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Protistology

ORIGINAL ARTICLE

Phytomonas vermiformis sp. n. (Kinetoplastea, Trypanosomatidae), a parasite of the shieldbug *Peribalus strictus vernalis* (Hemiptera, Pentatomidae)

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Summary

A new species of dixenous trypanosomatids, *Phytomonas vermiformis* sp. n., was discovered in the gut and salivary glands of the vernal shieldbug *Peribalus* (*P.*) *strictus vernalis* (Wolff, 1804) in the Novgorod Region and in Krasnodar Krai of the Russian Federation. Phylogenetic analysis based on SSU rRNA gene revealed a high level of similarity between the European isolates of *P. vermiformis* sp. n. and the isolate G24 (TU76) from the bug *Aeptus singularis* Dallas, 1851 (Pentatomidae) in Ghana. In insect hosts, the cells of *P. vermiformis* sp. n. were represented by two morphotypes: promastigotes and endomastigotes. Both morphotypes were found in the gut, the dominant one being represented by worm-like promastigotes with long flagella. Very long promastigotes with the body twisted several times along its length were noted in the hemolymph. Some of them had long flagella, while others had no flagella at all. Promastigotes and small endomastigotes were detected in salivary glands. Endomastigotes of *P. vermiformis* sp. n. were also found in the homogenate obtained from inflorescences of nettle *Urtica dioica* L., on which the shieldbugs aggregated in autumn. In culture, the cells of *P. vermiformis* sp. n. were represented by promastigotes and endomastigotes. Large virus-like particles (VLP) of two morphotypes were found in the cytoplasm of *P. vermiformis* sp. n. This is the first report of these particles in trypanosomatids. VLP1 had a diameter of about 260 nm, possessed an icosahedral capsid and were localized in the central part of the cytoplasm. VLP2, spheroid particles about 210 nm in diameter, were mainly localized at the periphery of the cell in the submembrane zone.

Key words: *Phytomonas*, new species, phylogeny, morphology, ultrastructure, large virus-like particles, *Peribalus strictus vernalis*

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Introduction

The genus *Phytomonas* (Donovan, 1909) (Kinetoplastea, Trypanosomatida) comprises species that are dixenous, i.e. their life cycle involves two hosts. The first host is represented by hemipteran insects from four families of true bugs: Alydidae, Coreidae, Lygaeidae and Pentatomidae (Camargo, 1999; Jaskowska et al., 2015; Frolov et al., 2015, 2021). In these bugs, the flagellates undergo a complex development associated with the invasion of various host organs and several cycles of endogenous agglomeration (Frolov et al., 2015, 2021; Frolov, 2016). The second host (reservoir) of phytomonads is represented by vascular plants, in which the flagellates can survive for a long time, in some cases retaining the ability for reproduction (Camargo, 1999; Jaskowska et al., 2015).

The monophyly of *Phytomonas* has been confirmed in numerous molecular phylogenetic and phylogenomic studies (Porcel et al., 2014; Frolov et al., 2016, 2019; Butler et al., 2017; Seward et al., 2017; Lukeš et al., 2018; Kostygov et al., 2024). Its closest relatives are monoxenous genera *Lafontella* and *Herpetomonas* (Frolov et al., 2021; Kostygov et al., 2024). These three genera, which could have a common monoxenous ancestor, are currently united into the subfamily Herpetomonadinae (Yurchenko et al., 2021; Kostygov et al., 2021, 2024; Albanaz et al., 2023). The composition of *Phytomonas* itself appears to be heterogeneous. The clades detected within this genus with the use of various molecular markers generally do not correlate with the food preferences and ecology of their members or the biogeography of their hosts (Dollet et al., 2000, 2012; Zanetti et al., 2016).

The knowledge of diversity and biology of *Phytomonas* spp. has numerous gaps. This is partly due to the fact that the research on trypanosomatids in general and phytomonads in particular has traditionally been conducted in areas with hot climate (Westenberger et al., 2004; Maslov et al., 2007; Votýpka et al., 2010, 2012, 2019, 2020, 2024; Králová et al., 2019), and for many decades these flagellates have been known only from the tropics and the subtropics. This is unsurprising, considering the enormous diversity of insects in those areas, where the centres of origin and radiation of the main groups of insects serving as trypanosomatid hosts are presumably located. At the same time, vast areas with a temperate climate have been explored poorly, or not at all, in this respect.

Northern Eurasia is one such blank space on the map (Frolov et al., 2019). This region has long been ignored in faunistic studies of phytomonads. It has been assumed a priori that a harsh climate with long cold winters and a short vegetation season of potential host plants entails if not a complete absence then at least an extremely limited diversity of *Phytomonas* spp. For a long time, the only phytomonad described in the Palearctic to the north of 50° N was *Phytomonas nordicus* (Frolov and Malysheva, 1993). This species has reverted to a monoxenous life style in the course of evolution (Frolov et al., 2016). Its life cycle is fully implemented in predatory bugs from the family Pentatomidae and does not involve any plants.

Recently, however, things have started to change. Two more species of "northern phytomonads" have been discovered and described in the last five years. Moreover, while one of them, *P. borealis*, was found, similarly to *P. nordicus*, in predatory pentatomid bugs (*Picromerus bidens* Linnaeus, 1758), another one, *P. lipae*, turned out to be a parasite of the dock bug *Coreus marginatus* Linnaeus, 1758 (Coreidae), which is phytophagous (Frolov et al., 2019; Ganyukova et al., 2020).

In this paper, we describe *Phytomonas vermiformis* sp. n. from the phytophagous shieldbug *Peribalus strictus vernalis* and analyse its morphology and phylogeny. We also report the presence of their endomastigotes in the homogenate obtained from the inflorescences of nettle *Urtica dioica* L. (Urticaceae), on which *P. strictus vernalis* bugs aggregate before wintering. We hypothesise that *P. vermiformis* sp. n. may also be distributed on the African continent because a similar genotype (TU76) has been found earlier in isolate G24 obtained from one individual of the bug *Aeptus singularis* Dallas, 1851 (Pentatomidae) in Ghana (Votýpka et al., 2012).

Material and methods

Hosts

Peribalus strictus vernalis bugs were collected by hand from herbaceous plants at two sites located 5 km apart in the Novgorod Region (near Oksochi Village, 58°39' N; 32°47' E), Russia. Each site had an area of approximately 40 m^2. If there were bugs of other species on a plant with *P. strictus vernalis*, they were also collected to test for infection with the new phytomonad species (Table 1). In addition, one

Table 1. Infection of hemipterans with *Phytomonas vermiformis* sp. n.

Hosts \ Localization in hosts	Gut	Salivary glands	Hemolymph
Family Pentatomidae			
Peribalus strictus vernalis (Wolff, 1804)	44/38*	44/38	44/9
Eysarcoris aeneus (Scopoli, 1763)	4/1	4/0	4/0
Dolycoris baccarum (Linnaeus, *1758)*	3/0	3/0	3/0
Palomena prasine (Linnaeus, 1761)	10/0	10/0	10/0
Family Scutelleridae			
Eurygaster testudinaria (Geoffroy, 1785)	7/0	7/0	7/0

* Number of dissected/infected insects.

individual of *P. strictus vernalis* was brought to the laboratory in a container together with the larvae of bugs *Nezara viridula* L. from Krasnodar (45°4' N; 38°58' E), Russia.

Inflorescences of nettle *Urtica dioica*, on which individuals of *P. strictus vernalis* were observed to feed, were placed in a hand-held homogeniser and ground in a small amount of physiological solution. The liquid fraction was purified by filtering through gauze and viewed under a microscope with phase contrast.

Dissection of insects. The insects were chloroformed before dissection. Salivary glands and fragments of the gut isolated part by part were placed into separate wells with saline solution as described previously (Malysheva et al., 2020). They were viewed under a microscope and, if trypanosomatids were found, the infected organs were used for further work.

Culturing and cryopreservation of trypanosomatids. SDM medium (Sigma-Aldrich, St. Louis, USA) with an addition of 10% fetal bovine serum (BioloT, St. Petersburg, Russia) was used as the basic medium for obtaining cultures of *Phytomonas vermiformis* sp. n. Cultivation and cryopreservation techniques of trypanosomatid isolates have been described in one of our recent articles (Frolov et al., 2022). Two laboratory cultures were obtained from fragments of infected organs of *P. strictus vernalis* (F2-470; F2-478) and one culture (F2-485) was obtained from the intestine of *Eysarcoris aeneus* (Scopoli, 1763).

The cultures are kept in the Research Collection of Parasitic Protists at the Zoological Institute of the Russian Academy of Sciences (St. Petersburg, Russia).

DNA isolation, amplification, cloning and sequencing

Genomic DNA from the host tissues (salivary glands, gut) and flagellate cultures was extracted using PureLink Genomic DNA Kit (Invitrogen) for DNA extraction according to the manufacturer's instructions. To amplify a short fragment of small SSU rRNA (~800 bp) we used 1127F-1958R primer pair (Maslov et al., 1996), and to amplify a long fragment of this gene (~2200 bp) we used S762-S763 primer pair (Kostygov and Frolov, 2007). gGAPDH gene fragments were amplified using primers M200-M201 (Maslov et al., 2010). Sequencing of the amplicons was performed as described previously (Frolov et al., 2019). The resulting sequences were used for phylogenetic analysis.

Phylogenetic analyses

The sequences of SSU rRNA and gGAPDH genes obtained in this study were combined with those available in GenBank (NR and WGS databases) (Supplement). Sequences of both genes were aligned in MAFFT v. 7.520 with the L-INS-i algorithm (Katoh and Standley, 2013). After that, the sequence alignments were additionally adjusted manually. The resulting alignment of SSU rRNA gene comprised 46 sequences with 2131 nucleotide sites including indels. The maximum likelihood tree reconstruction was performed in IQ-TREE v.1.6 (Nguyen et al., 2015) with the best evolutionary model (TIM2e+I+G4) selected automatically. Branch support was estimated using the standard bootstrap method (1000 replicates). Bayesian inference was accomplished in MrBayes v.3.2.7

under the GTR+I+G model, with the analysis run for 2,000,000 generations, the trees sampled every 1000 generations, and the other parameters left in default states (Ronquist and Huelsenbeck, 2003). The resulting alignment of SSU rRNA gene included 29 sequences with 1011 nucleotide sites. The best partitioned model of nucleotide substitutions selected by PartitionFinder was HKY+I+G/GTR+I/GTR+I/GTR+G for the first, second, and third codon positions of the gene (Lanfear et al., 2012, 2017). The maximum likelihood tree reconstruction was performed in IQ-TREE v.1.6 (Nguyen et al., 2015), and the branch support was estimated using the standard bootstrap method (1000 replicates). Bayesian inference was accomplished in MrBayes v.3.2.7 with the analysis run for 1,000,000 generations, the trees sampled every 1,000 generations and the other parameters left in default states (Ronquist and Huelsenbeck, 2003).

Estimates of evolutionary divergence (p-distance) within *Phytomonas* spp. (Supplement) were obtained in MEGA X (Kumar et al., 2018). Twenty nucleotide sequences were involved in the analysis. There were a total of 2053 positions in the final dataset. All positions with less than 95% site coverage were eliminated, i.e., alignment gaps, missing data, and ambiguous bases constituting less than 5% were allowed at any position (partial deletion option). The rate of variation between sites was modelled with a gamma distribution (shape parameter = 5).

Light microscopy and morphometry

Smears of *Phytomonas vermiformis* sp. n. cultures as well as the contents of the gut fragments and salivary glands of *P. strictus vernalis* were fixed with 96% ethanol for 30 min after air-drying and stained with Giemsa stain for 30 min (pH 6.8). To visualise DNA-containing organelles, the smears were stained with 4',6-diamidino-2-phenylindole (DAPI) (1 mg/ml) as described previously (Yurchenko et al., 2006). Microphotographs were taken using a Leica DM 2500 microscope equipped with a 14-megapixel UCMOS14000KPA camera (ToupTek, Hangzhou, China) at ×1000 magnification. All measurements of *P. vermiformis* sp. n. cells (n = 31) were performed in ImageJ v.1.53e (Abramoff et al., 2004; Schneider et al., 2012). Comparison of cells of the same morphotype belonging to isolates of different *Phytomonas* spp. was performed using the Mann-Whitney test in STATISTICA v. 10 StatSoft, Inc.

Electron microscopy

Fixation and embedding of the material into synthetic resin for the TEM study was performed as described previously (Frolov et al., 2018). Ultrathin sections (60-70 nm) were prepared using an EM UC6 ultramicrotome (Leica Microsystems). The sections were contrasted with aqueous solutions of uranyl acetate (for 1 h) and lead citrate (for 5 min) and examined under a Morgagni 268-D microscope (FEI Company/ThermoFisher Scientific, Hillsboro, OR, USA) at an accelerating voltage of 80.00 kV.

Results

Shieldbugs *P. strictus vernalis* were examined in late September-October 2022 before the start of wintering, and in late April 2023 shortly after emergence from wintering. In autumn, the bugs were found in groups of 2-6 individuals on inflorescences of nettle *U. dioica*. In spring, the bugs were detected on various vegetating herbaceous plants. The infection of the other hemipterans was studied in autumn, when various other bugs were feeding together with *P. strictus vernalis* on inflorescences of *U. dioica*. After microscopic examination of the gut and salivary glands of 68 individuals of bugs from the families Pentatomidae and Scutelleridae in the Novgorod Region, we found promastigotes of *Phytomonas vermiformis* sp. n. in two species of shieldbugs: *P. strictus vernalis* and *Eysarcoris aeneus* (Table 1). These phytomonads were also found in the gut and salivary glands of the only examined individual of *P. strictus vernalis* from Krasnodar.

Phylogenetic analysis

We obtained 18S rRNA gene fragments ~2100 bp long for three natural isolates of *P. vermiformis* (761, F2-470, F2-473) as well as fragments ~1000 bp long for four natural isolates (F2-466, F2-472, F2-478 and F2-485) and all the laboratory cultures. GAPDH gene fragment ~1000 bp long was amplified for one natural isolate (761) and three laboratory cultures (F2-470, F2-478 and F485). The fragments of each gene were identical. The sequences were deposited in the GeneBank under assession numbers PP707600-PP707606 (SSU rRNA gene) and PP704705-PP704708 (GAPDH gene).

A search for similar sequences in GenBank using BLAST yielded the greatest identity of the 18S rRNA

gene fragments obtained in our study with those of *Phytomonas* sp. G24 isolated from the phytophagous bug *Aeptus singularis* in Ghana (West Africa). The level of identity was 99.81% (4 mismatches per fragment 2087 bp long). Among the GAPDH sequences, the isolates showing the highest level of similarity to the new species were *Phytomonas* sp. TCC299 and *Phytomonas* sp. TCC305, the level of identity being 97.84-97.73%.

We compared the level of evolutionary divergence of the 18 S rRNA sequences within the following species: *Phytomonas vermiformis* sp. n. (4 sequences and 1 sequence of *Phytomonas* sp. G24), *P. serpens* (6 sequences), *P. oxycareni* (2 sequences), *P. lipae* (6 sequences) and *P. borealis* (2 sequences) (S. Table 1). Isolates of *P. oxycareni, P. lipae* and *P. borealis* had completely identical sequences of this gene within the species (p-distance = 0). *Phytomonas serpens* showed the greatest values of intraspecific variability, p-distance between the isolates being 0.0016. The p-distance value for isolates of the new species was 0.0003, which corresponds to the level of intraspecific variability in phytomonads.

In the phylogenetic trees based on 18S rRNA gene (Fig. 1, A) and GAPDH gene (Fig. 1, B), all isolates of *P. vermiformis* sp. n. were clustered with a high support. The topology of the trees was not identical because different sets of species were used for the analyses, as GAPDH sequences of many *Phytomonas* spp. are not available in GenBank. Nevertheless, in both trees, the sister branch to the new species included *Phytomonas* spp. isolated previously from pentatomid bugs in the boreal region of the Palearctic: *P. borealis* and *P. nordicus* for the tree based on 18S rRNA gene and only *P. borealis* for the GAPDH tree.

Light microscopy

Phytomonads *Phytomonas vermiformis* sp. n. were found in the gut, the hemolymph and salivary glands of shieldbugs *P. strictus vernalis*. In the anterior midgut (M1-M2), we found a few endomastigotes (Fig. 2, A) as well as promastigotes at different growth stages (Fig. 2 B, C). In the posterior midgut, we mostly observed large promastigotes with long flagella (more than a half of the body length) (Table 2; Fig. 2, D, E). A characteristic feature of the promastigotes of *P. vermiformis* sp. n. in the gut was the localization of their nuclei in the middle part of the cells. Large promastigotes (L = 31.1 ± 2.0 μm) in the gut formed a pleomorphic micropopulation comprising two forms: broad (Fig. 2, D) and narrow (Fig. 2, E) promastigotes. The broad forms often contained two spherical nuclei and had a cylindrical body, whereas the narrow forms had one ellipsoidal nucleus and their body was twisted several times along its length.

In the hemolymph of *P. strictus vernalis*, the phytomonads were usually rare (Table 1); a large-scale infection of the host body cavity was observed only twice, in insects recently emerged from wintering. Promastigotes found in the hemolymph were the largest, reaching 60 μm in length or even more (Table 2; Fig. 2, F). Their cell bodies were twisted 7-10 times along the length. About a half of the promastigotes from the hemolymph was represented by cells without flagella, while the other half had flagella comparable in length with the cell body (Table 2).

In the salivary glands of *P. strictus vernalis, P. vermiformis* sp. n. was represented by two morphotypes: promastigotes (Fig. 2, G-J) and endomastigotes (Fig. 2, K). The micropopulation of promastigotes from the salivary glands was mainly represented by clavate cells (Fig. 2, I): medium-sized flagellates (L = 23.2 ± 1.1 μm) with a spherical nucleus, whose kinetoplast was located in the anterior third of the body (Table 2; Fig. 2, I). There were both hapto- and nectomonads among those cells. Besides, numerous smaller (15.2 ± 0.2 μm) actively dividing promastigotes were noted in the salivary glands of *P. strictus vernalis* (Fig. 2, J). Giant forms (L > 45 μm) of promastigotes of *Phytomonas vermiformis* sp. n. were occasionally found in smears of the salivary gland contents of *P. strictus vernalis* (Fig. 2, G, H). Endomastigotes (cells without flagella) were small (L = 8.7 ± 0.2 μm) (Table 2; Fig. 2, K). Similar cells were found in the homogenate obtained from inflorescences of *U. dioica* on which the infected bugs had been feeding (Fig. 2 L). When cultured in vitro, the phytomonads were mainly represented by small promastigotes (L = 11.4 ± 0.4 μm) and endomastigotes (Table 2; Fig. 2, M, O). Large promastigotes, reaching 45 μm in length and possessing the flagellum as long as the body, could be seen, although rarely, in aging cultures of *Phytomonas vermiformis* sp. n. (Fig. 2, N).

Taking into account the high level of heteromorphism of flagellar stages of the phytomonads, the characters of their most conservative (in terms of morphology) stages, i.e. endomastigotes, were used for comparative morphometric analysis of the "boreal" *Phytomonas* spp. distributed in the North-West of Russia. We considered five standard parameters (cell length, cell width, nucleus length,

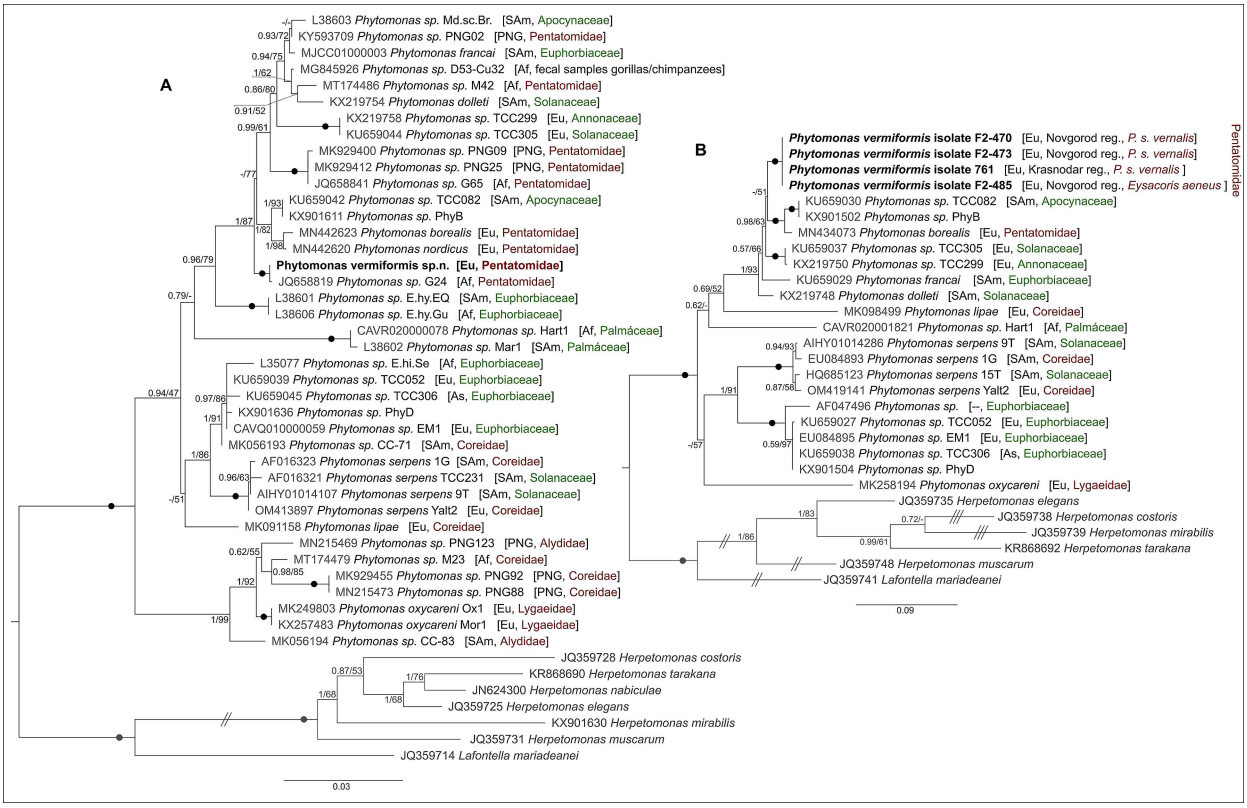

Fig. 1. Maximum likelihood phylogenetic trees reconstructed using different molecular markers. A – SSU rRNA gene; B – gGAPDH gene. Numbers at nodes indicate posterior probability and bootstrap percentage. Values less than 0.5 and 50% are replaced with dashes. Nodes having 1.0 posterior probability and 100% bootstrap support are marked with black circles. Double and triple crossed branches are 1/2 or 1/3 of the original length, respectively. The trees are rooted with the sequences of *Herpetomonas* spp. and *Lafontella* spp. (shown in grey). The scale bar represents the number of substitutions per site. Isolates of the new species are in bold.
The regions where isolates were obtained and the host families are indicated in square brackets. Families of hemipteran hosts are shown in red font, families of plant hosts are shown in green font. Double hyphen indicates missing data. Abbreviations: Af – Africa, As – Asia, Eu – Europe, PNG – Papua New Guinea, SAm – South America.

distance between the nucleus and the anterior end of the cell, distance between the kinetoplast and the anterior end of the cell) of endomastigotes of *P. nordicus* and *P. lipae* (data from: Frolov and Malysheva, 1993; Frolov et al., 1919) and *P. vermiformis* sp. n. (this study). Pairwise comparison with the use of Mann-Whitney criterion showed that there were statistically significant differences ($p < 0.001$, $n = 31$) in cell length and distance between the nucleus and the anterior end of the cell between all the three species. At the same time, no significant differences in cell width, nucleus length and distance between the kinetoplast and the anterior end of cell were found.

Electron microscopy

The ultrastructure of *Phytomonas vermiformis* sp. n. was studied using cells from cultures. The cells have a folded tubulemma (Fig. 3, A, B), with the width of the folds and the number of submembrane microtubules associated with them varying considerably (Fig. 3, B). The cytoplasm contains numerous ribosomes. The nucleus, the kinetoplast and the Golgi apparatus have a structure and localization characteristic of trypanosomatids (Fig. 3, A). The only dictyosome of the Golgi apparatus is located between the nucleus and the kinetoplast (Fig. 3, A). A compact kinetoplast (diameter 0.55 ± 0.11 μm, thickness 0.16 ± 0.02 μm) lies at the bottom of the flagellar pocket (Fig. 3, A, C, E). Glycosomes and mitochondrion profiles are present in the cytoplasm in large numbers, while acidocalcisomes are few (Fig. 3, A, E). We did not detect any large lipid droplets, multivesicular bodies, or smooth reticulum in cells of P. vermiformis sp. n. at this stage.

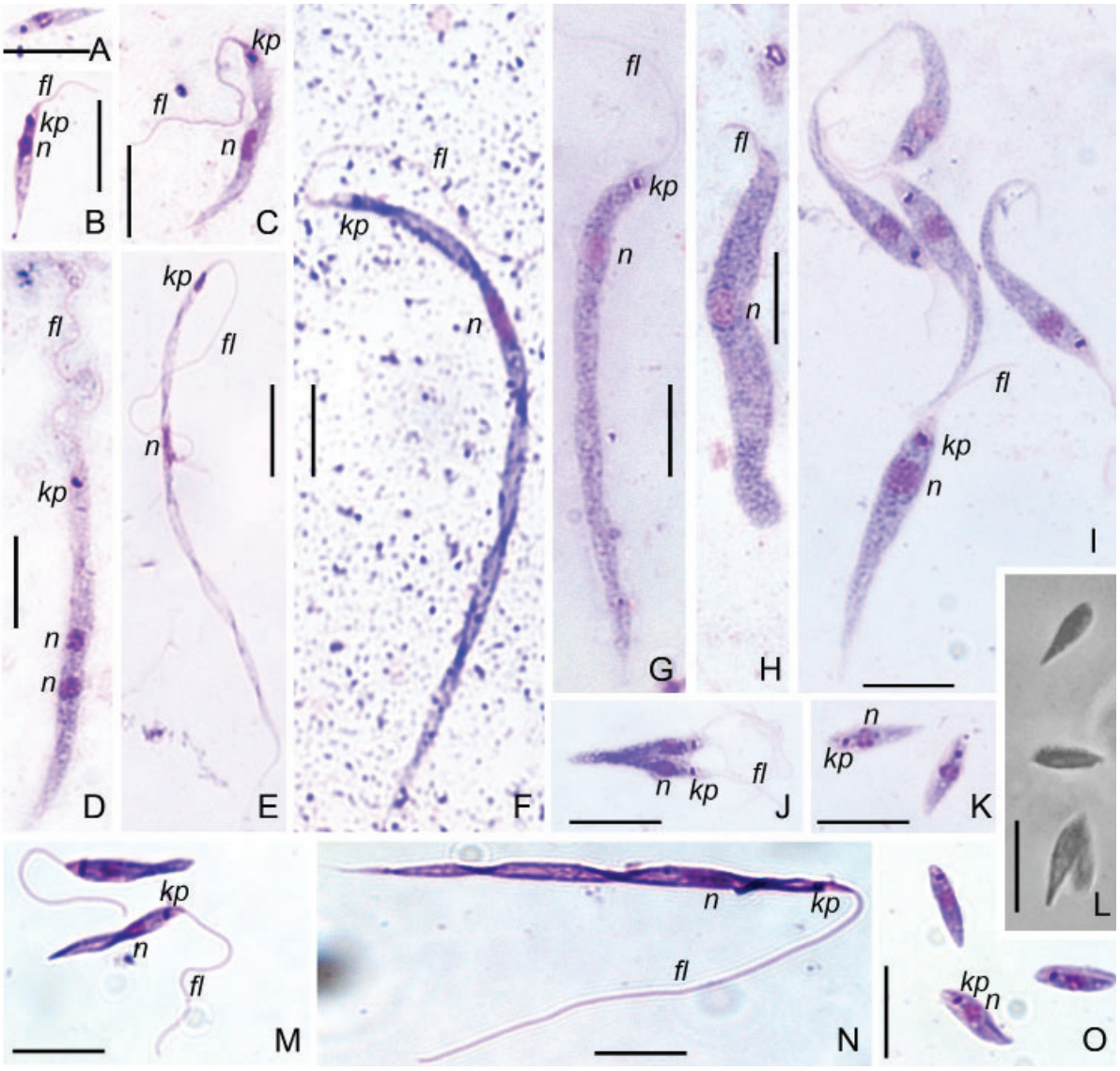

Fig. 2. Morphology of *Phytomonas vermiformis* sp. n. Light microscopy, L – phase contrast, others – Giemsa staining. A–E – Flagellates from the gut of *Peribalus strictus vernalis*; F – a promastigote from the host hemolymph; G–K – flagellates from salivary glands of *P. strictus vernalis*; L – endomastigotes from homogenate obtained from inflorescences of *Urtica dioica*; M–O – flagellates in culture. *Abbreviations*: fl – flagellum, kp – kinetoplast, n – nucleus. Scale bar: 10 μm.

The kinetosome of the flagellum lies near the frontal surface of the kinetoplast capsule of *P. vermiformis* sp. n. (Fig. 3, A, C, E). The basal part of the flagellar apparatus is well structured (Fig. 3, C). The transition zone bounded by the basal and the terminal lamina lies at the level of the flagellar pocket. The axoneme and the paraxial rod originate from the terminal plate. The flagellum of *P. vermiformis* sp. n. has an organization typical of phytomonads, i.e. with a massive paraxial rod (Fig. 3, C, D). A shallow and relatively broad flagellar pocket (diameter 0.42 ± 0.02 μm, depth 0.96 ± 0.06 μm) opens terminally at the anterior end of the flagellated cell (Fig. 3, A, C). The ultrastructure of small non-flagellated endomastigotes of *P. vermiformis* sp. n. is similar to that of promastigotes (Fig. 3, E), the only differences being a closed flagellar pocket and the reduction of the flagellum (Fig. 3, E).

Two types of large virus-like particles, VLP1 and VLP2, were sometimes detected in the cytoplasm of promastigotes of *Phytomonas vermiformis* sp. n. (Fig. 4). Giant VLP1s were found in the post-nuclear zone

Table 2. Morphometry (μm) of *Phytomonas vermiformis* sp. n. from different organs of shieldbugs *Peribalus strictus vernalis* and from culture (n = 31).

Parameters / Origin of cells	L	W	N	NA	KA	F
Midgut of *P. s. vernalis*	31.1±2.0	1.7±0,1	2.1±0.1	13.2±1.6	1.6±0.1	26.1±1.5
	(54.8-15.5)	(2.7-1.1)	(3.4-1.2)	(34.1-3.5)	(3.3-0.7)	(38.2-13.3)
Hemolymph of *P. s. vernalis*	48.3±1.6	1.6±0.1	3.0±0.1	14.1±0.7	2.2±0.1	
	(61.3-27.1)	(2.6-1.1)	(4.3-1.6)	(21.0-5.5)	(3.3-1.1)	(49.8-0)
Salivary glands of *P. s. vernalis*, promastigotes	23.2±1.1	2.1±0.05	2.1±0.05	4.0±0.2	1.5±0.1	9.8±0.4
	(35.9-12.6)	(2.7-1.5)	(3.2-1.6)	(5.5-1.9)	(2,6-0,9)	(14.1-6.5)
Salivary glands of *P. s. vernalis*, endomastigotes	8.7±0.2	1.8±0.1	1.7±0.1	2.4±0.1	1.1±0.1	
	(11.5-6.9)	(2.6-1.0)	(2.0-1.3)	(3.4-1.8)	(1.6-0.1)	
Culture of *P. s. vernalis*, promastigotes	11.4±0.4	1.8±0.04	1.6±0.04	3.5±0.1	1.3±0.1	10.8±1.1
	(44.9-7.5)	(2.0-1.5)	(3.4-1.1)	(4.3-2.2)	(3.0-0.9)	(45.6-2.4)
Culture of *P. s. vernalis*, endomastigotes	7.8±0.2	1.8±0.1	1.7±0.1	2.7±0.1	1.1±0.1	
	(9.9-5.8)	(2.6-1.2)	(2.1-1.3)	(3.2-2.2)	(1.5-0.7)	

Abbreviations: L – length, W – width, N – length of the nucleus, NA – distance between the nucleus and the anterior end of the cell, KA – distance between the kinetoplast and the anterior end of the cell, F – length of the flagellum.

of promastigotes (Fig. 4, A). Their capsid has an icosahedral shape and an outer diameter of about 260 nm (Fig. 4, C, D). A spherical core, about 125 nm in diameter, is surrounded by a relatively thick (ca. 40 nm) layer of loose material of medium electron density (Fig. 4, D). In VLP1 with an incomplete outer layer, a membrane lying on its top can be seen; when the formation of the outer layer is completed, the membrane is no longer visible (Fig. 4, C). The outer layer covering the capsid is approximately 20 nm thick (Fig. 4, D). Populations of VLP1, usually numbering 10-15 particles, are located in an altered area of the cytoplasm, which is filled with fine fibrillar material, lacks any organelles and contains very few ribosomes (Fig. 4, A, G).

Another morphotype of large virus-like particles, VLP2, is characterized by a smaller size (outer diameter of about 210 nm) and a spherical shape (Fig. 4, B, E, F, G). They have a core surrounded by two or three layers of material of variable electron density and an outer membrane (Fig. 4, B, E, F). Unlike VLP1, VLP2 particles do not form clusters in the central part of the cytoplasm. Most of them are located at the cell periphery, where they are embedded between submembrane microtubules so that the plasmalemma bulges out (Fig. 4, B, F, G, H). The membrane of VLP2 and that of the cell adhere tightly to each other at the points of contact (Fig. 4, F). The same cell could contain both VLP morphotypes or only one of them (Fig. 4, A, B, G). VLP1 and VLP2 were found both in the cells of *P. vermiformis* sp. n. from salivary glands of *P. strictus vernalis* (Fig. 4, G) and in cells that had undergone several passages in laboratory cultures (Fig. 4, A, B, H).

Discussion

Phytomonas vermiformis sp. n. described in this study is the fourth species of phytomonads discovered in the temperate zone of the Palearctic in the last decade (Frolov et al., 2016, 2019; Ganyukova et al., 2020). For comparison, a total of 14 species of phytomonads were described in the entire 20th century, and the existence of only five of them has been verified with the use of molecular methods (Jaskowska et al., 2015; Zanetti et al., 2016). The assessment of the actual diversity of *Phytomonas* spp. is aggravated by the fact that there are more than 200 laboratory strains of these flagellates, most of which have not been attributed to any of the species described so far (Jaskowska et al., 2015). In addition, there are several dozens of molecular 'proxy species' of these flagellates, the so-called Typing Units (TU), identified only based on sequences of some genes and the host species. The description of the TU within the framework of traditional systematics is extremely difficult if not impossible. Therefore, it is obvious that studies

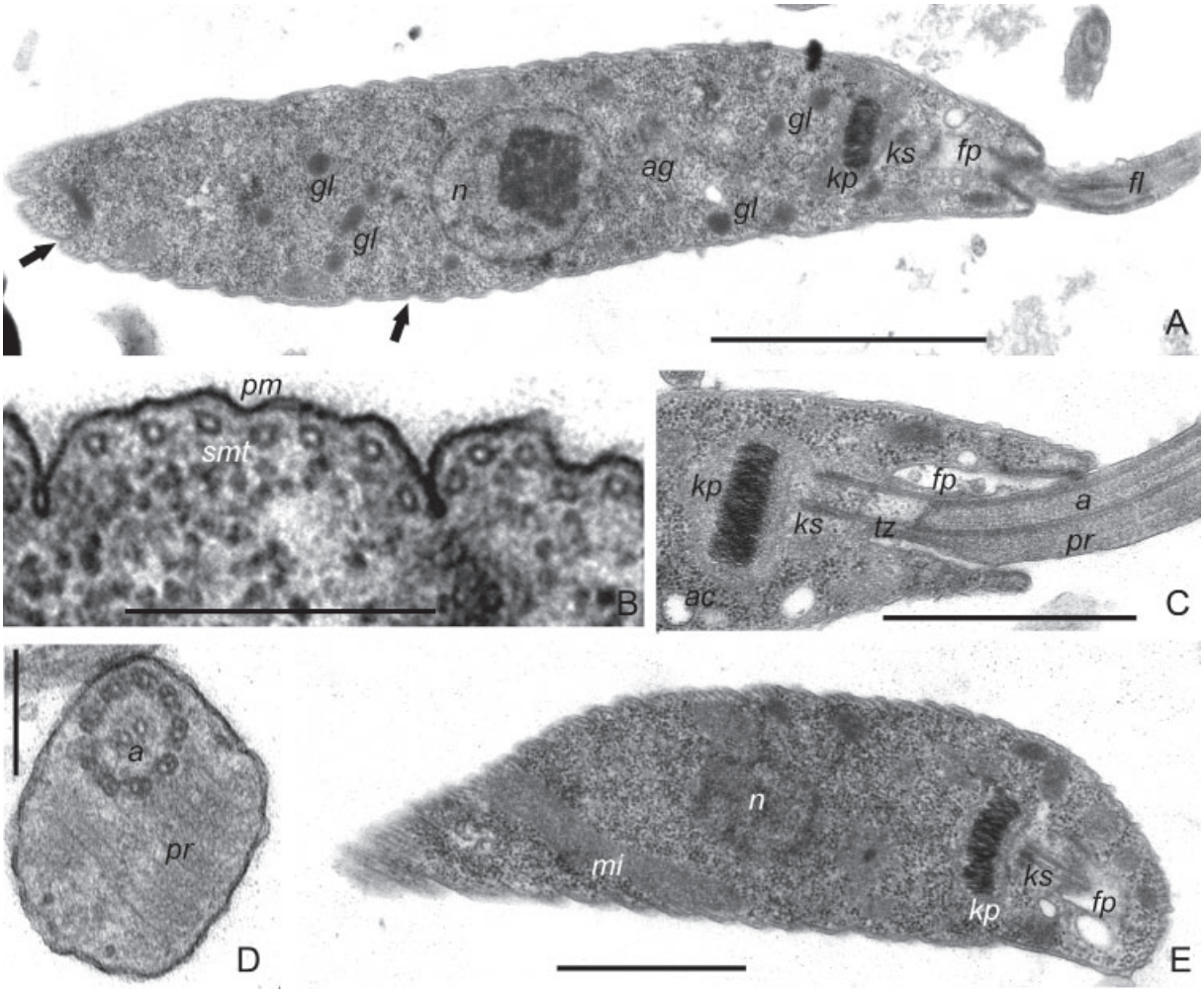

Fig. 3. Ultrastructure of *Phytomonas vermiformis* sp. n. in culture. A – Longitudinal section of the promastigote; B – folded tubulemma of the promastigote; C – longitudinal section of the anterior end and the flagellar pocket of the promastigote; D – transverse section of the flagellum of the promastigote; E – longitudinal section of the endomastigote. *Abbreviations*: a – axoneme, fl – flagellum, fp – flagellar pocket, gl – glycosome, kp – kinetoplast, ks – kinetosome, mi – mitochondrion, n – nucleus, pm – plasmalemma, pr – paraflagellar rod, smt – submembrane microtubules, tz – transitional zone; black *arrows* – folded tubulemma of the promastigote. Scale bar: A – 2 μm; B, C, E – 1 μm; D – 0.2 μm.

presenting complete descriptions of *Phytomonas* spp. and analysing information from previous works made with the use of different methods at different times are very important for the development of systematics of the phytomonads and the assessment of their diversity.

This particular approach was used in our study. Phylogenetic analysis of isolates from the gut and salivary glands of *Peribalus strictus vernalis* in the Novgorod Region of Russia (F2-470s, F2-473s) and in Krasnodar (761s) and the isolate from the intestine of *Eysarcoris aeneus* (F2-485s) from the Novgorod Region showed their complete (100%) identity by sequences of both marker genes (SSU rRNA and gGAPDH), which have been successfully used for this group of trypanosomatids (Zanetti et al., 2016; Frolov et al., 2019; Ganyukova et al., 2020, 2022). In addition, there was a high degree of similarity between *Phytomonas vermiformis* sp. n. and isolate G24 (TU76) that has been obtained earlier from the only examined individual of *Aeptus singularis* (Pentatomidae) in Ghana (Votýpka et al., 2012). While no sequence data on gGAPDH gene of isolate G24 are available in GenBank, the differences in the SSU rRNA gene sequences between this isolate and *P. vermiformis* sp. n. were insignificant

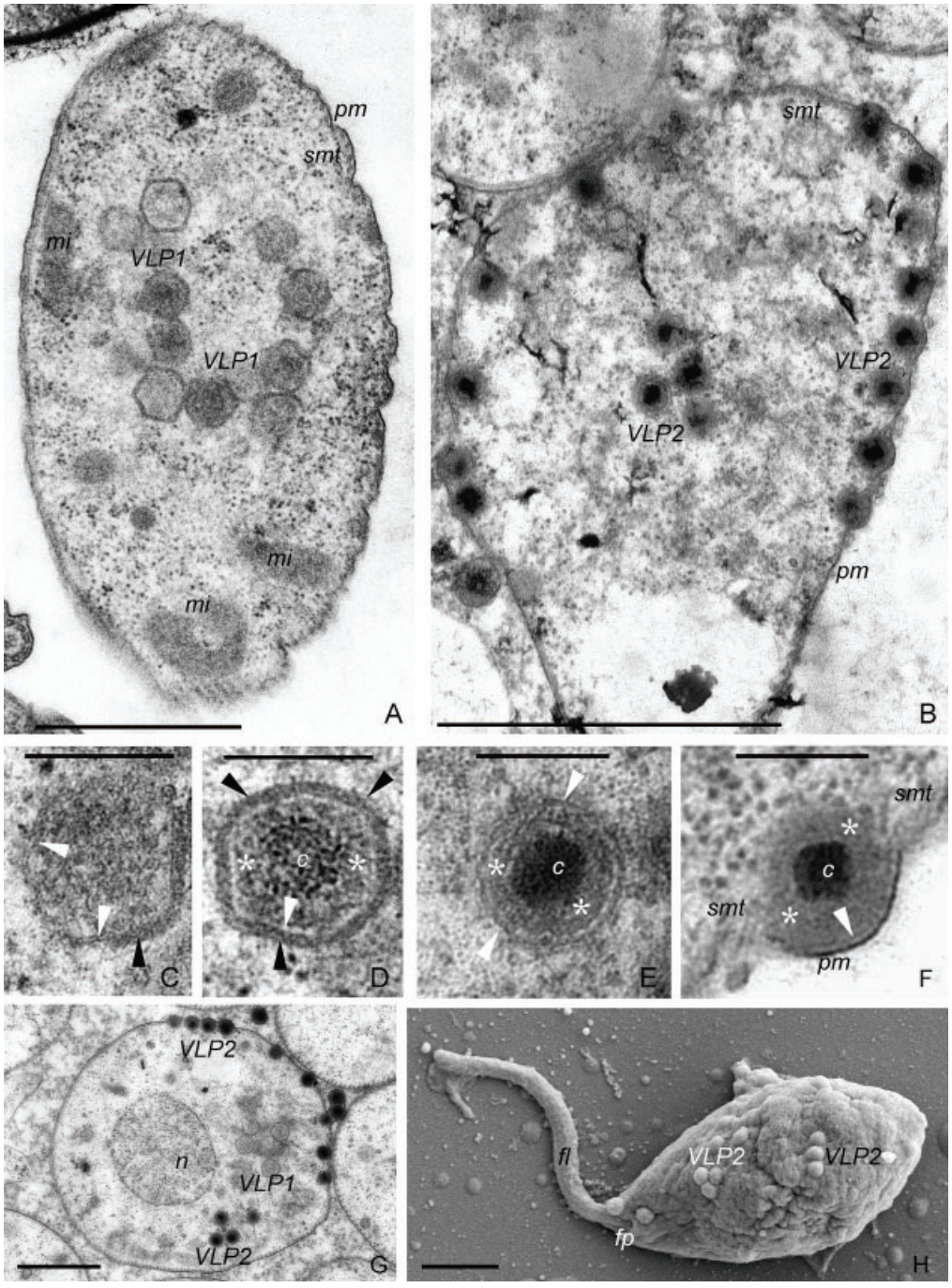

Fig. 4. Large virus-like particles VLP1 and VLP2 in cells of *Phytomonas vermiformis* sp. n. in culture and in the salivary glands (TEM and SEM). A, C, D – Large VLP1 with icosahedral capsids in the cytoplasm; B, E, F – large VLP2 in the cytoplasm and under the plasmalemma; G – two types of VLPs in the promastigote *Phytomonas vermiformis* sp. n. from the salivary glands of *P. strictus vernalis*; H – VLP2 on the surface of the promastigote *Phytomonas vermiformis* sp. n. in culture. *Abbreviations*: c – core, fl – flagellum, fp – flagellar pocket, mi – mitochondrion, n – nucleus, pm – plasmalemma, smt – submembrane microtubules. *Asterisk* – material surrounding the core of VLP1 and VLP2, white *arrowhead* – membrane, black *arrowhead* – outer layer of VLP1 capsid. Scale bar: A, G, H – 1 μm; B – 2 μm; C–F – 0.2 μm.

(4 nucleotides). This suggests that isolate G24 and *Phytomonas vermiformis* sp. n. either belong to one and the same species or represent two close species that have diverged only recently.

The first hypothesis is supported by two arguments. Firstly, both *P. vermiformis* sp. n. and isolate G24 parasitise bugs from the family Pentatomidae. Secondly, it has been shown using evidence from Brazilian isolates of *Phytomonas serpens* that the sequences of their SSU rRNA genes can differ from each other by 1-4 substitutions, which may reflect both the sequence polymorphism of the SSU gene itself and the differences between the strains of the same species (Hollar and Maslov, 1997).

The second hypothesis seems to be supported by the large distance between the southernmost finding of the European isolate of *P. vermiformis* sp. n. (761s, Krasnodar, Russian Federation, 45°4′52″ N, 38°58′15″ W) and the African isolate (G24, villages Abutia-Kloe, Ghana, 6°28′54″ N, 0°25′19″ E), which exceeds 5600 km (Votýpka et al., 2012; this paper), as well as by the geographical isolation of their ranges of their hosts (Belousova, 2007; Faúndez, 2017). However, the latter consideration can be challenged. Another subspecies of *Peribalus strictus, Peribalus strictus strictus* (Fabricius, 1803), whose range covers most of the area of the Mediterranean countries, is registered both in northern Africa and in south-eastern Europe (Belousova, 2007). In the latter case, its range partially overlaps with that of the Euro-Siberian subspecies, *Peribalus strictus vernalis*, the established host of *P. vermiformis* sp. n. Moreover, transitional forms between the two subspecies have been found in sympatric areas (Belousova, 2007). Therefore, the isolation of the African isolate (G24) and the European isolates of *P. vermiformis* sp. n. cannot be considered as an established fact. Our analysis of the level of evolutionary divergence of the 18S rRNA sequences between the isolates of different *Phytomonas* spp. also confirms the first hypothesis, i.e. that *P. vermiformis* sp. n. and isolate G24 belong to the same species.

As noted above, *P. vermiformis* sp. n. is the fourth species of this genus described in the boreal region of the Palearctic, and three of them are parasites of pentatomid bugs. They are *Phytomonas nordicus* from *Troilus luridus* (Fabricius, 1775) and *Picromerus bidens, Phytomonas borealis* from *P. bidens* and *Phytomonas vermiformis* sp. n. from shieldbugs *Peribalus strictus vernalis* and *Eysarcoris aeneus* (Frolov and Malysheva, 1993; Frolov et al., 2016; Ganyukova et al., 2020; this study). At the same time, a reservoir host, *U. dioica*, has been identified only for *Phytomonas vermiformis* sp. n. Considering that *P. strictus vernalis* is a broadly polyphagous species, it can be assumed that other plants may also play the role of reservoir hosts of the new species. According to our observations made in spring and summer, only a few individuals of *P. strictus vernalis* were found on nettle, and individuals collected from other plants showed consistently high infection levels. Individuals of *P. strictus vernalis* accumulated on inflorescences of *U. dioica* in autumn, together with bugs of other species. Interestingly, out of the fives species of pentatomid bugs examined in our study that fed on the same plant as infected individuals of *P. strictus vernalis*, the flagellates *Phytomonas vermiformis* sp. n. were found in the gut of only one individual of *Eysarcoris aeneus*. It is probable that *E. aeneus* is not the specific host of the new species and that these flagellates can survive in its digestive system only for a short time. A similar assumption has been made for P*hytomonas borealis*, which is found only in the gut of *P. bidens* (Ganyukova et al., 2020). Normal development of phytomonads in the hosts is known to be associated with their penetration into the salivary glands, the passage through the gut being transitory (Frolov et al., 2021). Development in the host gut is characteristic of monoxenous trypanosomatids, which have diverse morphofunctional adaptations for successful implementation of the life cycle in this organ (Frolov and Skarlato, 1995; Frolov et al., 2021).

The detection of large virus-like particles in the cytoplasm of *Phytomonas vermiformis* sp. n. was unexpected and intriguing. The structure and size of their capsids, which have an icosahedral shape and exceed 0.2 μm in diameter, as well as the character of their localization in the cytoplasm, suggest that these particles may be large DNA viruses (Mutsafi et al., 2014; Bajrai et al., 2016; Vávra et al., 2016; Colson et al., 2017). The fact that both VLP1 and VLP2 were detected not only in wild-type cells of *Phytomonas vermiformis* sp. n. but also in the cultured forms after several laboratory passages indicates that the viral infection was not accidental. Before our study, trypanosomatids have never been found to harbour large DNA viruses (Kostygov et al., 2021). Moreover, the only documented case of a large DNA virus in any protist group related to trypanosomatids has been a report of a virus in the free-living kinetoplastid *Bodo saltans* (Deeg et al., 2018). A reliable identification of the large VLPs of

the trypanosomatid *Phytomonas vermiformis* sp. n. requires further research with the use of genomic and other methods.

Taxonomic summary

Class Kinetoplastea Honigberg, 1963
Subclass Metakinetoplastia Vickerman, 2004
Order Trypanosomatida Kent, 1880
Family Trypanosomatidae Doflein, 1901
Subfamily Herpetomonadinae Alexeieff, 1911, emend. Kostygov and Yurchenko, 2021
Genus *Phytomonas* Donovan, 1909

Phytomonas vermiformis Frolov, Ganyukova et Malysheva sp. nov. (Fig. 2).

Hapantotype. Giemsa-stained slide F2-478 deposited in the Research Collection of Parasitic Protists at the Zoological Institute of the Russian Academy of Sciences (St. Petersburg, Russia).

Additional material. Smears F2-472 and F2-485 and cultures F2-470, F2- 478 and F2-485, deposited in the same collection.

Type locality. Oksochi Village, Novgorod Province, Russia.

Diagnosis. Cells in gut, hemolymph and salivary glands of host represented by promastigotes. Large intestinal promastigotes often binucleate. In salivary glands, small endomastigotes without flagella also present. Cells in culture shorter, also represented by two morphotypes: pro- and endomastigotes. The species can be unambiguously distinguished from other Phytomonas spp. by the sequences of 18S rRNA and gGAPDH genes (PP707600 - PP707606 and PP704705 - PP704708, respectively).

Differential diagnosis. *Phytomonas vermiformis* sp. nov. differs from the other two *Phytomonas* spp. described from pentatomid bugs in the North-West of Russia, *P. nordicus* and *P. borealis*, in morphometry of endomastigotes. However, confident species identification of "boreal phytomonads" can be done only based on the gene sequences (see above).

Hosts and localization. *Peribalus* (*P.*) *strictus vernalis* (Wolff, 1804) (Heteroptera: Pentatomidae), all parts of gut, hemolymph and salivary glands (type host); *Eysarcoris aeneus* (Scopoli, 1763) (Heteroptera: Pentatomidae), all parts of gut (probable host); *Aeptus singularis* Dallas, 1851 (Heteroptera: Pentatomidae), localization unknown (probable host).

Etymology. From Latin *vermis*, "worm", referring to the shape of promastigotes. Numerous long promastigotes with a worm-like cell body and without flagella can be seen in on Giemsa-stained smears of contents of host gut.

Addenda. Electronic supplementary material. GenBank accession numbers of sequences used in the phylogenetic analysis. File format: PDF.

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Supplementary materials

Table S1. GenBank accession numbers of all sequences used in phylogenetic analyses.