[1], Viktoria V. Spodareva[1,2], Vyacheslav Yurchenko[2,3] and Alexei Yu. Kostygov[1,2]

[1] Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia;

[2] Life Science Research Centre, Faculty of Science, University of Ostrava, Ostrava, Czech Republic;

[3] Martsinovsky Institute of Medical Parasitology, Tropical and Vector Borne Diseases, Sechenov University, Moscow, Russia

**Abstract:** Here we describe the new trypanosomatid, *Phytomonas borealis* sp. n., from the midgut of the spiked shieldbugs, *Picromerus bidens* (Linnaeus), collected in two locations, Novgorod and Pskov Oblasts of Russia. The phylogenetic analyses, based on the 18S rRNA gene, demonstrated that this flagellate is a sister species to the secondary monoxenous *Phytomonas nordicus* Frolov et Malysheva, 1993, which was concurrently documented in the same host species in Pskov Oblast. Unlike *P. nordicus*, which can complete its development (including exit to haemolymph and penetration into salivary glands) in *Picromerus bidens*, the new species did not form any extraintestinal stages in the host. It also did not produce endomastigotes, indispensable for transmission in other *Phytomonas* spp. These observations, along with the fact that *P. bidens* overwinters at the egg stage, led us to the conclusion that the examined infections with *P. borealis* were non-specific. Strikingly, the flagellates from the Novgorod population contained prokaryotic endosymbionts, whereas the parasites from the second locality were endosymbiont-free. This is a first case documenting presence of intracellular symbiotic bacteria in *Phytomonas* spp. We suggest that this novel endosymbiotic association arose very recently and did not become obligate yet. Further investigation of *P. borealis* and its intracellular bacteria may shed light on the origin and early evolution of endosymbiosis in trypanosomatids.

**Keywords:** new trypanosomatid species, prokaryotic endosymbionts, non-specific infection

This article contains supporting information (Fig S1) online at http://folia.paru.cas.cz/suppl/2020-67-004.pdf

The flagellates of the family Trypanosomatidae Doflein, 1901 parasitise insects, leeches; vertebrates, plants and ciliates (Podlipaev 1990). The members of the family are traditionally subdivided into two non-taxonomic groups: dixenous and monoxenous trypanosomatids (Maslov et al. 2019). The former group is characterised by the life cycles with an invertebrate vector and a vertebrate or a plant host. The representatives of the latter are restricted to one host, which is typically an insect. The transition to dixeny occurred independently in three different groups of monoxenous trypanosomatids (Lukeš et al. 2014, Frolov et al. 2015).

Dixenous trypanosomatids of the genera *Trypanosoma* Gruby, 1843 and *Leishmania* Ross, 1903 are among the most intensively studied parasitic protists' groups, since their representatives cause dangerous diseases in humans and domestic animals (Hoare 1972, Bruschi and Gradoni 2018). Meanwhile, the genus *Phytomonas* Donovan, 1909,

despite the fact that some of its members are pathogens of economically important cultivated plants (Camargo 1999), was effectively neglected and little is known about its diversity, biology and life cycles (Jaskowska et al. 2015).

Although the first species was described more than a century ago (Lafont 1909), the taxonomy of the genus is still underdeveloped. Only five out of fourteen species, described in the twentieth century, were verified by molecular markers (Jaskowska et al. 2015, Frolov et al. 2016, Zanetti et al. 2016) and two more species were described in the last few years (Seward et al. 2017, Frolov et al. 2019). However, in addition to this, there are more than two hundred laboratory strains, most of which do not belong to any described species (Jaskowska et al. 2015).

The genus *Phytomonas* is primarily interesting because of the peculiarities associated with its adaptation to parasitism in plants (Kořený et al. 2012, Porcel et al. 2014). However, this lineage also demonstrates another important
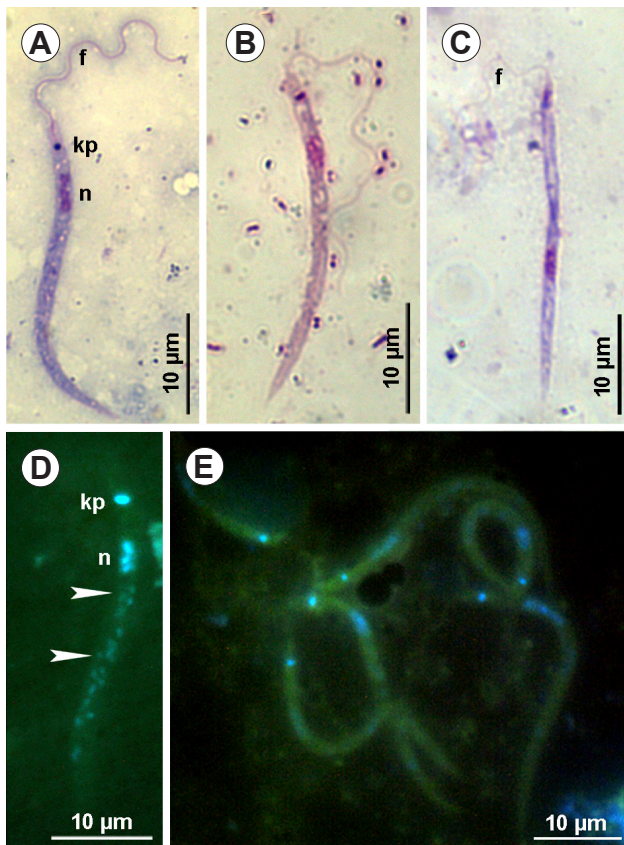
**Fig. 1.** Promastigotes of *Phytomonas* spp. from *Picromerus bidens* (Linnaeus) (light and fluorescent microscopy). **A**, **D** – *P. borealis* sp. n. from Novgorod Oblast; **B**, **E** – *P. borealis* from Pskov Oblast; **C** – *P. nordicus* Frolov et Malysheva, 1993 from Pskov Oblast; **A–C** – Giemsa staining; **D**, **E** – DAPI staining. *Abbreviations*: f – flagellum; kp – kinetoplast; n – nucleus; arrows show prokaryotic symbionts.

phenomenon: the secondary transition to monoxeny which has been described in one of its members, *Phytomonas nordicus* Frolov et Malysheva, 1993, parasitising the predatory pentatomid bug *Troilus luridus* (Fabricius) (Frolov and Malysheva 1993, Frolov et al. 2016).

Here we describe the closest relative of this trypanosomatid, documented in the intestine of another predatory pentatomid bug, *Picromerus bidens* (Linnaeus). The new species possesses a very interesting trait – the ability to host endosymbiotic bacteria. This phenomenon is rare in trypanosomatids and was never previously observed in *Phytomonas* spp.

## MATERIALS AND METHODS

### Collection of insects

The spiked shieldbugs *Picromerus bidens* were collected in 2016–2019 from herbs and bushes in two locations within North-West Russia: Pskov Oblast (village Lyady, 58°35'N; 28°55'E) and Novgorod Oblast (village Oksochi, 58°39'N; 32°47'E). From July to August and from August to September the bugs were represented by nymphs (4th and 5th instars) and imagines, respectively.

The insects were euthanised with the vapours of chloroform and dissected in saline solution. Their intestine, haemolymph and the salivary glands were prepared and analysed as described previously (Frolov et al. 2016). The material containing flagellates was used for the preparation of dry smears, establishing cultures, DNA isolation and fixation for electron microscopy.

### Cultivation

The cultivation of the new species was attempted on several biphasic media with blood agar overlaid with one of the following: Brain Heart Infusion, Schneider's Drosophila Medium, Grace's Insect Cell Culture Medium, TC-100 Insect Medium, RPMI 1640, and M199 (all from Sigma-Aldrich, St. Louis, USA). In all cases, the liquid phase was supplemented with 10% fetal bovine serum (BioloT, St. Petersburg, Russia) and 500 µg/ml of streptomycin and 500 Units/ml of penicillin (Sigma-Aldrich). The flagellates survived in xenic cultures no longer than 20 days and were invariably lost during the subsequent passage. Therefore, all studies, reported here, were performed in natural infections.

### Microscopy

The smears prepared from the contents of infected insects' organs were processed and stained with Giemsa and 4',6-diamidino-2-phenylindole (DAPI), as described earlier (Kostygov et al. 2014). Image acquisition was done at ×1,000 magnification using the DM 2500 microscope (Leica Microsystems GmbH, Wetzlar, Germany), equipped with UCMOS14000KPA 14-Mpx camera (Toup Tek, Hangzhou, China). Standard cell measurements, calculation of mean values and standard deviation were performed in UTHSCSA Image Tool for Windows v. 3.0. Processing of samples for transmission electron microscopy and their subsequent examination followed the earlier protocol established (Frolov et al. 2018).

### DNA isolation, amplification, cloning and sequencing

Total genomic DNA was extracted from the field samples with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplification of nearly full-length 18S rRNA gene was accomplished with the primers S762 and S763 (Maslov et al. 1996). If the trypanosomatid DNA content was insufficient for that, the primers 1127F (5'-AGG-CATTCTTCAAGGATACCTTCC-3') and 1958R (5'- TCTCG-TAGGCGCAGCTCATCA-3') were applied to obtain a shorter (~830 bp) fragment of 18S rRNA gene. The gene for glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) was amplified with the primers M200 and M201 (Maslov et al. 2010). The sequencing of full-length 18S rRNA gene amplicons was done as reported elsewhere (Gerasimov et al. 2012), while all other PCR products were sequenced with the amplification primers. All sequences obtained in this work were deposited to GenBank under accession numbers MN442620–MN442623 and MN434073–MN434074 for 18S rRNA and gGAPDH genes, respectively.

### Phylogenetic analyses

**18S rRNA gene.** The 18S rRNA gene dataset included the following sequences: i) that of the species under study; ii) all available from GenBank unique (non-identical) sequences of *Phytomonas* spp. longer than 600 bp; and iii) those of four outgroup species belonging to the related genera *Lafontella* Kostygov et Yurchenko, 2015 and *Herpetomonas* Kent, 1880 (see Yurchenko et al. 2016). The sequences were aligned in MAFFT v. 7.452

**Table 1.** Prevalence of two observed trypanosomatid species in *Picromerus bidens* (Linnaeus).

| Year | imagines | | nymphs |
| | *Phytomonas borealis* sp. n. | *P. nordicus* Frolov et Malysheva, 1993 | |
|---|---|---|---|
| Novgorod Oblast | | | |
| 2016 | 31% (5/16) | 0% (0/16) | n/a* |
| 2017 | 0% (0/23) | 0% (0/23) | 0% (0/21) |
| 2018 | 0% (0/37) | 0% (0/37) | 0% (0/15) |
| 2019 | 0% (0/19) | 0% (0/19) | 0% (0/10) |
| Pskov Oblast | | | |
| 2017 | 8% (2/26) | 15% (4/26) | n/a |
| 2018 | 13% (4/32) | 22% (7/32) | n/a |
| * n/a – not available | | | |

with the E-INS-i method (Katoh and Standley 2013). Alignment positions containing gaps in more than 50% sequences were removed using the online program Gap Strip/Squeeze V. 2.1.0 (https://www.hiv.lanl.gov/content/sequence/GAPSTREEZE/gap.html). Both original and processed alignments are included into the Supplementary materials.

Maximum likelihood analysis was performed in IQTREE v. 1.6.12 (Nguyen et al. 2015) under the TIM2e + I + G4 model as selected by the built-in ModelFinder (Kalyaanamoorthy et al. 2017). The 'standard' bootstrap test with 1,000 replicates was applied to estimate the statistical support of bipartitions.

Bayesian inference was accomplished using MrBayes 3.2.7 (Ronquist et al. 2012) under the GTR + I + G model with four gamma categories. The analysis was run for five million generations and sampling every 1,000th of them, whereas other parameters were set as default.

**gGAPDH gene.** The gGAPDH dataset was created in the same way as for the above gene. However, in order to preserve codon integrity, the alignment was processed in MEGA X (Kumar et al. 2018) using the integrated Muscle module (Edgar 2004) as described elsewhere (Frolov et al. 2019). The maximum likelihood and Bayesian tree reconstructions were performed in IQ-TREE v.1.68 (Nguyen et al. 2015) and MrBayes v.3.2.6 (Ronquist et al. 2012), respectively, with partitioning of protein-coding genes by codon position as described before (Spodareva et al. 2018) and other parameters of the analyses as specified above for the 18S rRNA gene. The best partitioned model for gGAPDH selected by ModelFinder was F81 + F + I / F81 + F + G4 / TVM + F + G4 for the three respective codon positions.

## RESULTS

### Occurrence of parasites

The prevalence of phytomonads in spiked shieldbugs varied over years and localities (Table 1). During the four-year observation period in Novgorod Oblast, only once (in 2016) five out of 16 examined imagines (31%) contained long vermiform promastigotes, which could not be assigned to any described trypanosomatid species, in the midgut (Fig. 1A). In Pskov Oblast, the presence of phytomonads was documented for two consecutive years, but in contrast to the first locality, two different infection types were observed. Some bugs (8–13%) had promastigotes, morphologically similar to those from Novgorod Oblast, in the midgut (Fig. 1B). In other host specimens (15–22%), midgut promastigotes had distinct morphology (Fig. 1C) and this was accompanied by infection of the hemolymph

and salivary glands, all reminiscent of *Phytomonas nordicus*, which has been previously characterised in the same region, from a locality about 50 km afar (Frolov et al. 2016).

### Phylogenetic analysis

The analysis of 18S rRNA gene sequences confirmed our morphological identification of the parasite with extra-intestinal stages and twisted promastigotes in the midgut as *P. nordicus* (100% identity). The sequences of the second flagellate were identical in both sampled localities. The closest BLAST hit in the GenBank database was *P. nordicus* with differences in eight nucleotide positions, indicating that the two trypanosomatids belong to separate species (see d'Avila-Levy et al. 2015). Both 18S rRNA gene-based maximum likelihood and Bayesian phylogenetic analyses supported the close relationship of these two species with high confidence (Fig. 2).

The *Phytomonas* sp. strain TCC084, which had been isolated from the latex of *Mandevilla scabra* in Surinam (see Zanetti et al. 2016), was identified as the closest relative to these two phytomonads. This association on the 18S rRNA gene-based tree had high posterior probability, but a moderate bootstrap support (Fig. 2), apparently due to the short length of the sequence for the isolate TCC084 (only 690 bp). Therefore, we also inferred phylogeny using the gGAPDH gene sequences, which showed high statistical support of this relationship by both methods (Fig. S1).

Subsequently, we analysed only the new species, which is hereafter referred to as *Phytomonas borealis* sp. n.

### Light and fluorescent microscopy

In all infected individuals of *Picromerus bidens*, the promastigotes of the new species formed a morphologically homogeneous micropopulation throughout the whole midgut, but were most abundant in the M2 segment. Cell divisions were very rare. All differences between morphometric parameters of the flagellates from both studied localities were negligible (Table 2). The promastigotes were vermiform and measured in average 35 × 1.3 μm with the flagellum being approximately equal in length to that of the cell body (Fig. 1A,B). The nucleus was oval and longitudinally oriented, with length nearly threefold larger than its width. The kinetoplast was located subterminally in the anterior portion of the cell, quite far from the nucleus. The staining with DAPI, in addition to the nucleus and the kinetoplast, revealed the presence of multiple (up to 20) small DNA-containing bodies, situated predominantly in the
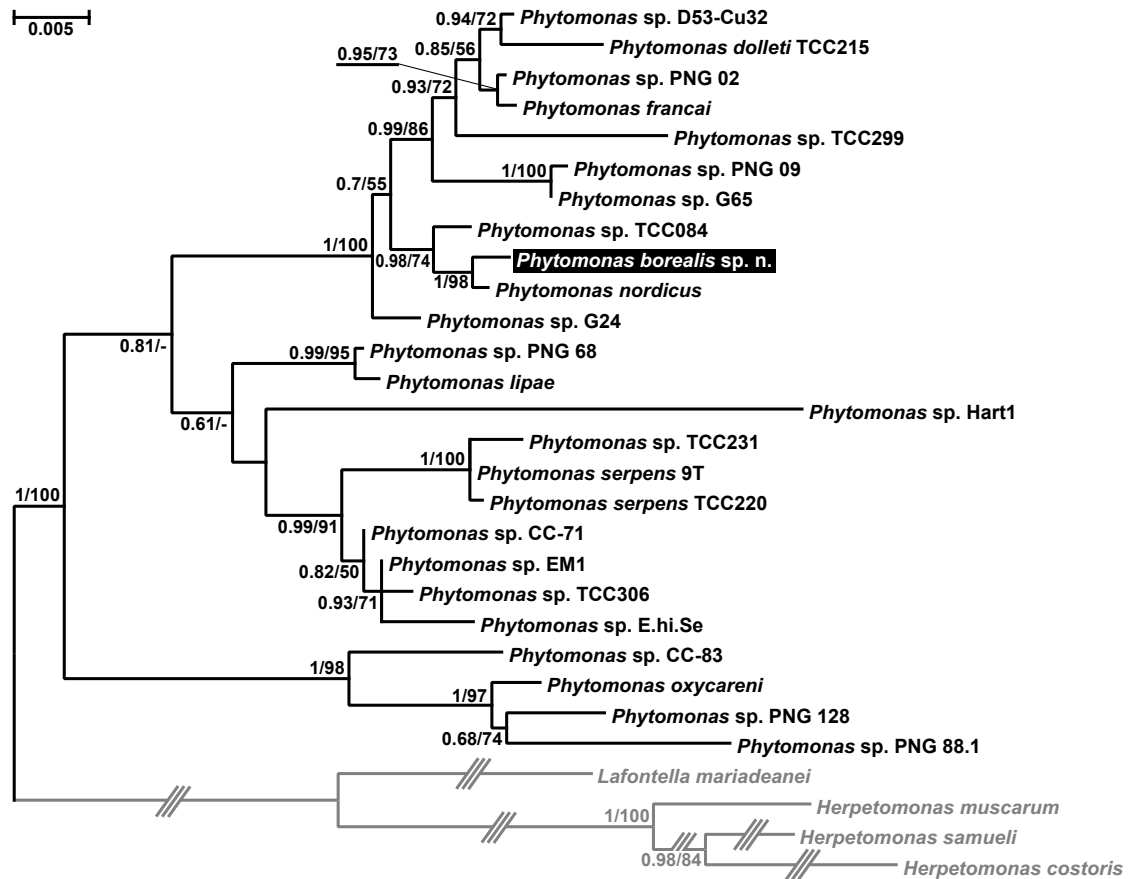
**Fig. 2.** Maximum likelihood phylogenetic tree based on 18S ribosomal RNA gene sequences. Numbers at nodes indicate posterior probability and bootstrap percentage, respectively. Values less than 0.5 and 50% are replaced with dashes or omitted. Triple-crossed branches are at 1/3 of their original lengths. The tree is rooted with the sequences of *Herpetomonas* spp. and *Lafontella* Kostygov et Yurchenko, 2015 (all shown in grey). The scale bar denotes number of substitutions per site. The species under study, *Phytomonas borealis* sp. n., is highlighted.

postnuclear part of the cell (Fig. 1D). These bodies were detected only in the isolates from the Novgorod Oblast, whereas no such entities were observed in the flagellates from the second locality (Fig. 1E).

**Electron microscopy**

The promastigotes of *P. borealis* formed no visible contacts with the intestinal epitheliocytes of the host (Fig. 3A). The ultrastructure of the flagellates was similar to that of previously studied *Phytomonas* spp. They had tubulemma with a regular microtubule corset, short terminally opening flagellar pocket, nucleus with parietal chromatin layer and central nucleolus, and typical compact kinetoplast with rod-shaped profile measuring $0.65 \pm 0.11 \times 0.2 \pm 0.05$ μm (Fig. 3B,C). As in the majority of trypanosomatids, the Golgi complex was located next to kinetoplast (Fig. 3B). The cytoplasm contained numerous ribosomes, glycosomes, acidocalcisomes and mitochondrial branches (Fig. 3C). The remarkable feature was the presence of symbiotic bacteria in the cytoplasm (Fig. 3C–G). The outer of the two membranes surrounding the endosymbionts appeared to be that of host origin, as judged by its contacts with the endoplasmic reticulum (Fig. 3F,G). Neither the

periplasmatic space, nor the cell wall were revealed in the bacterial cells (Fig. 3D,G).

**Taxonomic summary**

Class: Kinetoplastea Honigberg, 1963

Order: Trypanosomatida Kent, 1880

Family: Trypanosomatidae Doflein, 1901

Genus: *Phytomonas* Donovan, 1909

*Phytomonas borealis* Ganyukova, Frolov et Kostygov sp. n.

M o r p h o l o g y : long vermiform promastigotes in host midgut $34.8 \pm 7.7$ μm long and 1.3 μm $\pm$ 0.1 μm wide, flagellar length roughly equal to that of cell body; nucleus ($3.8 \pm 1.0$ μm $\times$ $1.3 \pm 0.1$ μm) and kinetoplast ($0.71 \pm 0.10 \times 0.2 \pm 0.05$ μm ) located at 7.3 μm $\pm$ 1.9 μm and 2.0 $\pm$ 0.6 μm from the anterior cell end, respectively.

G e n e   s e q u e n c e s : The species can be identified by the sequences of 18S rRNA and gGAPDH genes (GenBank accession numbers: MN442620 – MN442623 and MN434073 – MN434074, respectively) .

**Table 2.** Morphometry of promastigotes of *Phytomonas borealis* sp. n. (N = 31).

| Isolate | Length | Width | Nucleus | N-A | N-K | K-A | Flagellum |
|---|---|---|---|---|---|---|---|
| Pic3 | 34.8 ± 7.7 | 1.3 ± 0.1 | 3.8 ± 1.0 | 7.3 ± 1.9 | 4.8 ± 2.0 | 1.9 ± 0.6 | 33.5 ± 6.8 |
| Novgorod Oblast | 22.1–50.1 | 1.1–1.7 | 2.1–6.3 | 3.7–13.5 | 1.6–9.2 | 0.9–3.0 | 20.5–50.9 |
| Pic38 | 35.4 ± 7.5 | 1.3 ± 0.1 | 3.8 ± 0.6 | 7.2 ± 2.0 | 4.9 ± 1.5 | 1.9 ± 0.5 | 35.0 ± 8.6 |
| Pskov Oblast | 20.6–48.5 | 1.1–1.5 | 2.5–5.3 | 3.1–11.7 | 2.9–10.5 | 1.1–2.8 | 20.4–54.1 |

N-A – the distance between the nucleus and the anterior end of the cell; N-K – the distance between the nucleus and the kinetoplast; K-A – the distance between the kinetoplast and the anterior end of the cell. All measurements are in µm.

Type host: *Picromerus bidens* (Linnaeus, 1758) (Heteroptera: Pentatomidae) (non-specific host – see comments)

Location within host: midgut (see comments)

Type locality: Russia: Novgorod Oblast: village Oksochi (58°39'N; 32°47'E)

Other localities: Russia: Pskov Oblast: village Lyady (58°35'N; 28°55'E)

Type material: Giemsa-stained slide Pic3_16 (hapantotype) deposited in the research collection of Parasitic Protists of the Zoological Institute RAS (St. Petersburg, Russia) along with additional smears Pic1_16, Pic7_16; Pic9_16 (from Novgorod Oblast) and Pic38_17, Pb2_18; Pb7_18; Pb10_18 (from Pskov Oblast).

Etymology: the specific epithet *borealis* is a Latin adjective (boreālis) meaning "northern". It was selected to emphasise the presence of this species in northern European Russia.

Comments: This species differs from the closely related *P. nordicus*, documented in the same bug species, by the overall cell shape (vermiform versus spindle-shaped and twisted). The new species was found only in a non-specific host, therefore neither the specific host, nor the life cycle are known.

## DISCUSSION

*Picromerus bidens* belongs to the subfamily Asopinae, encompassing predatory members of the shieldbug family Pentatomidae (Schuh and Slater 1995). This species is widely distributed in the Palearctic and was also introduced to North America, where it successfully acclimatised. *Picromerus bidens* is a polyphagous predator with over 250 species of several insect orders recorded as prey (Schaefer and Panizzi 2000). This shieldbug has univoltine (one brood per year) life cycle with obligate embryonic diapause and overwinters primarily at the egg stage. Although rare cases of hibernation as adults (presumably associated with tachinid parasitoids) were also recorded (Saulich and Musolin 2014), these insects are unlikely to meet imagines of the new season.

All the above facts along with our observation, that nymphs in spiked stinkbugs were never infected with phytomonads, point to the non-specific nature of the studied host-parasite system. Another important argument in favour of this view is the absence of extraintestinal developmental stages in the observed infections with *P. borealis*. Even the secondarily monoxenous *Phytomonas nordicus* preserves the classical phytomonad developmental program in the insect host with exit to haemolymph and penetration into the salivary glands (Frolov et al. 2016). Endomastigotes, the resting stage, which is indispensable for transmission, were documented in all phytomonad species

studied *in vivo* thus far (Jankevicius et al. 1989, Freymuller et al. 1990, Frolov et al. 2016, 2019, Zanetti et al. 2016, Seward et al. 2017). However, they were not observed in any of the bugs infected with *P. borealis*.

Meanwhile, *P. nordicus*, whose main host is the predatory *Troilus luridus*, displayed normal development in spiked shieldbugs. Thus, we conclude that the studied infections of *P. bidens* with *P. borealis* are non-specific. The ability of predatory bugs to accumulate trypanosomatids acquired from their prey has been repeatedly discussed in the literature (Wallace 1966, Podlipaev 2003, Kozminsky et al. 2015). Given the extremely wide range of the spiked shieldbug's potential victims, identifying the specific host of this trypanosomatid is currently not possible.

The close relatedness of *P. borealis* to *P. nordicus* suggests an intriguing possibility that the new species is also a monoxenous one. The predatory nature of its host is of little importance in this respect, given that the infection is non-specific. Although acquisition of *P. borealis* by the spiked shieldbug from a phytophagous heteropteran is most parsimonious, it cannot be excluded that the specific host of this trypanosomatid is one more species of the subfamily Asopinae, such as *Arma custos* (Fabricius), *Jalla dumosa* (Linnaeus) or *Zicrona caerulea* (Linnaeus).

On the one hand, we never observed any of these species in the collection localities. On the other hand, the occasional occurrence of such bugs in the biotopes, to which they do not belong, would be a plausible explanation of low frequency and sporadic nature of the *P. bidens* infections. Even if the life cycle of *P. borealis* is normally dixenous, its detailed study should be interesting because of the evolutionary proximity to *P. nordicus* and could shed more light on the phenomenon of secondary monoxeny.

The majority of known phytomonad species and strains were isolated in South America (Jaskowska et al. 2015). This influenced the prevailing view on the geographical distribution of the genus. Meanwhile, *P. borealis* is already the third species discovered in the boreal zone of Eurasia north of 55°N (Frolov et al. 2016, 2019). To date, the association with plants was not confirmed for any of them. While for *P. nordicus* the restriction to only insect host was demonstrated (Frolov and Malysheva 1993, Frolov et al. 2016), the nature of the life cycles of *Phytomonas lipae* Frolov et Kostygov, 2019 and *P. borealis* remains obscure and requires further investigation.

The discovery of prokaryotic endosymbionts in the cytoplasm of promastigotes of *P. borealis* was unexpected, since none of the previously studied *Phytomonas* spp. has this trait. Until recently, symbiotic relationships were
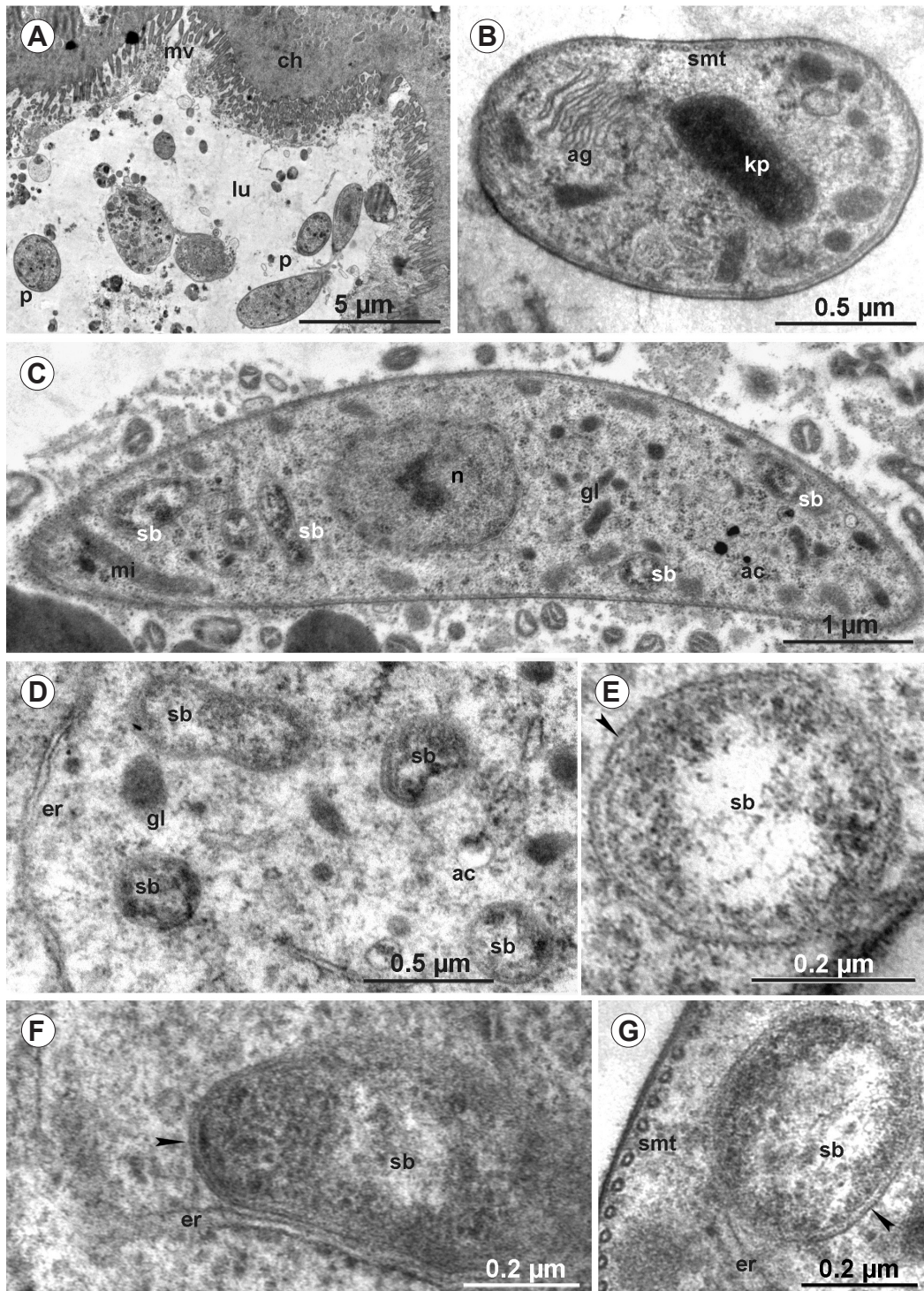
**Fig. 3.** Promastigotes of *Phytomonas borealis* sp. n. from *Picromerus bidens* (Linnaeus) (TEM). **A** – flagellates in the midgut lumen; **B** – cross section; **C** – longitudinal/tangential section; **D–G** – prokaryotic endosymbionts in the cytoplasm of flagellates. *Abbreviations*: ac – acidocalcisomes; ag – Golgi complex; ch – host's epithelial cell; er – endoplasmic reticulum; gl – glycosomes; kp – kinetoplast; lu – lumen of the gut; mi – mitochondrion; mv – microvilli; n – nucleus; p – promastigotes; sb – cell of prokaryotic symbiont; smt – subpellicular microtubules; arrows show the membrane of the symbyontophorous vacuole.

known only for one trypanosomatid lineage – the subfamily Strigomonadinae. All described species of all its three genera – *Strigomonas* Lwoff and Lwoff, 1931, *Angomonas* Souza and Corte-Real, 1991 and *Kentomonas* Votýpka, Yurchenko, Kostygov et Lukeš, 2014 – contain obligate intracellular β-proteobacteria *Candidatus* Kinetoplastibacterium spp. (one per host cell), with which they underwent a long-term coevolution (Du et al. 1994, Teixeira et al. 2011, Alves et al. 2013a, Votýpka et al. 2014, Silva et al. 2018). This resulted in accurate coordination of cell cycles and integration of metabolic pathways in these endosymbiotic associations (Motta et al. 2010, Alves et al. 2013b, Klein et al. 2013).

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An independently arisen endosymbiosis has been described in *Novymonas esmeraldas* Votýpka, Kostygov, Maslov et Lukeš, 2016 (subfamily Leishmaniinae), which harbours multiple (up to 15) cells of the β-proteobacterium *Ca.* Pandoraea novymonadis Kostygov et al. (2016). Neither the trypanosomatid, nor the bacterium have close relatives involved in such relationships, which indicates relatively recent origin of their association. Indeed, their cell cycles are not coordinated, resulting in unstable number of bacteria per trypanosomatid cell. Nevertheless, the genome of the endosymbiont has already undergone significant reduction and de-duplication of metabolic pathways, common with the host (Kostygov et al. 2017).

The multiplicity of endosymbionts in *P. borealis* is reminiscent of the situation in *N. esmeraldas*, where the relationships are not finely tuned. Moreover, the absence of bacteria in the cytoplasm of the flagellates from Pskov Oblast points to possibility that this endosymbiotic system is in 'infancy' and, therefore, not obligate. It is unclear, whether the bacteria-free population of *P. borealis* lost endosymbionts or never had them. In order to answer this question, as well as to determine the taxonomic position of the symbionts and understand their interaction with the trypanosomatid host, it is necessary to establish an axenic laboratory culture of *P. borealis*. Nevertheless, there is no doubt that this endosymbiotic system arose independently from those studied previously and its investigation should shed light on the origin and early evolution of endosymbioses in trypanosomatids.

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