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Back to monoxeny: *Phytomonas nordicus* descended from dixenous plant parasites

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Abstract

The trypanosomatid *Phytomonas nordicus* parasitizing the predatory bug *Troilus luridus* was described at the twilight of the morphotype-based systematics. Despite its monoxenous life cycle, this species was attributed to the dixenous genus *Phytomonas* due to the presence of long twisted promastigotes and development of flagellates in salivary glands. However, these characteristics were considered insufficient for proving the phytomonad nature of the species and therefore its description remained virtually unnoticed. Here, we performed molecular phylogenetic analyses using 18S ribosomal RNA (rRNA) gene and region containing internal trascribed spacers (ITS) 1 and 2 and convincingly demonstrated the affinity of *P. nordicus* to the genus *Phytomonas*. In addition, we investigated its development in the salivary glands. We argue that in many aspects the life cycle of monoxenous *P. nordicus* resembles that of its dixenous relatives represented by tomato-parasitizing *Phytomonas serpens*.

Keywords: Evolution; Monoxenous lifestyle; *Phytomonas*; Ultrastructure

Introduction

The genus *Phytomonas* Donovan, 1909 unites dixenous trypanosomatids with a life cycle split between two hosts: the plants and the herbivore bugs (Camargo 1999; Dollet 2001). In plants, the flagellates proliferate, accumulate, and differentiate into insect-infective stages. The development of phytomonads in the vector includes growth in the intestine, migration of the parasites to the salivary glands, and formation of cells capable of infecting plants (Freymuller et al. 1990; Jankevicius et al. 1989).

For a long time, the main criteria for assigning a species to the genus *Phytomonas* included the morphology of the promastigote stage and the presence in plants (Vickerman 1976). By the mid-1990s it became apparent that these traits were not sufficient for proper identification. In particular, some opportunistic developmental stages of parasites belonging to the genera *Herpetomonas*, *Leptomonas*, and *Crithidia* were documented in plants (Catarino et al. 2001; Conchon et al. 1989; Fiorini et al. 2001). However, phytophagous bugs can host different monoxenous parasites with similar morphology (Podlipaev 1990). Thus, biochemical, immunological, and molecular phylogenetic methods became widely used

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and generally accepted in the field (Hollar and Maslov 1997; Teixeira et al. 1996; Teixeira and Camargo 1989). Today "plant trypanosomatids" are considered monophyletic and include 8–10 well-defined subgroups (Dollet et al. 2012; Maslov et al. 2013; Sturm et al. 2007).

The trypanosomatid *Phytomonas nordicus* Frolov et Malysheva 1993 was isolated from the predatory bug *Troilus luridus* Fabricius 1775 and formally described at the twilight of the "morphotype-based" systematics. It was attributed to the genus *Phytomonas* based on its twisted promastigote morphology and the parasite's presence in the intestinal tract, hemolymph, and salivary glands of its host (Frolov and Malysheva 1993). Experiments testing different modes of horizontal transmission showed the monoxenous nature of this species. It was never observed in the sap of plants, but it could be transmitted between individuals of *T. luridus* through prey (serving as a temporary reservoir) or fecal contamination. Without molecular data, its affinity to other *Phytomonas* spp. was speculative and impossible to prove with any certainty. Therefore, this species was effectively neglected in the scientific literature. Meanwhile, if confirmed, this may be the first documented case of insect-only secondary monoxeny (evolvement of a monoxenous species from the dixenous predecessor). Indeed, predatory bugs have no trophic connection with plants (indispensable hosts of all previously described *Phytomonas* spp.). *P. nordicus* can be transmitted between insects either by coprophagy or while sucking the prey (Frolov and Malysheva 1993). The only documented example of a similar kind is *Trypanosoma equiperdum*, a tissue parasite of horses, donkeys, and their hybrids (Hoare 1973; Jensen et al. 2008; Lai et al. 2008).

In this work we performed molecular phylogenetic analyses using 18S ribosomal RNA (rRNA) gene and region containing internal trascribed spacers (ITS) 1 and 2 and conclusively demonstrated the affinity of *P. nordicus* to the genus *Phytomonas*. In addition, we investigated its development in the salivary glands, which is the phase of the life cycle that is indicative of this parasite's dixenous ancestry.

Material and Methods

Field work

Sixteen pentatomid bugs *T. luridus* were collected from leaves of goat willow (*Salix caprea*) and grey alder (*Alnus incana*) near Berezitsy (Pskov region, Russian Federation, 58°30′34″ N, 29°43′17″ E) in August 2014. The insects were immobilized by chloroform, and a drop of hemolymph from a leg was investigated by dark-field light microcopy using Leica DM 2500. Three individuals were found to be positive for parasites. They were dissected in normal saline solution, and their salivary glands were isolated. Only two insects showed infection in these organs. The principal glands of one individual was used for smears and tissue impressions followed by light microscopy and cultivation; the glands of the other were used for DNA isolation and ultrastructural studies.

Cultivation of trypanosomatids

Our attempts to cultivate *P. nordicus* in different media–brain–heart infusion (BHI, Becton, Dickinson and Co, Franklin Lakes, NJ, USA), Grace's Insect Cell Culture Medium (Life Technologies, Carlsbad, CA, USA), M199 (BioloT, St. Petersburg, Russia), or a combination thereof were not successful. The best result was obtained using diphasic blood agar media:liquid-phase BHI + 15% fetal bovine serum (FBS; Life Technologies). Under these conditions, flagellates survived for 15–20 days, actively proliferating for the first week. The cells died in subsequent passages. All studies reported here were conducted using populations of flagellates isolated from the salivary glands of insects.

Light microscopy

Smears and principal salivary gland impressions were air-dried on slides and fixed for 30 min in ethanol. Giemsa staining was performed as previously described (Kostygov et al. 2014; Yurchenko et al. 2008). All measurements were conducted on digital images obtained using the light microscope equipped with the 14-Mpx camera UCMOS14000KPA. In all cases, 25 cells were measured. Statistical analysis was carried out with the UTHSCSA Image Tool for Windows v. 3.0 software (http://compdent.uthscsa.edu/dig/itdesc.html).

Transmission electron microscopy

Transmission electron microscopy was performed as described earlier (Yurchenko et al. 2014). In brief, the salivary glands were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h on ice. Cells were washed thrice in 5% sucrose/0.1 M cacodylate buffer and post-fixed in 2% OsO_4/0.1 M cacodylate buffer for 30 min. The samples were dehydrated stepwise in an ascending alcohol series, and flat-embedded in epon–araldite resin. Ultrathin sections (70 nm) were cut using a Reichert–Jung Ultracut E ultramicrotome (Leica Microsystems), collected on copper grids, contrasted in ethanolic uranyl acetate and lead citrate, and observed under a Jeol 100C microscope (Jeol, Tokyo, Japan).

DNA isolation, amplification, cloning, and sequencing

Total genomic DNA was isolated from the field samples using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The 18S rRNA gene was amplified using primers S762 and S763 and sequenced directly as described previously (Maslov et al.

1996; Yurchenko et al. 2006). The ITS1-containing region was amplified with the primers IAMWE and Tc5.8SR (Dollet et al. 2012), and then cloned using InsTA PCR Cloning Kit (Thermo Fisher Scientific, Waltham, USA). Both strands of two clones were sequenced. The GenBank accession numbers for the new sequences determined in this work are KT223609 (18S rRNA), KT223610, and KT223611 (ITS1-containing region).

Phylogenetic analyses

The 18S rRNA sequences of 16 isolates of *Phytomonas* spp. and five species of *Herpetomonas* spp. retrieved from GenBank (Suppl. Table 1) were aligned with the sequence of *P. nordicus* using Muscle 3.8.3.1 (Edgar 2004). The resulting alignment was refined manually using the BioEdit sequence alignment (Hall 1999). The data matrix for the ITS-containing region was prepared similarly, with sequences of *P. nordicus*, 20 isolates of other phytomonads and *Herpetomonas muscarum* being included (Suppl. Table 1). As this region was much more difficult to align properly, many ambiguous alignment positions were removed manually. The final dataset thus contained only 780 nucleotides (nt). Maximum likelihood-based phylogenetic inference was performed in PhyML (Guindon et al. 2010) under TIM2ef + I + G and TPM3uf + G models (for 18S rRNA gene and ITS-containing region, respectively) as selected in jModeltest 2.1.6 (Darriba et al. 2012) with four gamma categories and the subtree pruning and regrafting (SPR) branch-swapping algorithm. The edge support was estimated using the bootstrap test (1000 replicates). Bayesian inference was accomplished in MrBayes 3.2.5 (Ronquist et al. 2012) with the analysis run for two million generations in the GTR + I + G or GTR + G models (for 18S rRNA gene and ITS-containing datasets, respectively), with four gamma categories and every 500 generations being sampled. Other parameters were left in their default states.

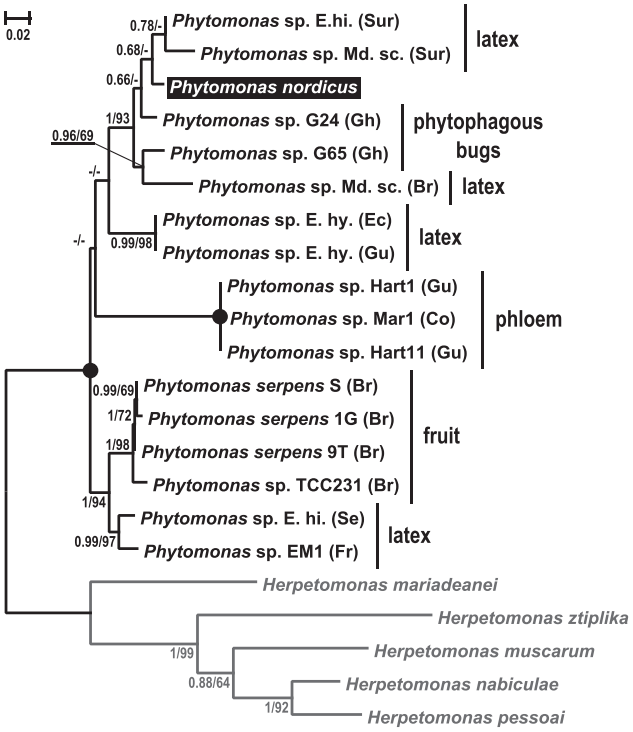

Fig. 1. Maximum likelihood phylogenetic tree reconstructed using 18S ribosomal RNA gene sequences. Numbers at nodes indicate posterior probability and bootstrap percentage, respectively. Values <0.5 and <50% are replaced with dashes. Nodes having 1.0 posterior probability and 100% bootstrap support are marked with black circles. The tree is rooted with the sequences of *Herpetomonas* spp. (shown in gray). The scale bar represents the number of substitutions per site. The species under study (*Phytomonas nordicus*) is highlighted. The geographic origin of all strains is included in their names with the following abbreviations used: Br = Brazil, Co = Colombia, Ec = Ecuador, Fr = France, Gh = Ghana, Gu = French Guiana, Se = Senegal, and Sur = Suriname.

Results

Phylogenetic analyses

The maximum likelihood and Bayesian phylogenetic trees inferred using 18S rRNA gene and the ITS-containing region were similar to those published before, with paraphyly of the latex-derived isolates and polyphyly of the fruit parasites (Figs 1 and 2). The trees reconstructed using both markers were congruent with each other, despite significant differences in taxa composition. The most important result was the unambiguous clustering of *P. nordicus* within one of the subgroups of the genus *Phytomonas*.

As seven out of 17 *Phytomonas* 18S rRNA gene sequences were extremely short (309–358 bp in length), the part of the tree holding these sequences allowed only very poor resolution (Fig. 1). Nevertheless, the presence of two full-length sequences of flagellates obtained from phytophagous bugs (isolates G24 and G65) allowed delineating a group of isolates closely related to *P. nordicus*. This clade, which also includes some parasites of latex, showed high bootstrap support.

Because the resolution within the clade of interest was low, we decided to estimate these relationships using the ITS1/ITS2-containing region (Fig. 2). Although only some isolates were shared between the two phylogenetic datasets, the affinity of *P. nordicus* to the same group of phytomonads was evident. In addition to flagellates from latex, the *P. nordicus*-containing clade also included one isolate from the fruit (tomato). Interestingly, we detected close relationships of the species under study with some flagellates parasitizing the latex of different plants from Suriname. These relationships were supported by high bootstrap percentage and posterior probability values.

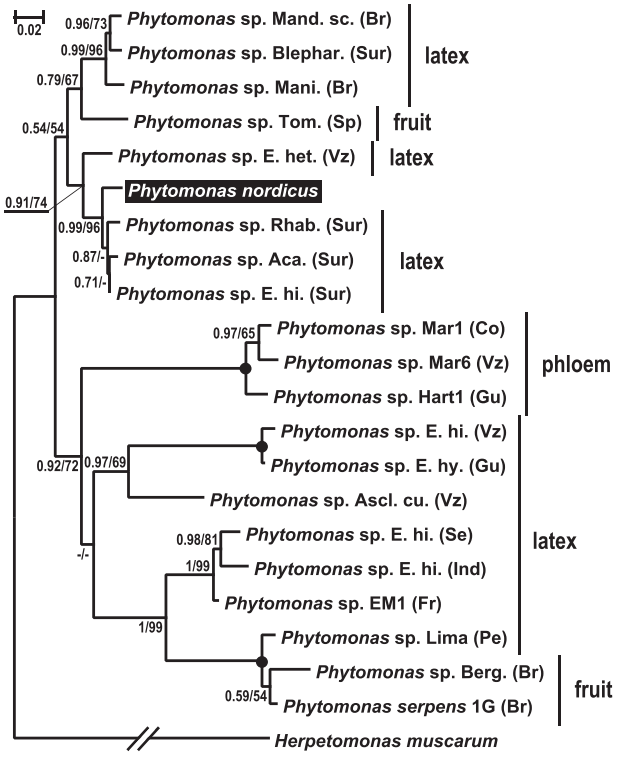

Fig. 2. Maximum likelihood phylogenetic tree reconstructed using ITS-containing ribosomal RNA sequences. Double-crossed branch appears at 50% of its original length. Numbers at nodes indicate posterior probability and bootstrap percentage, respectively. Values <0.5 and <50% are replaced with dashes. Nodes with 1.0 posterior probability and 100% bootstrap support are marked with black circles. The tree is rooted with the sequence of *Herpetomonas muscarum*. The scale bar represents the number of substitutions per site. The species under study (*Phytomonas nordicus*) is highlighted. The geographic origin of all strains is included in their names with the following abbreviations used: Br = Brazil, Co = Colombia, Fr = France, Gu = French Guiana, Ind = India, Pe = Peru, Se = Senegal, Sp = Spain, Sur = Suriname, and Vz = Venezuela.

Development of *P. nordicus* in salivary glands of *T. luridus*

Light microscopy

Smears of hemolymph and impressions of the salivary glands allowed us to study different morphotypes and the development of intracellular and extracellular forms of *P. nordicus*. The predominant morphotype in hemolymph was a large, twisted (three to seven twists) promastigote (Fig. 3A). All standard measurements are summarized in Suppl. Table 2. The intensity of infection was usually low with only a few slow-moving cells detected in each microscopic field. In rare cases of high infection (dozens of cells per microscopic filed), about 5–10% of promastigotes were found captured by the host's hemocytes (data not shown).

Analysis of the principal salivary glands revealed four major morphotypes corresponding to the developmental stages of *P. nordicus*: free promastigotes, promastigotes within vacuoles, haptomonads attached to the microvilli of the salivary gland epithelium, and endomastigotes. Free promastigotes were often U-shaped and reached 60 μm in length with the flagellum adjoining the cell body. The nucleus was localized at the posterior end of the cell, with the kinetoplast lying near the bottom of the flagellar pocket (Fig. 3B). Promastigotes within vacuoles occupied their whole volume; cells were interwoven, thus preventing accurate measurements (Fig. 3C–E). The nucleus was close to the anterior end of the cell. These promastigotes proliferated actively. The size of a parasitophorous vacuole correlated with the number of flagellates within it. The largest conglomerates contained up to 50 parasites (Fig. 3E). Club-shaped haptomonads multiplied on the surface of the salivary gland epithelium and covered it entirely (Fig. 3F, G). The free flagellum was short and used for attachment. The anterior part of the parasite cell was widened, containing an adjacent nucleus and kinetoplast. The posterior part was extended forming a long-tail fiber. The numerous endomastigotes were significantly smaller than the promastigotes and divided in the lumen of the salivary gland (Fig. 3H, I).

Transmission electron microscopy

Here, we describe the fine details of the salivary gland infection of *T. luridus* by *P. nordicus*. From the hemocoel, parasites enter the myocytes and the epithelial cells situated on the border of the salivary gland. Then they perforate the basal lamina and invade the salivary gland tissue. Proliferating flagellates advance through the gland epithelium finally reaching the lumen where they form the final infective stages.

Traversal of the salivary gland by *P. nordicus*: The flagellates were observed inside the cells lining the salivary gland from the hemocoel side. U-shaped parasites with flagellum adjacent to the cell surface were enclosed in parasitophorous vacuoles (Fig. 4A). There were multiple glycosomes and acidocalcisomes in the trypanosomatid's cytoplasm. Most of the myocytes and the epithelial cells were infected and exhibited different stages of decay. The part of the parasitophorous vacuole membrane that faced the decomposing basal lamina came in tight contact with the flagellates (Fig. 4A, B). Thinning and breaking of the basal lamina caused the parasitophorous vacuole to advance into the cell of the salivary gland epithelium (Fig. 4B).

Proliferation of *P. nordicus* in the salivary gland of *T. luridus* and formation of the infective stages: parasitophorous vacuoles within the cells of the principal salivary glands of *T. luridus* contained either singular large nondividing or multiple small actively proliferating promastigotes (Fig. 5A, B). They differed in the number of glycosomes and acidocalcisomes, which were more abundant in the cells of the first type. During migration of the parasitophorous vacuoles to the apical part of the host cells, the quantity of the flagellates increased (Fig. 5B, C).

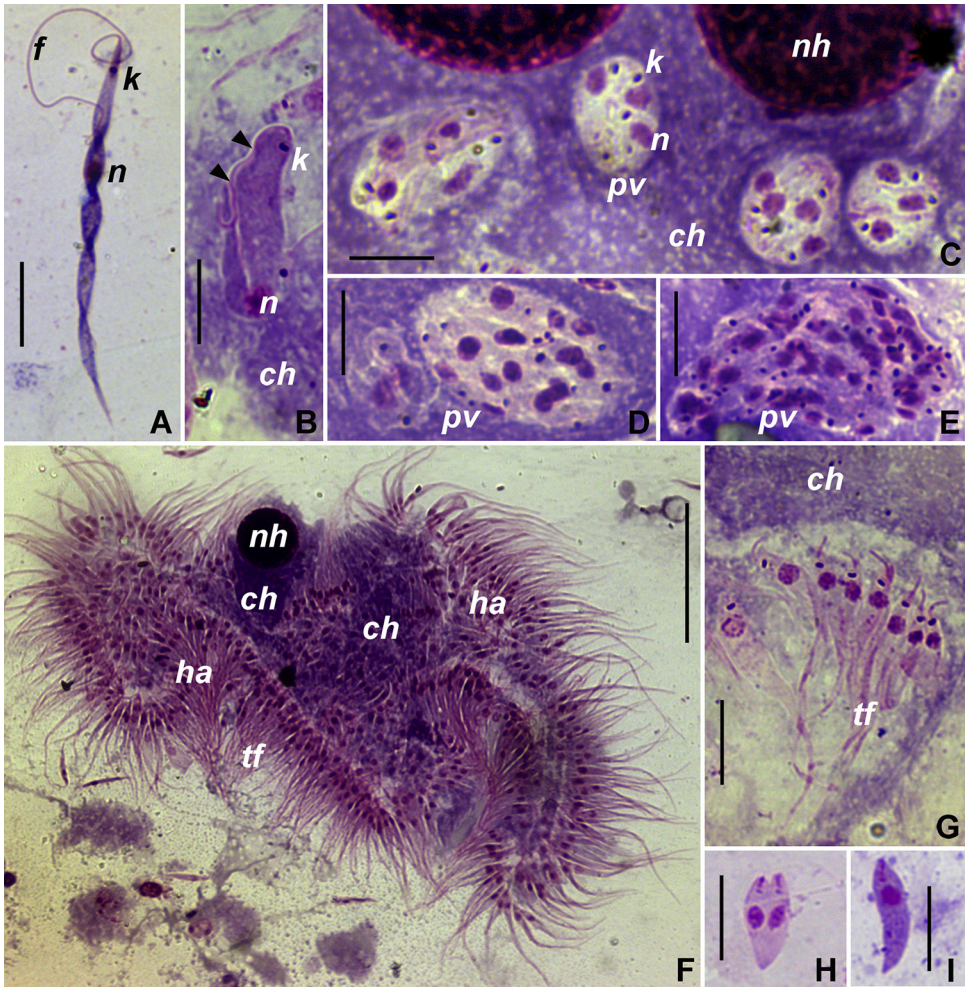

Fig. 3. *Phytomonas nordicus* in the hemolymph and salivary glands of *Troilus luridus*, light microscopy, Giemsa stain. **A**, **H**, **I**, dry smears; **B–G**, impressions of the salivary gland tissue. **A,** promastigote in the bug's hemolymph. **B**, U-shaped promastigote with the flagellum adjacent to the cell body (arrowheads) from the salivary gland epithelium. **C–E**, promastigotes during various stages of proliferation within parasitophorous vacuoles of the salivary gland epithelial cells. **F**, **G**, impressions of the inner surface of salivary gland epithelium covered by haptomonads. **H**, **I,** endomastigotes in the salivary gland lumen. **ch** – cytoplasm of the host cell (salivary gland epithelium); **f** – flagellum; **ha** – haptomonads on the surface of the salivary gland epithelium; **k** – kinetoplast; **n** – flagellate's nucleus; **nh** – nucleus of the host cell; **pv** – parasitophorous vacuole; and **tf** – tail fibers of haptomonads. Scale bars: **A–E**, **H**, and **I** – 10 μm; **F** – 30 μm; and **G** – 20 μm.

On the inner surface of the salivary glands, haptomonads were found to be attached to the microvilli of the host cells using modified flagella (Fig. 5C, D). The flagellum was bulb-shaped with a shortened axoneme and a reduced paraflagellar rod (Fig. 5D). Acidocalcisomes and glycosomes were scarce.

Small promastigotes and endomastigotes were observed in the lumen of the salivary glands (Fig. 5E). No acidocalcisomes were present in their cytoplasm. The flagellum lacking a paraflagellar rod plugged up the narrow flagellar pocket.

Discussion

Until now, the description of *P. nordicus* has remained virtually unnoticed, which is due to its dubious affiliation to the genus *Phytomonas* (Frolov and Malysheva 1993). Without a proven phytomonad origin, this species was considered nothing but one more monoxenous trypanosomatid from a true bug. The presence of long, twisted promastigotes and development in salivary glands were indeed reminiscent of *Phytomonas* spp. However, two members of the unquestionably monoxenous genus *Herpetomonas* (*H. mirabilis* and recently described *H. wanderleyi*) are characterized by the same morphology (Borghesan et al. 2013). The presence of parasite cells in salivary glands was reported for some typical monoxenous trypanosomatids and therefore does not imply a direct origin from the dixenous ancestors (Frolov et al. 2014; Nascimento et al. 2010). Sufficient data for comprehensive comparative morphological analyses of *P. nordicus* and other *Phytomonas* spp. were not available. Although phytomonads are always considered to be of great practical significance, they remain poorly studied and the overwhelming majority of known isolates remains undescribed (Jaskowska et al. 2015). Meanwhile, this genus is of great interest, not only because

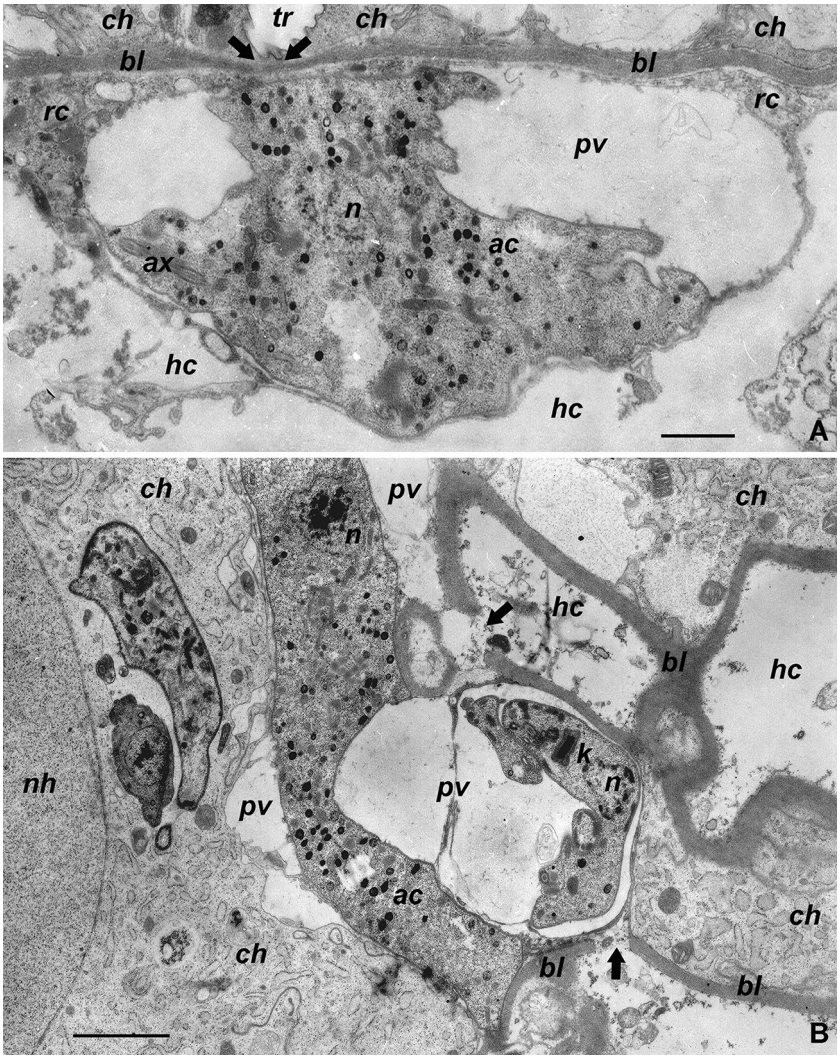

Fig. 4. Traversal of the salivary gland by *Phytomonas nordicus*. **A**, flagellates within a parasitophorous vacuole of a degrading epithelial cell. Arrows indicate the thinned basal lamina in the zone of the parasite adjoining. **B**, penetration of parasites into the cells of the salivary gland epithelium through basal lamina. Arrows show breaches of the basal lamina. **ac** – acidocalcisomes; **ax** – flagellar axoneme; **bl** – basal lamina; **ch** – cytoplasm of the host cell (salivary gland epithelium); **hc** – hemocoel; **n** – parasite's nucleus; **nh** – nucleus of the host cell; **rc** – residual cytoplasm of the host cell; and **tr** – trachea. Scale bars: **A** – 1 μm; **B** – 2 μm.

of its dixenous lifestyle but also because parasitism in plants is quite uncommon for protists (Adl et al. 2012).

The results of molecular phylogenetic analysis performed here definitely confirm that *P. nordicus* descended from phytoparasites. The most closely related isolates originated from South America (Fig. 2), whereas its bug host (*T. luridus*) is considered to be mainly Palearctic species with some records from India and Indonesia (Rider and Zheng 2002), which is not paradoxical as phytomonads have mostly been sampled in the New World thus far (Jaskowska et al. 2015). More comprehensive probing of their diversity would likely reveal even closer kins of the species under study.

Most of the known relatives of *P. nordicus* were isolated from lactiferous plants. This clade seems to be archetypal for all phytomonads. It is reasonable to suggest that this group is the least specialized and, under certain conditions, its representatives can give rise to new forms with life cycles adapted to new environments. Indeed, parasites of fruits independently emerged at least twice in the evolutionary history of the genus *Phytomonas* (Figs 1 and 2). In the case of *P. nordicus*, we speculate that at some point the infection of the predatory bug *T. luridus* did not become a deadlock for the development of a phytomonad and its life cycle changed to adjust to the new conditions. Since transmission to plants from such an insect host was no longer possible, the trypanosomatid turned to monoxeny.

In the current work, we also studied the development of *P. nordicus* in salivary glands, which is reminiscent of this parasite's dixenous ancestry. We attempted to correlate this phase of its life cycle with that of typical phytomonads. Infection with *P. nordicus* begins in the intestine of the bug host and proceeds into the hemolymph and then to

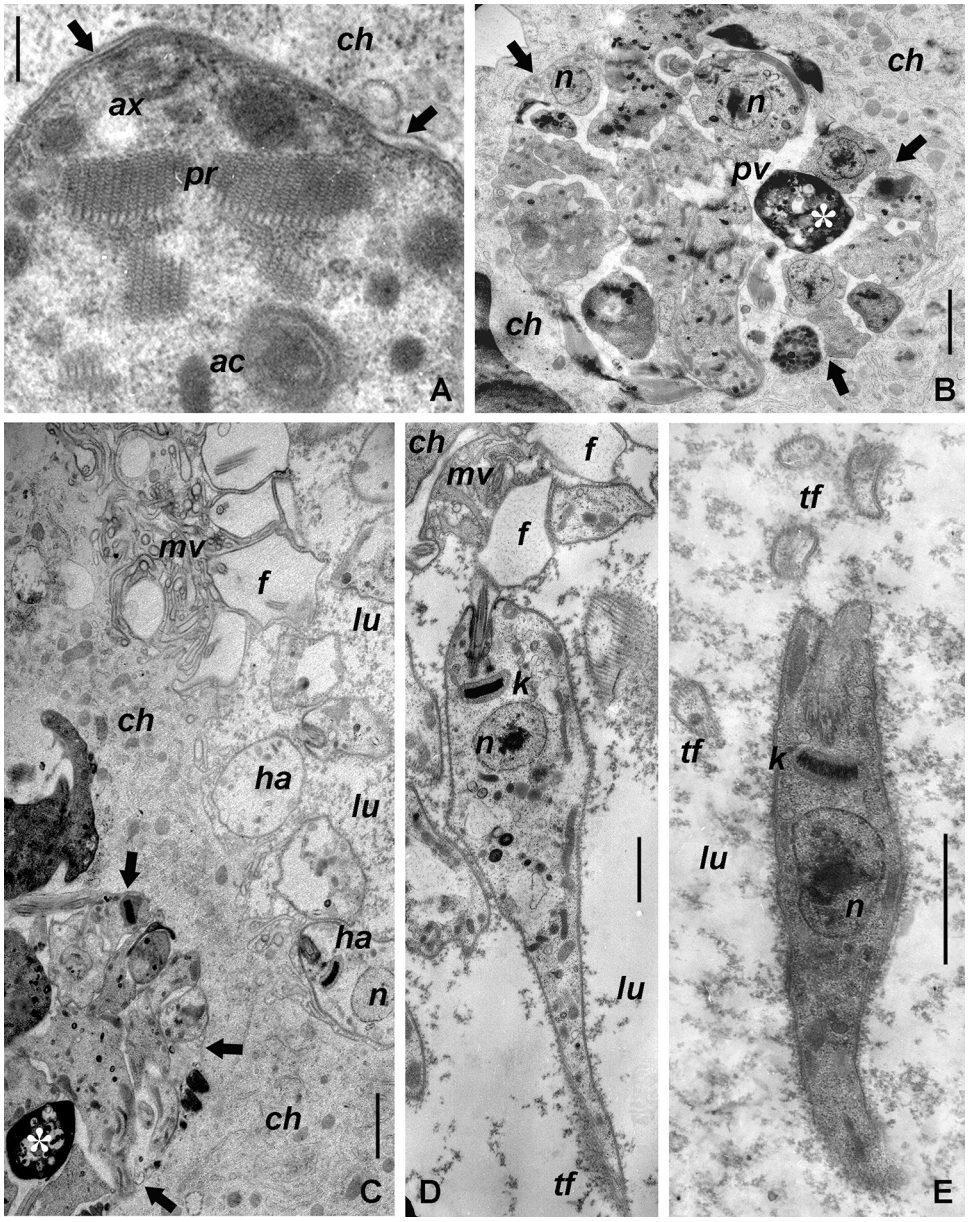

Fig. 5. Proliferation of *Phytomonas nordicus* in the salivary gland of *Troilus luridus* and formation of the infective stages. **A**, flagellate in the parasitophorous vacuole of the salivary gland cell. **B**, dividing parasites within the parasitophorous vacuole. **C**, giant parasitophorous vacuole in the apical part of the salivary gland cell with multiple promastigotes and numerous haptomonads in the lumen. **D**, haptomonad in the lumen of the salivary gland attached to an epithelial cell. **E**, endomastigote. Arrows indicate the membrane of the parasitophorous vacuole, whereas asterisks denote the residual body after division of the flagellates. **ax** – axoneme; **ac** – acidocalcisomes; **ch** – cytoplasm of the host cell; **f** – flagellum; **ha** – haptomonads on the surface of the salivary gland epithelium; **k** – kinetoplast; **lu** – lumen of the salivary gland; **mv** – microvilli of the salivary gland epithelium; **n** – parasite's nucleus; **pr** – paraflagellar rod; **pv** – parasitophorous vacuole; and **tf** – tail fibers of haptomonads. Scale bars: **A** – 0.2 μm; **B**, C – 3 μm; **D** – 2.5 μm; and **E** – 2 μm.

the salivary glands (Frolov and Malysheva 1993). We consider hemolymph as a transitional route in the course of *P. nordicus* dispersal within the host. The number of flagellates detected was significantly lower in the hemolymph than in the intestine or salivary glands, with only few of them dividing. Infection of the hemolymph is usually coupled with colonization of the salivary glands (Hecker et al. 1990; Jankevicius et al. 1989; Nascimento et al. 2010; Tobie 1965). Traversal through the salivary gland epithelium varies among different species of trypanosomatids. *Phytomonas serpens* can migrate by the intercellular space or through epithelial cells either in vacuoles or in direct contact with the host cell cytoplasm (Freymuller et al. 1990). All *P. nordicus* promastigotes crossing this tissue were detected only in vacuoles. In both species, those cells are rich in acidocalcisomes. In the gland lumen, promastigotes of *P. nordicus* are localized on the

epithelial surface using their modified flagella for attachment, whereas promastigotes of *P. serpens* are not attached. Both species undergo extensive cell proliferation resulting in the formation of infective stages in the shape of endomastigotes (Jankevicius et al. 1989).

For the dixenous trypanosomatids of genera *Trypanosoma* and *Phytomonas*, the development in salivary glands is an inherent phase of their life cycle ensuring transmission of parasites to a vertebrate or plant host, respectively (Hoare 1972; Jankevicius et al. 1989). When monoxenous trypanosomatids, such as *Blastocrithidia culicis* in the mosquito *Aedes aegypti* (Diptera: Culicidae) or *Leptomonas pyrrhocoris* in the true bug *Pyrrhocoris apterus* (Hemiptera: Pyrrhocoridae), are concerned, the biological rationale for infecting the salivary glands is not evident (Frolov et al. 2014; Nascimento et al. 2010). As for *P. nordicus*, its development in the salivary glands, accompanied by intensive multiplication and accumulation of infective endomastigotes, is necessary for the autoinvasion of bugs. When feeding on a caterpillar, *T. luridus* injects saliva with endomastigotes of *P. nordicus* into the prey. When the bug starts sucking, it retrieves the parasites, which then pass into the intestine in order to replenish the gut population. Without this process, intestinal flagellates would vanish as they do not attach to the gut walls and are constantly removed with fecal masses. *P. serpens* also produces endomastigotes in the salivary glands of the insect host, but its purpose is considered to be the establishment of a flagellate population in a tomato (Freymuller et al. 1990). Nevertheless, the possibility of autoinvasion, as in the case of *P. nordicus*, cannot be excluded, because the bug host should ingest back some of the endomastigotes during feeding. It is further justified by the fact that gut-dwelling stages of *P. serpens* are not attached to the intestinal walls as well (Jankevicius et al. 1989).

The horizontal transmission of *P. nordicus* also occurs by means of endomastigotes. However, in this case, endomastigotes are formed in the hindgut and disseminated with feces (Frolov and Malysheva 1993). The parasite can survive a winter only by persisting in hibernating adults. We observed that infected females appearing in the field after hibernation laid eggs smeared with feces. After hatching, the young nymphs of *T. luridus* soon became infected with *P. nordicus* (Frolov, unpublished). We also found that keeping infected adult bugs together with the nymphs resulted in rapid infestation of the latter (Frolov and Malysheva 1993). These facts are easily explained by the coprophagous habit of the young bugs, which is implicated in the effectiveness of the contaminative mode of transmission in trypanosomatids. Occasionally, horizontal transmission of the parasites can be achieved when two or more bugs feed simultaneously on one caterpillar. By contrast, *P. serpens* uses its plant host to infect new bugs. However, the presence of endomastigotes in the urine and feces (Jankevicius et al. 1989) indicates that the traditional mode of transmission inherited from monoxenous ancestors remains fully operational. Thus, in many aspects, the life cycle of the monoxenous *P. nordicus* still resembles that of its dixenous relatives, as represented by the tomato-parasitizing *P. serpens*. The switch from a phytophagous host to a predatory host rendered the proliferation of the parasite outside the bug impossible. Nevertheless, the developmental stages inhabiting salivary glands remained to be in high demand for other purposes (primarily for autoinvasion), thus preventing a reduction of this phase of the life cycle.

We believe that the case of *P. nordicus* demonstrates the high evolutionary plasticity of trypanosomatids. Unlike *Trypanosoma equiperdum*, which became monoxenous when it stopped to develop in the insect host and thus lost the basic part of its life cycle (Carnes et al. 2015; Lai et al. 2008), *P. nordicus* exhibits reversal to a state that appear close to the ancestral one. The data regarding the life cycle of *P. nordicus* provides insight into the biology of its dixenous relatives and the evolutionary history of the whole genus *Phytomonas*.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejop.2015.08.002.

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