Protistology 16 (1): 30–41 (2022) | doi:10.21685/1680-0826-2022-16-1-4

Protistology

ORIGINAL ARTICLE

A puzzling finding: the Brazilian tomato parasite *Phytomonas serpens* in the western conifer seed bug *Leptoglossus occidentalis* in Crimea

Anna I. Ganyukova*, Marina N. Malysheva, Ludmila V. Chistyakova and Alexander O. Frolov

Zoological Institute RAS, 199034 St. Petersburg, Russia

| Submitted January 3, 2022 | Accepted February 1, 2022 |

Summary

The trypanosomatid flagellates of the genus *Phytomonas* were found in the salivary glands and gut of the invasive western conifer seed bug *Leptoglossus occidentalis* (Hemiptera, Coreidae) in Crimea. The parasites were isolated into axenic culture and identified by phylogenetic analysis based on the 18S rRNA and gGAPDH genes as *P. serpens*. Previously, all isolates of this species have been obtained in Brazil, from either tomato fruits or their pest bugs *Phthia picta* (Hemiptera, Coreidae). We investigated the morphology of trypanosomatids from the new isolate in the host bug, laboratory culture, and experimentally infected tomato fruits using light and transmission electron microscopy. We propose hypothetical scenarios of *L. occidentalis* involvement in the life cycle of *P. serpens* in the territory, which is new for both species.

Key words: invasive species, *Phytomonas serpens*, Trypanosomatidae, *Leptoglossus occidentalis*

Introduction

Trypanosomatids (Euglenozoa, Kinetoplastea) are represented in the classification of eukaryotes by the order Trypanosomatida containing only one family, Trypanosomatidae (Vickerman, 1994; Simpson et al., 2006; Flegontov et al., 2013; Kostygov et al., 2021). The analysis of the evolutionary history of systems that include trypanosomatids and their hosts based on the data on flagellates' life cycles and the results of phylogenetic, population-genetic, genomic and other studies indicates that the modern diversity of these parasites is based on host-switching (Hamilton et al., 2007, 2012; Stevens, 2008; Maslov et al., 2013; Lukeš et al., 2014, 2018; Frolov, 2016; Frolov et al., 2015, 2021). The study of this phenomenon appears particularly important in the light of the recent global developments such as climate change, the environmental effects of transport, transformation of ecosystems under the increasing anthropogenic impact and so on (Frolov, 2016). Under these conditions, the ability to make horizontal transitions to new hosts offers great opportunities to trypanosomatids. By overcoming host barriers, they can also expand to new territories with their new hosts. Given that trypanosomatids include a number of actual and potential pathogens of humans, animals and cultivated plants, the phe-

https://doi.org/10.21685/1680-0826-2022-16-1-4

***Corresponding author**: Anna Ganyukova. Zoological Institute RAS, Universitetskaya Emb. 1, 199034 St. Petersburg, Russia; **anna.ganyukova@gmail.com**

nomenon of host-switching deserves the closest attention.

In this paper, we report the discovery of dixenous flagellates *Phytomonas serpens* in an invasive insect, the western conifer seed bug *Leptoglossus occidentalis* (Heteroptera, Coreidae), in southern Russia. The history of expansion of this North-American bug in Europe and the timeline of its progression from west to east is well documented (Gapon, 2012). *L. occidentalis* reached southern Russia and Ukraine in 2010, and has more recently spread to Krasnodar Territory of Russia. The development of the western conifer bug is closely associated with conifers of the families Pinaceae and Cupressaceae. The bugs feed on ripe and ripening seeds of the conifers and on the sap of their apical shoots (Gapon, 2012).

Trypanosomatid flagellates *Phytomonas serpens* parasitize the fruit of tomato (*Solanum lycopersicum*) in Brazil (Jankevicius et al., 1989) and use the bugs *Phthia picta* (Heteroptera, Coreidae) as vectors. These bugs feed on various plants but have never been recorded to feed on conifers.

Material and methods

Two individuals of *Leptoglossus occidentalis* (both ♂) were caught in a human dwelling in the Nikita Settlement, the Republic of Crimea (44° 31' 04" N; 34° 13' 44" E), in November 2020. They were denominated as samples Yalt1 and Yalt2. The insects were euthanized with the chloroform vapors and dissected in saline solution (Frolov et al., 2018; Malysheva et al., 2020). The dissection and subsequent microscopic examination revealed infection with trypanosomatids: flagellate cells were found in the gut of both individuals and in the salivary glands of the sample Yalt2.

Cultivation of phytomonads

Fragments of the bug gut infected with trypanosomatids were placed into 2 ml test tubes (isolates Yalt1 and Yalt2) filled to the brim with Schneider's Insect Medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% Fetal Bovine Serum (Biolot, St. Petersburg, Russia), 500 μg/ml of streptomycin and 500 units/ml of penicillin (Sigma-Aldrich, St. Louis, USA). Later, after the sustainable development of the cultures had been achieved, they were maintained without the antibiotic solution at the temperature of 22 °C and passaged every 10-14 days. One culture (Yalt2) was purified from fungal contamination using a previously described device consisting of two glass tubes with a V-shaped cannular connector (Podlipaev and Frolov, 1987). The two cultures (Yalt1 and Yalt2) were cryopreserved and stored at -86 °C in the growth medium supplemented with 10% DMSO (Sigma-Aldrich). The cultures are currently deposited 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 was extracted from the host tissues — the infected salivary glands (sample Yalt2-sg), fragments of the infected gut (sample Yalt1-int), and flagellate culture (Yalt2) — using PureLink Genomic DNA Kit (Invitrogen) for DNA extraction according to the manufacturer's instructions. DNA isolated from fragments of the host digestive tract (Yalt1-int, Yalt2-sg) was used for amplification of the 18S rRNA gene with primers 1127F and 1958R (generating ~900 bp fragment) (Kostygov and Frolov, 2007). The amplicons were sequenced using the PCR primers and the resulting sequences were used for species identification of parasites from these samples.

The nearly full-length SSU rRNA and gGAPDH genes were amplified using DNA isolated from the culture Yalt2 and the respective primer pairs: S762 and S763 (Maslov et al., 1996), M200 and M201 (Maslov et al., 2010). Amplification protocol and reaction mixture composition were described earlier (Kostygov and Frolov, 2007). Sequencing of the amplicons with the PCR primers was performed as described previously (Frolov et al., 2019). The resulting sequ-ences were used for phylogenetic analysis. The GenBank accession numbers for the new sequences determined in this work are: OM413897-OM413898 (SSU rRNA gene) and OM419141 (gGAPDH gene).

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) (Table S1). The alignment of SSU rRNA

gene sequences was performed in MEGA7 using MUSCLE under the default parameters (Kumar et al., 2016). The dataset for gGAPDH gene was processed in MEGA7 as follows: translated into amino acids, aligned with the built-in MUSCLE and then reverse translated to nucleotides.

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 using Bayesian information criterion by the built-in ModelFinder (Kalyaanamoorthy et al., 2017). Branch support was estimated using the ultrafast bootstrap method (1000 replicates) (Hoang et al., 2017). Bayesian inference was accomplished in MrBayes v.3.2.7 under the GTR+I+G model, with analysis run for 2,000,000 generations, trees sampled every 1000 generations and other parameters left in default states (Ronquist et al., 2003).

The resulting alignment of SSU rRNA gene included 42 sequences with 2138 nucleotide sites including indels, and the final alignment of gGAPDH gene included 30 sequences with 887 nucleotide sites. The resulting alignment was used for phylogenetic inference in IQ-TREE and MrBayes. The analyses were done generally as described above with the best evolutionary model for the maximum likelihood tree (GTR+I+G4+F).

Light microscopy

The smears prepared from the cultures, from fragments of infected gut, salivary glands and from infected tomato fruits were air-dried, fixed with 96% ethanol for 30 min, and Giemsa-stained for 30 min (pH 6.8). To visualize DNA-containing structures, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (1 mg/ml) as described earlier (Yurchenko et al., 2006). Microphotographs were taken using Leica DM 2500 microscope equipped with UCMOS14000KPA 14-Mpx camera (Toup Tek, Hangzhou, China) at ×1,000 magnification. All cell measurements (n=27) and the statistical analysis were performed in UTHSCSA Image Tool for Windows v.3.0. The statistical significance of differences in the average values of morphological characteristics was evaluated using Student's t criterion (n=27) (significance level of $P<0.01$).

Transmission electron microscopy

A previously centrifuged five-day culture of *Phytomonas serpens* (Yalt2) was prepared for TEM as described earlier (Frolov et al., 2016). Ultrathin sections were examined in Morgagni 268-D microscope (FEI Company/Thermo Fisher Scientific, Hillsboro, OR, USA) with accelerating voltage of 80.00 kV.

Experimental infection of tomatoes

Tomatoes for the experiment were bought in the supermarket. Six-day culture of phytomonads (~105 cells/ml) was introduced with the help of a capillary pipette into the tomato fruit at a depth of ~3-5 mm. The volume of the inoculant was V = 0.01 ml. The injection site was marked with a paper sticker. After infection, the tomatoes were stored in a fridge at +10 °C.

Results

The two individuals of *Leptoglossus occidentalis* (Yalt1 and Yalt2) were dissected and examined for infection. Motile promastigotes and immotile endomastigotes of trypanosomatids were found in the midgut of both individuals (Fig. 1, A, B) and in the salivary glands of Yalt2 (Fig. 1, C). Two laboratory cultures of trypanosomatids were isolated from the infected fragments of the midgut of Yalt1 and Yalt2 and assigned the same abbreviations as their hosts. Xenic culture Yalt1, contaminated with fungi, was cryopreserved. The culture Yalt2 was purified from concomitant organisms and used in further research.

Phylogenetic analysis

The resulting SSU rRNA gene sequences (~900 bp fragments obtained from the digestive tract of the Yalt1-int and Yalt2-sg specimens, as well as a ~2100 bp fragment from a laboratory culture of Yalt2) were identified as *Phytomonas serpens*. The resulting sequences of the nearly full-length SSU rRNA and gGAPDH genes obtained from the culture Yalt2 were used for phylogenetic analysis. The phylogenetic inferences based on SSU rRNA and gGAPDH genes, the two molecular markers traditionally used for trypanosomatids (Votypka et al., 2015), reliably demonstrated the position of the isolate Yalt2 in the cluster of different isolates of *Phytomonas serpens*. Both maximum likelihood and Bayesian analyses based on these genes demonstrated well-supported branches on the phylogenetic trees (Fig. 3).

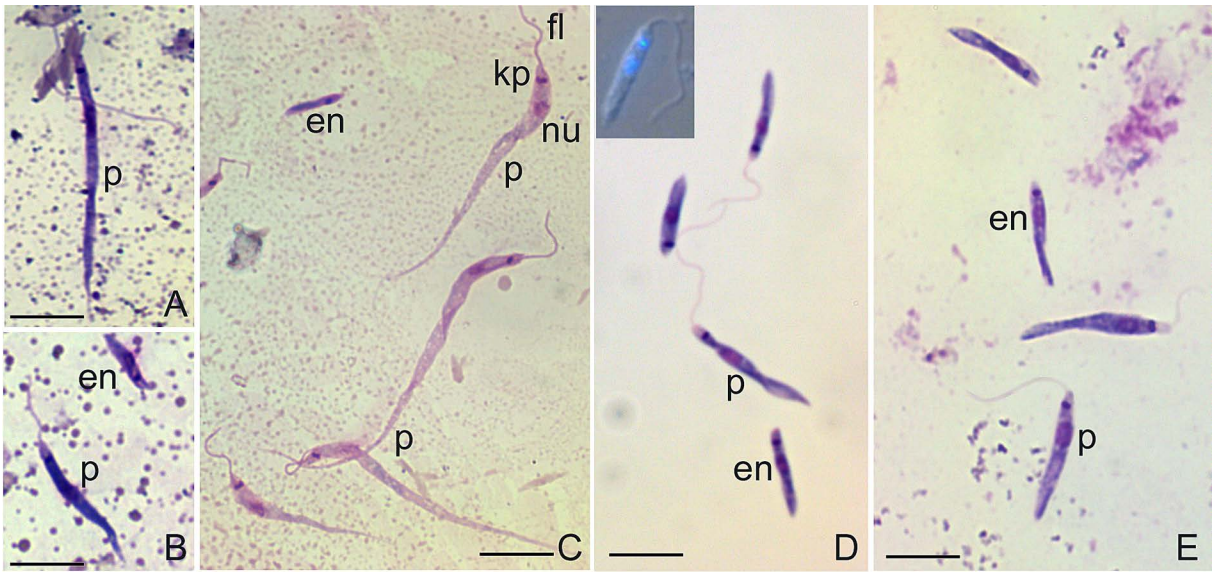

Fig. 1. Morphology of *Phytomonas serpens* Yalt2 (light microscopy: Giemsa stained; inset: DIC + DAPI). A, B – Flagellates from intestine of *Leptoglossus occidentalis*; C – flagellates from salivary glands of *L. occidentalis*; D – flagellates from culture Yalt2; E – flagellates from tomatoes. *Abbreviations*: en – endomastigotes, fl – flagellum, kp – kinetoplast, nu – nucleus, p – promastigotes. Scale bars: 10 μm

Morphology of flagellates

Flagellates *Phytomonas serpens* Yalt2 were found to form heteromorphic populations in all investigated cases: (1) in the gut and the salivary glands of the host, (2) in laboratory cultures, and (3) in the juice of experimentally infected tomatoes (see below).

Two main morphotypes of the cells were detected in all these cases: motile flagellated promastigotes (Fig. 1, Table 1) and immotile non-flagellated endomastigotes (Fig. 1, Table 2). Both promastigotes and endomastigotes were capable of division. Since the cells of the two morphotypes can undergo transformations into each other, many transitional forms, differing in the cell size and the flagellum length, were always present in the micropopulations.

This flagellate polymorphism correlates with the localization of the parasite. In this way, the largest promastigotes reaching 50 μm or more (36.1±8.4) μm were observed in the salivary glands of the host (Table 1). They had a characteristic shape with an expanded anterior third of the body and an elongated whip-like posterior end (Fig. 1, C). The length of their flagella usually did not exceed ¼ of the cell length.

Promastigotes from the host gut had a worm-like body shape (Fig. 1, A, B). Their length was on average 10 μm less than that of the largest promastigotes described above (25.8±5.1) μm, while the length of their flagella was equal to their body length (Table 1).

Promastigotes from Yalt2 culture and from the juice of tomato fruit experimentally infected with this culture (Fig. 1, D, E) were the smallest in size, 10.2±2.1 and 12.4±1.7 μm, respectively, with flagella of both being approximately of the same length as their cells (Table 1).

In salivary glands and the gut of *L. occidentalis* endomastigotes of *P. serpens* Yalt2 were much shorter than promastigotes (Fig. 1, A-C; Tables 1, 2). However, in Yalt2 culture and in the juice of experimentally infected tomatoes (Fig. 1, D, E; Table 2) the differences in mean cell length values of these two morphotypes were not statistically significant ($P > 0.01$).

The ultrastructure of *P. serpens* was studied using cells from Yalt2 culture (Fig. 2). The organization of pro- and endomastigotes was generally similar. The main cell organelles, the nucleus and kinetoplast, had a structure typical of all trypanosomatids and were located along the longitudinal axis of their cells (Fig. 2, A-D). The kinetoplast was shaped as a slightly concave disc (~ 0.67 × 0.15 μm). The nucleus was located behind the kinetoplast. Profiles of mitochondria, glycosomes and acidocalcisomes were revealed in the cytoplasm (Fig. 2). The main differences in the morphology of the pro- and endo-

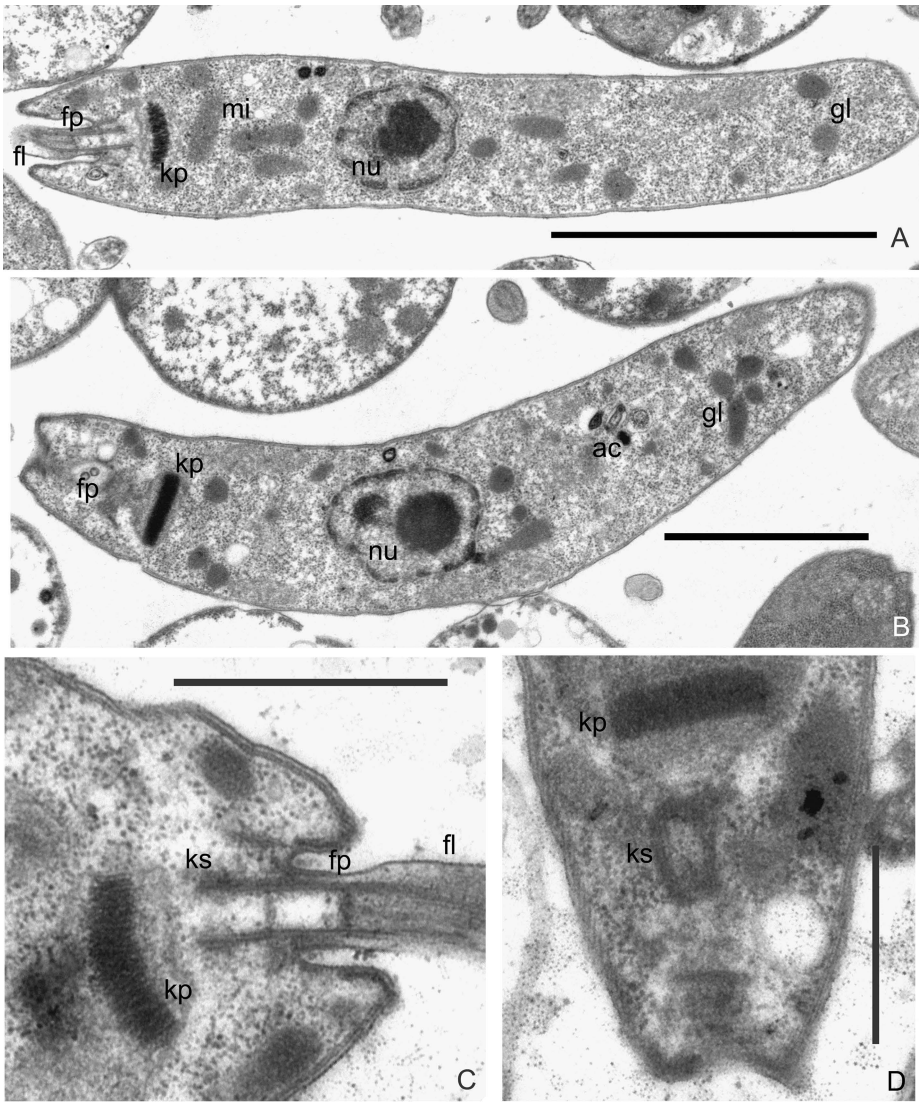

Fig. 2. Morphology of *Phytomonas serpens* in the culture Yalt2 (TEM). A, C – Promastigotes, B, D – endomastigotes. *Abbreviations*: ac – acidocalcisomes, fl – flagellum, fp – flagellar pocket, gl – glycosomes, kp – kinetoplast, ks – kinetosome, mi – mitochondrion, nu – nucleus. Scale bars: A – 3 μm, B – 2 μm, C – 0.8 μm, D – 0.6 μm

mastigotes of P. serpens Yalt2 were associated with the organization of anterior ends of their cells. Promastigotes had a short and rather wide cylindrical flagellar pocket (Fig. 2, A, C); its diameter did not exceed 0.8 μm. The flagellum exits the flagellar pocket of promastigotes freely, with no contacts being formed between the plasmalemma of the flagellum and that of the flagellar pocket. In endomastigotes, the flagellar pocket was reduced (Fig. 2, B, D). The outer opening of the pocket was smaller in diameter, and its basal part was fragmented (Fig. 2, B) and then resorbed and replaced by finely granular matrix of medium electron density (Fig. 2 D). The flagellar apparatus was also reduced, so that only kinetosomes could be seen in the cells of endomastigotes (Fig. 2 D).

Experimental infection of tomatoes

Six tomatoes were infected with a six-day culture of *Phytomonas serpens* Yalt2. In all cases the infection was successful. Starting from day 5 post infection, a large number of flagellates, both motile promastigotes and endomastigotes, were observed in tomato juice collected with a sterile capillary. Gradually, the flagellates spread throughout the

Table 1. Morphometry of *P. serpens* promastigotes (Yalt2) from different organs of the *L. occidentalis*, Tomatoes fruits and culture (N = 27).

Origin of cells	Length	Width	Flagellum	Nucleus	NA	KA	NK
Midgut *L. occidentalis*	25,8±5,1	1,2±0,2	24,7±5,1	2,6±0,6	5,4±0,9	1,9±0,3	2,8±1,0
	(36,8–17,9)	(1,7–0,7)	(30,9–17,1)	(3,2–1,7)	(6,8–4,3)	(2,6–1,1)	(4,4–1,2)
Saliv. glands *L. occidentalis*	36,1±8,4	1,8±0,6	8,9±1,9	2,1±0,4	5,5±0,8	1,9±0,3	2,6±0,5
	(53,4–19,0)	(3,7–1,2)	(12,1–5,5)	(3,0–1,3)	(7,0–3,8)	(2,6–0,9)	(3,9–1,8)
Tomato fruits	12,4±1,7	1,5±0,2	9,6±4.0	2,0±0,4	2,4±0,8	1,1±0,2	0,9±0,3
	(15,4–9,5)	(1,9–0,9)	(16,4–2,6)	(2,9–1,1)	(3,4–0,8)	(1,5–0,8)	(1,4–0,4)
Culture	10,2±2,1	1,5±0,2	12,4±6,0	1,7±0,3	3,3±0,6	1,1±0,2	1,5±0,4
	(14,5–4,9)	(1,8–1,1)	(19,9–2,6)	(2,2–1,2)	(4,8–2,2)	(1,4–0,7)	(2,5–0,7)

Notes: NA – The distance between the nucleus and the anterior end of the cell; KA – the distance between the kinetoplast and the anterior end of the cell; NK – the distance between the nucleus and the kinetoplast. All the measurements are in μm.

tomato fruit. Live cells were maintained in infected tomatoes for up to 3.5 months, until the fruit decomposed completely or dried up.

Discussion

Leptoglossus occidentalis Heidemann, 1910 is an invasive species of true bugs that originally inhabited western North America (Gapon, 2012). In Europe, the species was first discovered in Italy in 1999, and later on, it spread rapidly across the continent. In the south of Russia and in Ukraine, *L. occidentalis* was first noted in 2010 (Gapon, 2012). Recent genetic studies of different European populations of *L. occidentalis* have shown that this species is most likely to invade new territories by multiple introductions (Lesieur et al., 2019). The origin of the *L. occidentalis* population in southern Russia and Ukraine is yet unknown. The western conifer seed bug is a polyphagous conifer pest feeding on seeds and sap of apical shoots of conifers of the family Pinaceae (*Abies, Cedrus, Picea, Pinus, Pseudotsuga, Tsuga*) and the family Cupressaceae (*Calocedrus, Cupressus, Juniperus*). More than 40 species of conifers have been recorded as its forage plants (Werner, 2011; Fent and Kment, 2011).

Dixenous flagellates of the genus *Phytomonas* are distributed worldwide (Camargo, 1999). An overwhelming majority of the currently available *Phytomonas* isolates have been isolated from their hosts at the American continent (Jaskowska et al., 2015). Meanwhile, recent studies have shown that the fauna of these flagellates also appears to be fairly diverse in the Palaearctic (Seward et al., 2016; Frolov et al., 2016, 2019; Ganyukova et al., 2020). Phytomonads are parasites of Flowering plants, capable of developing in fruits, seeds, the milky sap or the phloem of host plants (Camargo, 1999). They have been recorded in plants from 24 families

Table 2. Morphometry of *P. serpens* endomastigotes (Yalt2) from different organs of the *L. occidentalis*, Tomatoes fruits and culture (N = 27).

Origin of cells	Length	Width	Flagellum	Nucleus	NA	KA	NK
Midgut *L. occidentalis*	9,0±1,7	1,5±0,2	–	1,8±0,3	3,5±0,6	1,4±0,1	1,5±0,5
	(12,1–5,7)	(1,8–1,1)		(2,3–1,2)	(4,7–2,6)	(1,7–1,1)	(2,8–0,9)
Saliv. glands *L. occidentalis*	9,3±1,4	1,6±0,2	–	1,8±0,4	4,0±0,8	1,2±0,5	1,6±0,5
	(11,5–6,7)	(1,9–1,2)		(2,2–1,1)	(4,3–2,1)	(2,0–0,9)	(3,0–0,8)
Tomato fruits	11,6±1,3	1,3±0,2	–	1,7±0,5	2,8±0,4	1,0±0,2	0,9±0,3
	(14,4–9,1)	(1,6–1,0)		(2,3–0,2)	(3,9–2,1)	(1,3–0,7)	(1,5–0,5)
Culture	7,6±1,1	1,4±0,2	–	1,3±0,2	2,4±0,4	0,7±0,2	1,1±0,4
	(9,7–5,9)	(1,9–1,1)		(1,7–0,9)	(3,3–1,6)	(1,2–0,4)	(2,2–0,7)

Notes: NA – The distance between the nucleus and the anterior end of the cell; KA – the distance between the kinetoplast and the anterior end of the cell; NK – the distance between the nucleus and the kinetoplast. All the measurements are in μm.

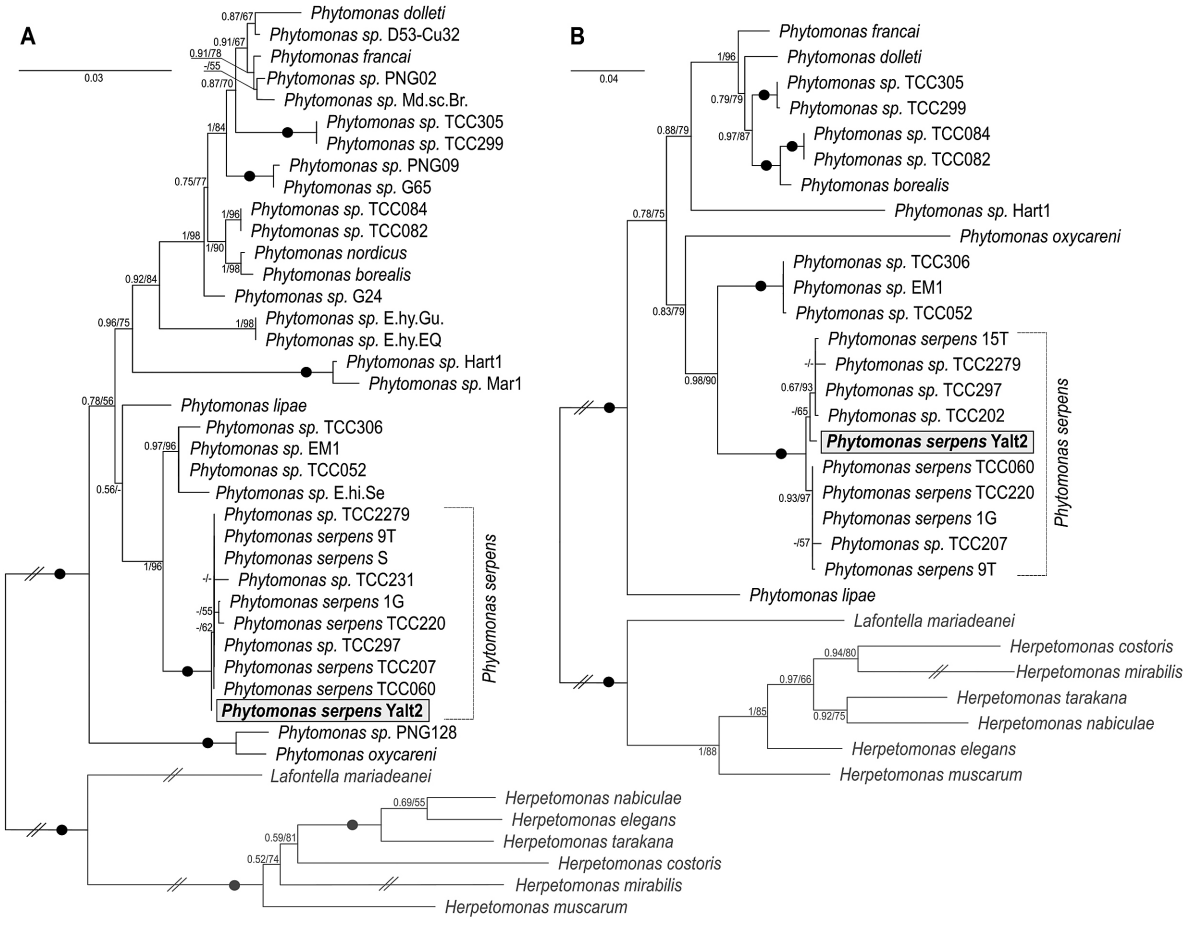

Fig. 3. Maximum likelihood phylogenetic trees reconstructed using different molecular markers. A – SSU rRNA gene; B – gGAPDH gene. Numbers at nodes indicate posterior probability and bootstrap percentage, respectively. Values less than 0.5 and 50% are replaced with dashes. Nodes having 1.0 posterior probability and 100% bootstrap support are marked with black circles. Double-crossed branches are at 50% of their original lengths. The trees are rooted with the sequences of *Herpetomonas* and *Lafontella* spp. (shown in grey). The scale bar represents number of substitutions per site. *Phytomonas serpens* Yalt2 is highlighted in bold.

(Jaskowska et al., 2015). However, in the course of more than a century of research, phytomonads have never been found in conifers. *Phytomonas* spp. is transmitted by phytophagous bugs from the families Lygaeidae, Coreidae and Pentatomidae. In bugs, flagellates undergo a complex succession of developmental stages, successively infecting the gut, the hemolymph, and the salivary glands of their hosts (Freymuller et al., 1990; Frolov et al., 2016, 2021).

We performed a molecular phylogenetic analysis of isolate Yalt2 from the invasive bug species *L. occidentalis* in the Republic of Crimea using sequences of the two molecular markers commonly used for trypanosomatids – SSU rRNA and gGAPDH genes. The results showed that the isolate belonged to the species *Phytomonas serpens* known as "trypanosomatid parasite of tomatoes" (Jankevicius et al., 1989). The comparison of this nearly full-length small SSU rRNA with the sequences from GenBank revealed the new isolate was identical to *P. serpens* isolate 9T (WHS sequence) differing only by one substitution in the variable region V4 of the gene. SSU rRNA sequences of the known Brazilian *P. serpens* isolates have also been shown to differ by 1–4 substitutions, which may reflect both SSU sequence polymorphism and differences between individual *P. serpens* strains (Hollar and Maslov, 1997).

Trypanosomatids parasitizing tomatoes became known after the pioneering study of Gibbs (1957). He found flagellates in the bugs *Nezara viridula* (Hemiptera, Pentatomidae) from the Cape Peninsula of the South Africa and in the juice of tomatoes

fruit *Solanum lycopersicum*, which these bugs fed on. Gibbs (1957) named these flagellates *Leptomonas serpens* and described the morphology of *L. serpens* from the gut and salivary glands of the bugs and from the juice of infected tomatoes. He identified two morphotypes of these flagellates in both hosts: promastigotes (= leptomonads *sensu* Gibbs) and endomastigotes (= metacyclic form). Unfortunately, laboratory culture of these flagellates has not been isolated, and nowadays it is not possible to unambiguously identify the taxonomic affiliation of these trypanosomatids using molecular markers.

Numerous species of both monoxenic trypanosomatids and phytomonads are known to occur and persist in tomato fruit (Conchon et al., 1989; Sanchez-Moreno et al., 1995). The species epithet "*serpens*" was used for tomato parasites for a second time by Brazilian researchers in *Phytomonas serpens* (Jankevicius et al., 1989). These flagellates are morphologically similar to the trypanosomatids described in South Africa (Gibbs, 1957) and they also have two hosts in their life cycle: the fruit of tomato *Solanum lycopersicum* and the neotropical phytophagous bug *Phthia picta* (Hemiptera, Coreidae) as vectors. The authors obtained axenic cultures of *P. serpens* isolated from both tomato fruit and experimentally infected insects (Jankevicius et al., 1989). Tests with monoclonal antibodies specific to the genus *Phytomonas* (Teixeira and Camargo, 1989) performed on all isolates confirmed that *P. serpens* indeed belonged to that genus (Jankevicius et al., 1989). Later, the number of isolates of this species from Brazil increased, and *P. serpens* became a "model" species in diverse trypanosomatid studies (Hollar and Maslov, 1997; Camargo, 1999; Alves e Silva et al., 2013; Dollet et al., 2012; Kořený et al., 2012; Verner et al., 2014; Zanetti et al., 2016; dos Santos Júnior et al., 2018).

Interestingly, large-scale studies of trypanosomatid biodiversity using molecular barcoding techniques carried out during the last decade in tropical and subtropical regions on different continents and focused mainly on the identification of trypanosomatids from hemipterans have not yet revealed the presence of *P. serpens* in any bug species (Maslov et al., 2007; Votýpka et al., 2010, 2012, 2020; Dollet et al., 2012; Kozminsky et al., 2015; Zanetti et al., 2016; Králová et al., 2019; Boucinha et al., 2020). Moreover, only isolates from Brazilian tomatoes have been attributed to the "serpens" group out of all known isolates of phytomonads from plants on different continents identified using the main marker genes (18S, gGAPDH, SL) (Jaskowska et al., 2015).

It is noteworthy that in 1995, in Europe, in the south of Spain, the similar flagellates were found in tomato fruits, and the results of a lectin-agglutination test revealed their belonging to the genus *Phytomonas* (Sanchez-Moreno et al., 1995). However, a later molecular genetic study of these phytomonads (isolate TCC 305E) showed that they did not belong to the group of *P. serpens* isolates but clustered in a separate group together with European flagellates isolated from *Annona cherimola*, *Trifolium* sp. and *Amaranthus retroflexus* (Serrano et al., 1999; Zanetti et al., 2016). To sum up, the species name *P. serpens* has been retained only for flagellates from tomato fruit and *Phthia picta* coreid bugs in Brazil (Jankevicius et al., 1989; Jaskowska et al., 2015).

In this study, we provide the first record of *P. serpens* on the European continent. We showed experimentally that isolate Yalt2 could infect tomato fruit and form stable micropopulations inside, while flagellates retain their proliferative activity for a long time.

Taking into account that tomatoes are broadly cultivated in the south of Russia and elsewhere in southern Europe, we can suggest that *P. serpens* is either already distributed in the southern Palaearctic region (and has escaped the researchers' attention so far) or is currently colonizing new territory. A prerequisite for both assumptions is the presence of a specific vector for these phytomonads. *Phytomonas* spp. widely use coreid bugs as vectors (Dollet et al., 1982; Jankevicius et al., 1989, 1993; Brasil et al., 1990; Frolov et al., 2019), which means that these bugs are "comfortable hosts" for phytomonads. However, western conifer seed bug *Leptoglossus occidentalis*, whose diet is normally restricted to conifers, can hardly be a specific vector of *P. serpens* (Fent and Kment, 2011; Werner, 2011).

In the light of these considerations, we tend to regard the detection of *P. serpens* in *L. occidentalis* as a consequence of stochastic host-switching resulting from spontaneous disruption of the host diet. *L. occidentalis* bugs could become infected with *P. serpens* flagellates after feeding, e.g., on the faeces or the corpse of a specific vector. Both coprophagy and necrophagy are common in insects including phytophagous bugs, and thus may serve as a primary and a secondary mode of transmission of trypanosomatids between hosts (Frolov et al., 2021). It is also known that the gut stages of phytomonads, including *P. serpens*, which do not migrate to the

salivary glands of the host, get into the hindgut and are excreted with the faeces into the environment, where they can serve as a source of new infections (Jankevicius et al., 1989; Frolov et al., 2016).

Another pathway that might hypothetically lead to the emergence of the unusual host-parasite association described in this paper could be a for-ced or accidental change in the plant diet of *L. occidentalis*, triggered by food shortage and/or new climatic conditions for this invasive bug. There is evidence that *L. occidentalis* may feed on ripening fruit of pistachio (Anacardiaceae), though in rare cases and under experimental conditions (Rice et al., 1985; Uyemoto et al., 1986). Therefore, it cannot be ruled out that these bugs might occasionally feed on other nonconiferous plants, too. This scenario does not exclude the feeding of *L. occidentalis* on tomato fruits and could explain the fact that the bugs were infected with the flagellates *P. serpens*.

The question about the specific vector of *P. serpens* on the European continent and, in particular, in the Crimean Peninsula is still open. In our opinion, the most probable candidate for this role is another invasive hemipteran species *Nezara viridula* L. (Hemiptera, Pentatomidae), also known as "southern green stink bug" or "green vegetable bug". *N. viridula* is thought to be native in the Ethiopian zoogeographical region, from where it has spread into Asia, Europe and the Americas (Musolin and Saulich, 2012). In the Crimea, *N. viridula* is a harmful pest of vegetable crops, including tomatoes (Stryukova and Stryukov, 2020). The early work of Gibbs (1957) and the reports that the bugs *N. viridula* could be infected by Brazilian isolates of *P. serpens* in their preliminary experiments (Jankevicius et al., 1989) lend credibility to this hypothesis.

Acknowledgements

This work was supported by the Russian Science Foundation (grant No 22-24-00033 https://rscf.ru/project/22-24-00033/). The research was carried out using the equipment of the Core Facilities Centre "Taxon" at the Zoological Institute, Russian Academy of Sciences (St. Petersburg, Russia). We thank Dr. D. A. Gapon for help in identifying L. occidentalis.

References

Alves e Silva T.L., Vasconcellos L.R.C., Lopes A.H. and Souto-Padron T. 2013. The immune response of hemocytes of the insect *Oncopeltus fasciatus* against the flagellate *Phytomonas serpens*. PLoS One. 8 (8): e72076. doi:10.1371/journal.pone.0072076

Boucinha C., Caetano A.R., Santos H., Helaers R. et al. 2020. Analysing ambiguities in trypanosomatids taxonomy by barcoding. Mem. Inst. Oswaldo Cruz. 115: e200504. doi: 10.1590/0074-02760200504

Brazil R.P., Fiorini J.E. and Faria-e-Silva P. 1990. *Phytomonas* sp. a trypanosomatid parasite of tomato, isolated from salivary glands of *Phthia picta* (Hemiptera: Coreidae) in southeast Brazil. Mem. Inst. Oswaldo Cruz. 85 (2): 239–40.

Camargo E.P. 1999. *Phytomonas* and other trypanosomatid parasites of plants and fruit. Adv. Parasitol. 42: 29–112. doi: 10.1016/s0065-308x(08)60148-7

Conchon I., Campaner M., Sbravat C. and Camargo E.P. 1989. Ttypanosomatids, other than *Phytomonas* spp., isolated and cultured from fruit. J. Protozool. 36 (4): 412–414. https://doi.org/10.1111/j.1550-7408.1989.tb05537.x

Dollet M., Cambrony D. and Gargani D. 1982. Culture axйnique *in vitro* de *Phytomonas* sp. (Trypanosomatidae) d'Euphorbe, transmis par *Stenocephalus agilis* Scop (Coreide). C. R. Acad. Sci. III. 295: 547–550.

Dollet M., Sturm N.R. and Campbell D.A. 2012. The internal transcribed spacer of ribosomal RNA genes in plant trypanosomes (*Phytomonas* spp.) resolves 10 groups. Infect. Genet. Evol. 12: 299–308. doi:10.1016/j.meegid.2011.11.010

dos Santos Júnior A.C.M., Ricart C.A.O., Pontes A.H., Fontes W. et al. 2018. Proteome analysis of *Phytomonas serpens,* a phytoparasite of medical interest. PLoS One. 13 (10): e0204818. https://doi.org/10.1371/journal.pone.0204818

Fent M. and Kment P. 2011. First record of the invasive western conifer seed bug *Leptoglossus occidentalis*, (Heteroptera: Coreidae) in Turkey. North-West. J. Zool. 7 (1): 72–80.

Flegontov P., Votýpka J., Skalický T., Logacheva M.D. et al. 2013. *Paratrypanosoma* is a novel early-branching trypanosomatid. Curr. Biol. 23: 1787–1793. doi:10.1016/j.cub.2013.07.045

Freymuller E., Milder R., Jankevicius J.V., Shiduca I. and Plessmann E. 1990. Ultrastructural studies on the trypanosomatid *Phytomonas serpens* in the salivary glands of a phytophagous hemipteran. J. Protozool. 37 (3): 225–229. https://doi.org/10.1111/j.1550-7408.1990.tb01132.x

Frolov A.O. 2016. Trypanosomatids (Kinetoplastea: Trypanosomatida) and insects (Insecta):

patterns of co-evolution and diversification of the host–parasite systems. Proc. Zool. Inst. RAS. 320 (S4): 16–75 (in Russian with English summary). https://www.zin.ru/journals/trudyzin/doc/vol_320_s4/TZ_320_4_Supplement_Frolov.pdf

Frolov A.O., Malysheva M.N. and Kostygov A.Y. 2015. Transformations of life cycles in the evolutionary history of trypanosomatids. Macrotransformations. Parazitologiya. 49: 233–256 (in Russian with English summary). https://www.zin. ru/journals/parazitologiya/content/2015/prz_2015_4_1_Frolov.pdf

Frolov A.O., Malysheva M.N., Yurchenko V. and Kostygov A.Yu. 2016. Back to monoxeny: *Phytomonas nordicus* descended from dixenous plant parasites. Eur. J. Protistol. 52: 1–10. https://doi.org/10.1016/j.ejop.2015.08.002

Frolov A.O., Malysheva M.N., Ganyukova A.I., Yurchenko V. and Kostygov A.Y. 2018. Obligate development of *Blastocrithidia papi* (Trypanosomatidae) in the Malpighian tubules of *Pyrrhocoris apterus* (Hemiptera) and coordination of host-parasite life cycles. PLoS ONE 13 (9): e020 4467. https://doi.org/10.1371/journal.pone.0204467

Frolov A.O., Malysheva M.N., Ganyukova A.I., Spodareva V.V.et al. 2019. Development of *Phytomonas lipae* sp. n. (Kinetoplastea: Trypanosomatidae) in the true bug *Coreus marginatus* (Heteroptera: Coreidae) into the evolution of the life cycles in the genus *Phytomonas*. PLoS ONE. 14 (4): e0214484. doi:10.1371/journal.pone.0214484

Frolov A. O., Kostygov A. Y. and Yurchenko V. 2021. Development of monoxenous trypanosomatids and phytomonads in insects. Trends Parasitol. 37 (6): 538–551. doi:10.1016/j.pt.2021.02.004

Ganyukova A., Frolov A., Malysheva M., Spodareva V. et al. 2020. A novel endosymbiont-containing trypanosomatid *Phytomonas borealis* sp. n. from the predatory bug *Picromerus bidens* (Heteroptera: Pentatomidae). Folia Parasitol. 67: 004. doi:10.14411/fp.2020.004

Gapon D. A. 2012. First records of the western conifer seed bug *Leptoglossus occidentalis* Heid. (Heteroptera, Coreidae) from Russia and Ukraine, regularities in its distribution and possibilities of its range expansion in the Palaearctic Region. Ėntomologicheskoe Obozrenie. 91 (3): 559–568 (in Russian with English summary).

Gibbs A.J. 1957. *Leptomonas serpens* n. sp., parasitic in the digestive tract and salivary glands of *Nezara viridula* (Pentatomidae) and in the sap of *Solanum lycopersicum* (tomato) and other plants. Parasitology. 47 (3–4): 297–303. doi:10.1017/s0031182000021983

Hamilton P., Gibson W. and Stevens J. 2007. Patterns of co-evolution between trypanosomes and their hosts deduced from ribosomal RNA and protein-coding gene phylogenies. Mol. Phylogenet. Evol. 44: 15–25. doi:10.1016/j.ympev.2007.03.023

Hamilton P.B., Cruickshank C., Stevens J.R., Teixeira M.M.G. and Mathews F. 2012. Parasites reveal movement of bats between New and Old Worlds. Mol. Phylogenet. Evol. 63: 521–526. doi:10.1016/j.ympev.2012.01.007

Hoang D.T., Chernomor O., von Haeseler A. and Minh B.Q. 2017. UFBoot2: improving the ultrafast bootstrap approximation. Mol. Biol.Evol. 35 (2): 518–522. doi:10.1093/molbev/msx281

Hollar L. and Maslov D.A. 1997. A phylogenetic view on the genus *Phytomonas*. Mol. Biochem. Parasitol. 89: 295–299. https://doi.org/10.1016/S0166-6851(97)00117-5

Jankevicius J.V., Jankevicius S.I., Campaner M., Conchon I. et al. 1989. Life cycle and culturing of *Phytomonas serpens* (Gibbs), a trypanosomatid parasite of tomatoes. J. Protozool. 36 (3): 265–271. https://doi.org/10.1111/j.1550–7408.1989.tb05361.x

Jankevicius S.I., de Almeida M.L., Jankevicius J.V., Cavazzana M. et al. 1993. Axenic cultivation of trypanosomatids found in corn (*Zea mays*) and in phytophagous hemipterans (*Leptoglossus zonatus*, Coreidae) and their experimental transmission. J. Eukar. Microbiol. 40: 576–581. https://doi.org/10.1111/j.1550-7408.1993.tb06110.x

Jaskowska E., Butler C., Preston G. and Kelly S. 2015. *Phytomonas*: trypanosomatids adapted to plant environments. PLoS Pathog. 11 (1): e1004484. doi:10.1371/journal.ppat.1004484

Kalyaanamoorthy S., Minh B.Q., Wong T.K.F., von Haeseler A. and Jermiin L.S. 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods. 14 (6): 587–589. doi:10.1038/nmeth.4285

Kořený L., Sobotka R., Kovářová J., Gnipova A. et al. 2012. Aerobic kinetoplastid flagellate *Phytomonas* does not require heme for viability. Proc. Natl. Acad. Sci. U.S.A. 109: 3808–3813. https://doi.org/10.1073/pnas.1201089109

Kostygov A.Y., Karnkowska A., Votýpka J., Tashyreva D. et al. 2021. Euglenozoa: taxonomy, diversity and ecology, symbioses and viruses. Open Bio. 11: 200407. https://doi.org/10.1098/rsob.200407

Kostygov A.Y. and Frolov A.O. 2007. *Leptomonas jaculum* (Leger, 1902) Woodcock 1914: a leptomonas or a blastocrithidia? Parazitologiya. 41: 126–136 (in Russian with English summary). https://www.zin.ru/journals/parazitologiya/content/2007/prz_2007_2_4_Kostygov.pdf

Kozminsky E., Kraeva N., Ishemgulova A., Dobáková E.et al. 2015. Host-specificity of monoxenous trypanosomatids: statistical analysis of the distribution and transmission patterns of the parasites from Neotropical Heteroptera. Protist. 166 (5): 551–568. https://doi.org/10.1016/j.protis.2015.08.004

Králová J., Grybchuk-Ieremenko A., Votýpka J., Novotný V. et al. 2019. Insect trypanosomatids in Papua New Guinea: high endemism and diversity. Int. J. Parasitol. 49 (13-14): 1075–1086. doi:10.1016/j.ijpara.2019.09.004

Kumar S., Stecher G. and Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33 (7): 1870–1874. doi:10.1093/molbev/msw054

Lesieur V., Lombaert E., Guillemaud T., Courtial B. et al. 2019. The rapid spread of *Leptoglossus occidentalis* in Europe: a bridgehead invasion. J. Pest. Sci. 92 (1): 189–200. https://doi.org/10.1007/s10340-018-0993-x

Lukeš J., Skalický T., Týc J., Votýpka J. and Yurchenko V. 2014. Evolution of parasitism in kinetoplastid flagellates. Mol. Biochem. Parasit. 195 (2): 115–122. doi:10.1016/j.molbiopara.2014.05.007

Lukeš J., Butenko A., Hashimi H., Maslov D.A. et al. 2018. Trypanosomatids are much more than just trypanosomes: clues from the expanded family tree. Trends Parasitol. 34 (6): 466–480. doi:10.1016/j.pt.2018.03.002

Malysheva M.N., Ganyukova A.I. and Frolov A.O. 2020. *Blastocrithidia frustrata* sp. n. (Kinetoplastea, Trypanosomatidae) from the brown marmorated stink bug *Halyomorpha halys* (Stel) (Hemiptera, Pentatomidae). Protistology. 14: (3) 130–146. doi: http://dx.doi.org/10.21685/1680-0826-2020-14-3-3

Maslov D.A., Lukeš J., Jirku M. and Simpson L. 1996. Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: implications for the evolution of parasitism in the trypanosomatid protozoa. Mol. Biochem. Parasit. 75 (2): 197–205. doi:10.1016/0166-6851(95)02526-x

Maslov D.A., Westenberger S.J., Xu X., Campbell D.A. and Sturm N.R. 2007. Discovery and barcoding by analysis of Spliced Leader RNA gene sequences of new isolates of Trypanosomatidae from Heteroptera in Costa Rica and Ecuador. J. Eukar. Microbiol. 54: 57–65. doi:10.1111/j.1550-7408.2006.00150.x

Maslov D.A.,Yurchenko V.Y., Jirkü M. and Lukeš J. 2010. Two new species of trypanosomatid parasites isolated from Heteroptera in Costa Rica. J. Euk. Microbiol. 57: 177–188. doi:10.1111/j.1550-7408.2009.00464.x

Maslov D., Votýpka J., Yurchenko V. and Lukeš J. 2013. Diversity and phylogeny of insect trypanosomatids: all that is hidden shall be revealed. Trends Parasitol. 29 (1): 43–52. doi:10.1016/j.pt.2012.11.001

Musolin D.L. and Saulich A.K. 2012. Responses of insects to the current climate change: from physiology and behaviour to range shifts. Entomol. Rev. 92: 715–740 (in Russian with English summary).

Nguyen L.T., Schmidt H.A., von Haeseler A. and Minh B.Q. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32: 268–27. doi:10.1093/molbev/msu300

Podlipaev S.A. and Frolov A.O. 1987. Description and laboratory cultivation of *Blastocrithidia miridarum* sp. n. (Mastigophora, Trypanosomatidae). Parazitologiya. 21: 545–552 (in Russian with English summary). https://www.zin.ru/journals/parazitologiya/content/1987/prz_1987_4_5_Podlipaev.pdf

Rice R.E., Uyemoto J.K., Ogawa J.M. and Pemberton W.M. 1985. New findings on pistachio problems. Calif. Agric. 39: 15–18. https://calag.ucanr.edu/download_pdf.cfm?article=ca.v039n01p15

Ronquist F. and Huelsenbeck J. 2003. MRBA YES 3: Bayesian Phylogenetic inference under mixed models. Bioinformatics. 19 (12): 1572–1574. doi:10.1093/bioinformatics/btg180

Sanchez-Moreno M., Fernandez-Becerra C., Entrala E., Opperdoes F.R. et al. 1995. In vitro culture of *Phytomonas* sp. isolated from *Euphorbia characias*. Metabolic studies by 1NMR. J. Euk. Microbiol. 43 (2): 314–20. https://doi.org/10.1111/j.1550-7408.1995.tb01586.x

Serrano M.G., Camargo E.P. and Teixeira M.M.G. 1999. *Phytomonas*: analysis of polymorphism and genetic relatedness between isolates from plants and phytophagous insects from different geographic regions by RAPD fingerprints and synapomorphic markers. J. Euk. Microbiol. 46 (6): 618–625. doi:10.1111/j.1550-7408.1999.tb05138.x

Seward E., Votýpka J., Kment P. and Lukeš J. 2016. Description of *Phytomonas oxycareni* n. sp. from the salivary glands of *Oxycarenus lavaterae*.

Protist. 168 (1): 71–79. doi:10.1016/j.protis.2016.11.002

Simpson A., Stevens J. and Lukeš J. 2006. The evolution and diversity of kinetoplastid flagellates. Trends Parasitol. 22 (4): 168–174. doi:10.1016/j.pt.2006.02.006

Stevens J. 2008. Kinetoplastid phylogenetics, with special reference to the evolution of parasitic trypanosomes. Parasite. 15 (3): 226–232. doi:10.1051/parasite/2008153226

Stryukova N.M. and Stryukov A.A. 2020. New data on invasive insects in the republic of Crimea. Plant biology and horticulture: theory, innovation. 4(157): 56–66 (in Russian with English summary). https://doi.org/10.36305/2712-7788-2020-4-157-56-66

Teixeira M.M.G. and Camargo E.P. 1989. Monoclonal antibodies for the identification of trypanosomatids of the genus *Phytomonas*. J. Protozool. 36: 262–264.

Uyemoto J.K., Ogawa J.M., Rice R.E., Teranishi H.R. et al. 1986. Role of several true bugs (Hemiptera) on incidence and seasonal development of pistachio fruit epicarp lesion disorder. J. Econ. Entomol. 79: 395–399. https://doi.org/10.1093/jee/79.2.395

Verner Z., Čermáková P., Kovacova B. and Škodová I. 2014. Comparative analysis of respiratory chain and oxidative phosphorylation in *Leishmania tarentolae, Crithidia fasciculata, Phytomonas serpens* and procyclic stage of *Trypanosoma brucei*. Mol. Biochem. Parasitol. 193 (1): 55–65. doi:10.1016/j.molbiopara.2014.02.003

Vickerman K. 1994. The evolutionary expansion of the trypanosomatid flagellates. Int. J. Parasitol. 24 (8): 1317–1331. doi: 10.1016/0020-7519(94)90198-8

Votýpka J., Maslov D.A., Yurchenko V., Jirků M. et al. 2010. Probing into the diversity of trypanosomatid flagellates parasitizing insect host in South-West China reveals both endemism and global dispersal. Mol. Phylogenet. Evol. 54 (1): 243–253. doi: 10.1016/j.ympev.2009.10.014

Votýpka J., Klepetkova H., Jirků M., Kment P. and Lukeš J. 2012. Phylogenetic relationships of trypanosomatids parasitizing true bugs (Insecta: Heteroptera) in sub-Saharan Africa. Int. J. Parasitol. 42: 489–500. doi: 10.1016/j.ijpara.2012.03.007

Votýpka J., d'Avila-Levy C.M., Grellier P., Maslov D.A. et al. 2015. New approaches to systematics of Trypanosomatidae: criteria for taxonomic (re) description. Trends Parasitol. 31 (10): 460–469. doi: 10.1016/j.pt.2015.06.015

Votýpka J., Kment P., Yurchenko V. and Lukeš J. 2020. Endangered monoxenous trypanosomatid parasites: a lesson from island biogeography. Biodivers. Conserv. 29: 3635–3667. doi:10.1007/s10531-020-02041-2

Werner D.J. 2011. Die amerikanische Koniferen-Samen-Wanze *Leptoglossus occidentalis* (Heteroptera: Coreidae) als Neozoon in Europa und in Deutschland: Ausbreitung und Biologie. Entomologie heute. 23: 31–68. https://www.zobodat.at/pdf/Entomologie-heute_23_0031-0068.pdf

Yurchenko V., Lukeš J., Jirků M., Zeledon R. and Maslov D. A. 2006. *Leptomonas costaricensis* sp. n. (Kinetoplastea: Trypanosomatidae), a member of the novel phylogenetic group of insect trypanosomatids closely related to the genus *Leishmania*. Parasitology. 133, 537–546. doi:10.1017/S0031182006000746

Zanetti A., Ferreira R.C., Serrano M.G., Takata C.S. et al. 2016. *Phytomonas* (Euglenozoa: Trypanosomatidae): phylogenetic analyses support infrageneric lineages and a new species transmitted to Solanaceae fruits by a Pentatomid hemipteran. Eur. J. Protistol. 56: 232–249. doi:10.1016/j.ejop.2016.09.004

Supplementary material

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