Protist, Vol. 168, 71–79, xx 2017
http://www.elsevier.de/protis
Published online date 30 November 2016

Protist

ORIGINAL PAPER

# Description of *Phytomonas oxycareni* n. sp. from the Salivary Glands of *Oxycarenus lavaterae*

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Submitted May 20, 2016; Accepted November 12, 2016
Monitoring Editor: Dmitri Maslov

***Phytomonas*** **spp. (phytomonads) are a diverse and globally distributed group of unicellular eukaryotes that parasitize a wide range of plants and are transmitted by insect hosts. Here we report the discovery and characterisation of a new species of *Phytomonas*, named *Phytomonas oxycareni* n. sp., which was obtained from the salivary glands of the invasive species of true bug *Oxycarenus lavaterae* (Heteroptera). The new *Phytomonas* species exhibits a long slender promastigote morphology and can be found both within the lumen of the insect host's salivary glands as well as within the cells of the salivary gland itself. Sampling multiple individuals from the same population post-winter hibernation on two consecutive years revealed that infection was persistent over time. Finally, phylogenetic analyses of small subunit ribosomal RNA genes revealed that this species is sister to other species within the genus *Phytomonas*, providing new insight into the evolutionary history of the clade.**


**Key words:** Kinetoplastid; *Phytomonas*; phylogeny; intracellular; vector; trypanosomatid.

## Introduction

Trypanosomatids are single-celled eukaryotic parasites that collectively cause a large burden on human health and livelihood, infecting an estimated 20 million people worldwide as well as livestock and crops (da Silva et al. 2013). One large and diverse sub-group of trypanosomatids known as *Phytomonas* (Donovan, 1909), are parasites and pathogens of plants (Camargo 1999; Jaskowska et al. 2015). *Phytomonas* are globally distributed, however little is known of their biology, host range or evolutionary history and comparatively limited sampling has been conducted outside of South America, where several species cause economically important plant pathologies (Jaskowska et al. 2015; Votýpka et al. 2010).

Species in the genus *Phytomonas* are descended from a single adaptation of monoxe-

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http://dx.doi.org/10.1016/j.protis.2016.11.002
1434-4610/

nous insect parasites to a plant host about 400 million years ago (Lukeš et al. 2014). Following this event different species have evolved to colonise a large diversity of plant species and can be found in multiple plant tissues including phloem, latex ducts, fruit, flowers and seeds as reviewed in (Jaskowska et al. 2015). In doing so they have evolved to inhabit both extracellular and intracellular plant environments, spanning a wide range of contrasting biochemical compositions. *Phytomonas* are transmitted between plant hosts by insect vectors of the suborder Heteroptera (order Hemiptera) and there is evidence that *Phytomonas nordicus,* a parasite of the predatory stink bug *Troilus luridus* Fabricius, 1775 (Pentatomidae), has reverted back to a monoxenous lifestyle, completing its entire life cycle in the insect host (Frolov et al. 2016). Consistent with their colonisation of plants, *Phytomonas* have also been found to inhabit both extracellular and intracellular environments within their insect hosts (Freymuller et al. 1990; Frolov et al. 2016). Thus, given this wide range of contrasting host tissues and cellular environments, it is likely that there is a large diversity of life cycles and transmission strategies that remain unexplored in this group.

Though the genus *Phytomonas* encompasses the majority of plant-infecting trypanosomatids, the diversity of these protists is not accurately represented in the literature. To improve this the creation of the subfamily Phytomonadinae has been proposed to include the genera *Herpetomonas, Phytomonas* and *Lafontella* (Yurchenko et al. 2015). Historically, species classification of insect and plant trypanosomatids depended on morphology and host specificity (Vickerman and Preston 1976). However, these criteria are not sufficient for species descriptions as opportunistic non-*Phytomonas* trypanosomatids have been found in plants and there is extensive size and shape polymorphism within a single species depending on both host and culture conditions (Catarino et al. 2001; Jaskowska et al. 2015; Wheeler et al. 2011). Thus as there is potential uncertainty in using a morphotype-orientated approach, recent classifications rely on comparatively data-rich molecular methods for taxonomic assignment (Votýpka et al. 2015).

In this paper we characterize a new species, *Phytomonas oxycareni* n. sp., obtained from the salivary gland of the heteropteran insect *Oxycarenus lavaterae* Fabricius, 1787. This true bug is native to the Western Mediterranean, however its range in Europe has expanded both eastwards and westwards since the 1980s (Nedvěd et al. 2014). This finding highlights the potential for the migration of herbivorous insects to be associated with the migration of associated *Phytomonas* parasites. *O. lavaterae* feeds primarily on plants in the Malvaceae family, including both herbaceous representatives of the subfamily Malvoideae (e.g., *Abelmoschus*, *Abutilon*, *Gossypium*, *Hibiscus*, *Lavatera*, *Malva*) as well as lime trees (Tilioideae: *Tilia* spp.). However, in the Mediterranean it may also feed on other plants (e.g. apricots, peaches, *Citrus* spp.) as reviewed in (Kment et al. 2006). Though the plants it feeds on include several important crops (cotton, okra, apricots, peaches) and ornamentals (hibiscus, lime trees) it is rarely reported as an agricultural pest. During the winter, the species hibernates by forming tight aggregations of several hundred individuals on the sunny side of lime tree trunks. These aggregations also occasionally form on other structures such as buildings or fences, causing a public nuisance (Nedvěd et al. 2014).

Based on phylogenetic data we classified the new parasite as *Phytomonas* and propose the species name *oxycareni* to reflect its insect host.

## Results

### Material Collection and Primary Characterisation of a New Trypanosomatid Species from the True Bug *O. lavaterae*

*Oxycarenus lavaterae* bugs were sampled from a single large population overwintering on the trunk of a *Tilia cordata* tree in May 2015 and March 2016 in Sedlec, Czech Republic. Individual insects within this population were dissected and examined by light microscopy to search for the presence of trypanosomatid cells. In total, trypanosomatids were found in ~80% of the salivary glands that were examined. Trypanosomatid cells failed to be detected consistently in any other tissue type that was dissected from the insect. However, trypanosomatid cells were detected in the mid-gut in a small number of dissections but not in sufficient quantities to facilitate further analysis. This very low level of detectable presence in the mid-gut could be due to the life cycle of *O. lavaterae* bugs, which at the time of collection were still aggregated on the trunk of the tree and so had not fed in many months (Nedvěd et al. 2014). Unfortunately, despite repeated attempts using different media, a culture was not established. Thus all analyses reported here were conducted using trypanosomatids isolated directly from the salivary glands of the insects.

## Phylogenetic Analysis Places the New Trypanosomatid Species in the Genus *Phytomonas*

Identical 18S rRNA gene sequences were obtained from trypanosomatids that originated from three infected *O. lavaterae* bugs. These bugs were collected from the same population sampled in two subsequent years. This indicates that infection by the same species of trypanosomatid was pervasive within the population and that infection was likely maintained within the population over multiple generations of the host insect. The alignable region of the 18S rRNA gene of the new trypanosomatid (GenBank Acc. Number KX257483) shared 96% identity with both the 18S sequence of *Phytomonas serpens* (GenBank acc. Number U39577, PRJNA80957) and *P. nordicus* (GenBank Acc. No. KT2236609) (Frolov et al. 2016).

To confirm the taxonomic classification of the new species, a phylogenetic tree of 18S sequences was reconstructed using the new species as well as 40 published species sampled from across the trypanosomatids. The topology of the resultant maximum likelihood tree (Fig. 1) showed that the newly identified trypanosomatid species was monophyletic with previously characterised *Phytomonas* and thus part of the newly proposed subfamily, Phytomonadinae (Yurchenko et al. 2015). This grouping received 100% bootstrap support and a posterior probability of 1.

To independently verify the phylogenetic position of the new species, the spliced leader (SL) RNA gene from a range of representative trypanosomatid species were compared (Supplementary Material Fig. S1). In support of the 18S rRNA analysis, the SL sequence showed that the newly identified trypanosomatid species had 90% bootstrap support and a posterior probability of 0.99 supporting its position as a sister species to previously characterised *Phytomonas* species. The exon of the SL sequence of the new trypanosomatid species differs from previously characterised *Phytomonas* sequences by only one nucleotide and has cytosine at position 15 of the sequence, as is the case for other *Phytomonas* species. Therefore, given the new species forms a monophyletic group with the genus *Phytomonas*, shares features such as a prolonged cell morphology and typical tissue localization (salivary glands) with other *Phytomonas* spp., and is found in a typical insect host for *Phytomonas*, we have assigned the new species to the genus *Phytomonas*. Though it is impossible to determine, this may possibly be the same flagellate that was observed previously in *Oxycarenus*

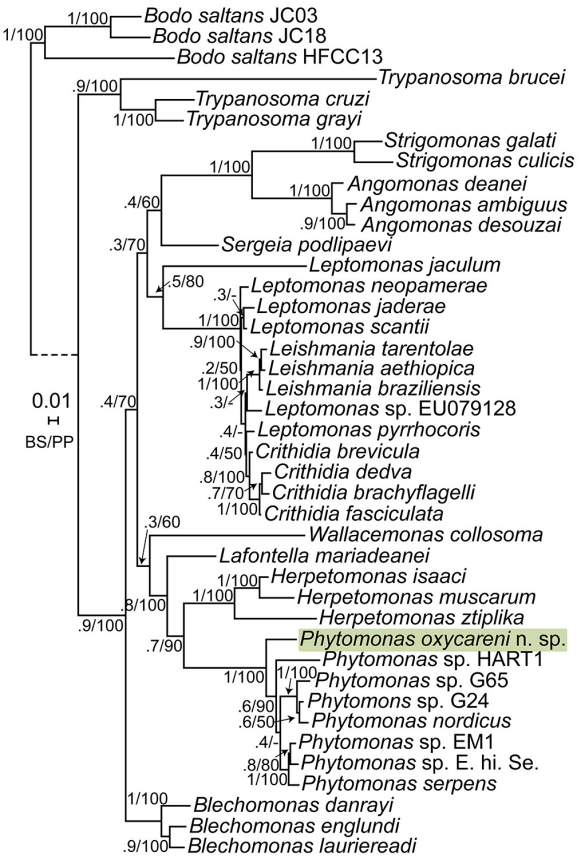


**Figure 1.** The new kinetoplastid parasite belongs to the subfamily Phytomonadinae. Maximum likelihood phylogenetic tree of kinetoplastids obtained using RaxML on 18S ribosomal RNA gene sequences. The tree is rooted on the branch that separates *Bodo saltans* isolates and trypanosomatids. The scale bar represents the number of substitutions per site. Values on each branch represent bootstrap values/posterior probabilities for that branch. Posterior probabilities were calculated using Mr Bayes. For display purposes the dashed branch has been reduced in length by 75%. The new species described in this study (*Phytomonas oxycareni* n. sp.) is highlighted.

*lavaterae* in Italy (Franchini 1922). Thus, based on the phylogenetic information, host and tissue localisation, and its cell morphology (described below), we have named the new species *Phytomonas oxycareni* n. sp.

## Parasites Within the Salivary Gland Exhibit Promastigote Morphology and are Proliferative

Light microscopic examination of *P. oxycareni* cells isolated from salivary glands revealed slender

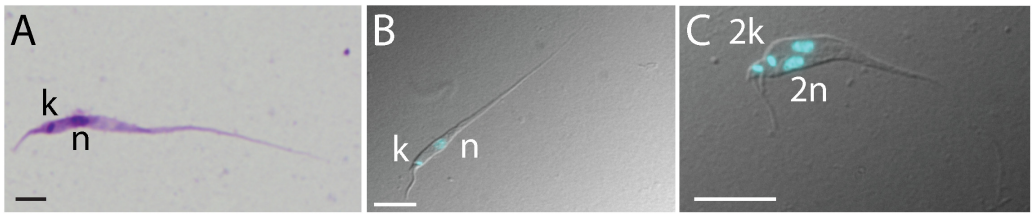


**Figure 2.** Light microscopy images of *Phytomonas oxycareni* n. sp. **A)** A Giemsa-stained cell. **B-C)** DAPI-stained cells displayed as the differential interference contrast image of the cell overlaid with the (blue) fluorescence microscopy image of the DNA stained with DAPI. **B)** Single cell with elongated slender morphology **C)** Cells in the process of dividing (2k 2n). Scale bars are 5 μm. Cells are oriented with the flagellum on the left of each image. "k" denotes the kinetoplast, "n" denotes the nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

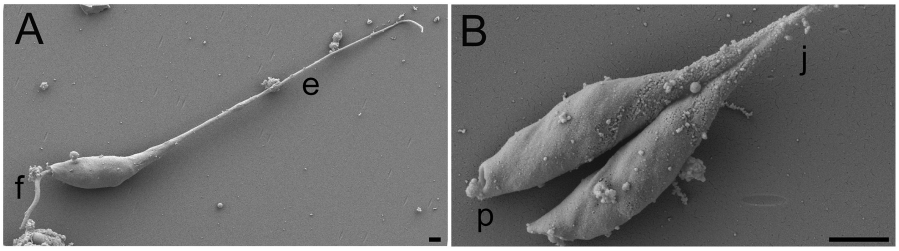


**Figure 3.** Scanning electron microscopy of *Phytomonas oxycareni* n. sp. Cells were obtained directly from infected salivary glands of *Oxycarenus lavaterae*, thus images contain particulate material derived from the salivary gland. **A)** Elongated slender promastigote with flagellum [f] oriented on the left of the image. [e] highlights the elongated cell body **B)** Dividing cells with no external flagella. [p] denotes the flagellar pocket. [j] shows where the cells are still joined. Scale bars are 1 μm.

promastigotes with an elongated cell body and short flagellum that is detached from the body of the cell (Fig. 2A-B). This morphology is similar to that of the corresponding stages observed for promastigotes of the monoxenous species *P. nordicus* (Frolov et al. 2016), and is broadly consistent with several previous morphological descriptions within this genus (Camargo 1999; Jaskowska et al. 2015; Wheeler et al. 2011). Analysis of DAPI stained slides revealed that cells within the population were undergoing cell division. That is, several cells were identified that had already completed DNA replication and segregation of the nuclei and kinetoplasts but had not yet undergone cytokinesis (Fig. 2C).

Higher resolution imaging of cells by scanning electron microscopy (SEM) revealed the full extent of the long slender cell body (Fig. 3A). Although SEM cannot image nuclei and kinetoplasts, cells in late stage cytokinesis were identified as they were still joined at the posterior end (Fig. 3B). The observed lack of external flagella (Fig. 3B) is likely due to the point in cell division captured in this image rather than a diagnostic characteristic of the species (Wheeler et al. 2011).

## *Phytomonas oxycareni* Can be Found Inside Host Cells of the Salivary Gland

Transmission electron microscopy analysis of whole fixed salivary glands readily identified cells within the lumen of the gland (Fig. 4). The parasite cells did not appear to be attached to the surface of the lumen but were instead distributed throughout (Fig. 4A–C). There were also cells that appeared to be undergoing cytokinesis within these sections as indicated by the presence of two axonemal profiles within the same cell (Fig. 4B). Given that two different species of *Phytomonas* have previously been found to also reside within cells of the insect host (Freymuller et al. 1990; Frolov et al. 2016), the cells of the salivary gland tissue were inspected for the presence of *Phytomonas* cells. Indeed, *Phytomonas* flagellates located inside the cells of the salivary gland lumen were identified in multiple instances (Fig. 5). In all cases that were examined, the protists were determined to be inside two distinct membranes (Fig. 5A–B). This indicates that the parasite resided inside a vacuole. It was noteworthy that some intracellular *Phytomonas* cells

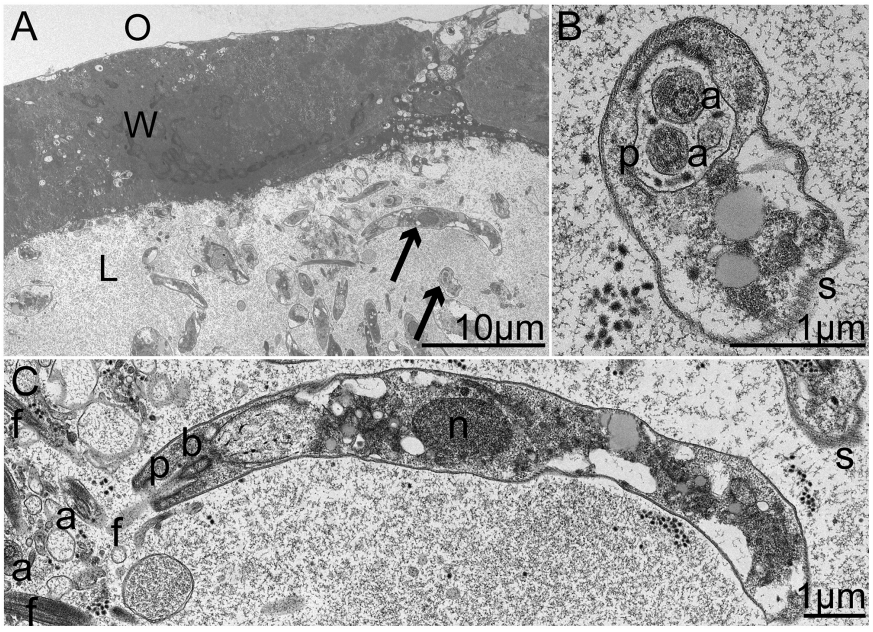


**Figure 4.** Transmission electron microscopy of an infected salivary gland containing *Phytomonas oxycareni* n. sp. **A)** A low magnification image of a section of the gland and the encompassed lumen of an infected *Oxycarenus lavaterae* salivary gland. (O) is outside the gland, (W) is the tissue of salivary gland (L) is the salivary gland lumen. Multiple parasites are visible in the salivary gland lumen and do not appear attached to the epithelium of the salivary gland. **B, C)** High magnification of the parasites highlighted with black arrows in (A) [n] nucleus. [s] subpellicular microtubules. [f] flagellum. [b] basal body. [p] flagellar pocket.

appeared to be in the process of cytokinesis within these vacuoles (Fig. 5C–D). This intracellular proliferation of *Phytomonas* would be consistent with observations of dividing *P. nordicus* within the parasitophorous vacuole of a salivary gland cell (Frolov et al. 2016). However, it is unknown whether this process was initiated before or after the parasite entered the host cell, so it is unknown whether *P. oxycareni* is capable of proliferation within the insect host cells.

## Taxonomic Summary

Class: Kinetoplastea (Honigberg, 1963) Vickerman, 1976

Subclass: Metakinetoplastina Vickerman, 2004

Order: Trypanosomatida (Kent, 1880) Hollande, 1952

Family: Trypanosomatidae (Doflein, 1901) Grobben, 1905

Subfamily: Phytomonadinae Yurchenko, Kostygov, Votypka et Lukes, 2015

Genus: *Phytomonas* Donovan, 1909

## *Phytomonas oxycareni* Votypka, Seward, Kment, Kelly et Lukes n. sp.

### Diagnosis

The species is identified by the unique sequence of 18S rRNA (GenBank accession number: KX257483) and splice leader RNA (GenBank accession number KX611848).

### Species description

Elongated slender promastigotes in the salivary glands were on average 30.9 ± 8.6 µm (19.7–52.7 µm) long and 1.76 ± 0.62 µm (0.93–2.99 µm) wide, with a single short external flagellum that was on average 3.7 ± 1.2 µm (2.2–6.4 µm). Cells had a dilated anterior portion (measuring 13-52% of the total body length) that contains the nucleus and kinetoplast. The posterior part of the cell is narrowed and pointed at the end. The cell body is twisted 0-2 times. The kinetoplast disk is compactly packed, on average 0.78 ± 0.17 µm (0.54–0.93 µm) in length and 0.165 µm (0.165–0.166 µm) in diameter and 0.68 ± 0.08 µm (0.58–0.83 µm) from the anterior of the cell. The nucleus was on average 2.15 ± 0.62 µm (1.34–2.88 µm) in length and

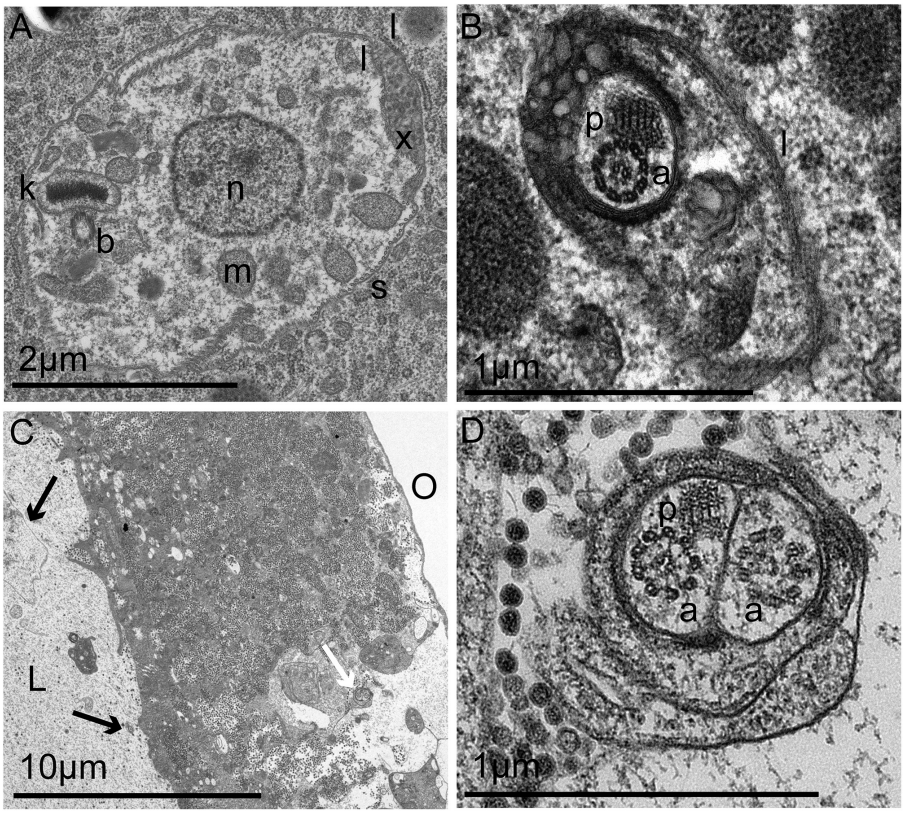


**Figure 5.** Transmission electron micrographs of three intracellular *Phytomonas oxycareni* n. sp. within the salivary glands of the insect host *Oxycarenus lavaterae*. **A)** An intracellular *Phytomonas oxycareni* [k] kinetoplast [n] nucleus [s] subpellicular microtubules adjacent to two membranes. [b] basal body, [l] two lipid membranes, [x] granular material in the posterior part of the parasitophorous vacuole, [m] mitochondria. **B)** A second intracellular *P. oxycareni* [a] axoneme. [p] paraflagellar rod (PFR) with characteristic lattice [l] two membranes indicating that the parasite is inside a vacuole. **C)** A third intracellular *P. oxycareni* (white arrow) near the outside (O) of the salivary gland, multiple parasites (black arrows) are visible in the salivary gland lumen [L]. **D)** High magnification of the cell in (C): [a] axoneme, [p] PFR, [s] subpellicular microtubules. This is likely to be a cell in the process of cytokinesis as indicated by the presence of two axonemal profiles.

$3.92 \pm 1.06\,\mu m$ (2.19–5.00 $\mu$m) from the anterior of the cell.

## Type host

*Oxycarenus lavaterae* (Fabricius, 1787) (Heteroptera: Oxycarenidae). The xenotype, collected on *Tilia cordata* (Malvaceae), is deposited at the Department of Parasitology, Charles University, Prague.

## Location within host

Found both within the midgut and lumen of the salivary gland, as well as within the cells of the salivary gland itself.

## Type locality

Vicinity of Sedlec, Czech Republic, South Moravia (48°46′44.43″N; 16°41′55.13″E), 180 meters above sea level.

## Type material

The name-bearing type, a hapantotype, is a Giemsa-stained slide of the dissected salivary glands, deposited in the research collection of the Department of Parasitology at Charles University in Prague. An axenic culture was not established.

## Etymology

The specific epithet, *oxycareni*, is derived from the generic name of its host; noun in genitive case given in apposition.

## Discussion

We report the identification of a new species of *Phytomonas*, named *Phytomonas oxycareni* that was found in the salivary glands of the heteropteran insect *Oxycarenus lavaterae*. The new species exhibits a long slender promastigote morphology and can be found both within the lumen of the salivary glands, as well as within the cells of the salivary gland itself. Furthermore, sampling different individuals from the same bug population in two consecutive years revealed that infection was likely persistent through several generations of bugs and that the protists overwinter in the insect host/vector.

Interestingly, this newly characterised species is the earliest branching *Phytomonas* species that has been identified to date. However, we were unable to confirm the presence of this species, either by cultivation or by PCR of homogenized leaves and fine branches, within the *Tilia* spp. on which the insect was found (data not shown). Therefore we could not ascertain whether it is dixenous or monoxenous, like the recently discovered *P. nordicus* (Frolov et al. 2016). While *P. nordicus* only parasitizes insect hosts and is spread between hosts via contaminated faeces and autoinfection rather than plants, *O. lavaterae* feeds primarily by drawing fluid from plant tissues such as leaves and seeds of plants in the Malvaceae family. Thus, while it is possible that *P. oxycareni* is directly transmitted between insect hosts through contact with contaminated faecal matter, it is more parsimonious to assume that it is dixenous and is transmitted between insect hosts via a plant infective stage. In this context it is interesting to note that *P. oxycareni* does not appear to attach to the epithelia of the salivary gland like the monoxenous *P. nordicus* (Frolov et al. 2016), but can be found throughout the lumen of the gland like the promastigotes of the dixenous *P. serpens* which infects tomato plants (Jankevicius et al. 1989). However, irrespective of the confirmation of this lifestyle habit, the discovery that an early diverging species of *Phytomonas* inhabits the cells of the salivary gland suggests that this ability to survive inside host cells is ancestral and may be widespread within the genus.

Of note is the difficulty to detect parasites in the mid-gut of the insect host. As described above, this could be due to the life cycle of *O. lavaterae*. These insects form large overwintering aggregations on the trunks and branches of *Tilia* trees during which time they presumably do not feed (Nedvěd et al. 2014). The individuals from these aggregations begin to disperse in spring, coincident with the flowering of lindens, and they then feed on a range of plants in the Malvaceae (Nedvěd et al. 2014). Therefore it may not be the case that the putative plant host of the newly described parasite is the same species as the plant on which the insect host hibernates. Given the range of plant species that *O. lavaterae* can feed on (Kalushkov and Nedvěd 2010), and the difficulty of obtaining *Phytomonas* parasites from plant tissue (Jaskowska et al. 2015), it may be difficult to ascertain the true plant host range of this species.

In summary the work presented here identifies a new species within the *Phytomonas* clade that is a sister species to all currently sequenced phytomonad species and thus provides new insight into the ancestral biology of the genus (i.e. long slender cell morphology, host cell invasion). Furthermore identification of parasites associated with herbivorous insects that are migrating through Europe highlights the need to focus not only on the movement of insects but also on the presence of associated parasites.

## Methods

**Collection and dissection of bug hosts:** Several hundred individuals of *Oxycarenus lavaterae* were collected from the trunks of several lime trees (*Tilia cordata*, and to a lesser extent *T. platyphyllos*) in the South Moravian region of the Czech Republic in May 2015 and March 2016. *O. lavaterae* remains aggregated on the trunks of trees until late May when the trees begin to flower, this facilitated ease of collection. Population infection rate varied but one bug population in particular, near Sedlec (Mikulov vicinity; 48°46′46.157″N, 16°41′54.389″E, 180 m a.s.l.) was >80% positive for parasites. This host population is the focus of this paper. The insects were euthanized in 70% ethanol, washed in 96% ethanol and a saline solution, and dissected in a saline solution to isolate the salivary glands.

**Cultivation and light microscopy:** Smears of infected salivary glands were fixed with methanol, hydrolysed in 5N HCl for 15 minutes at room temperature and stained with either Giemsa or 4′,6-diamidino-2-pheylindole (DAPI) as has been described previously (Yurchenko et al. 2006). These stained slides were then visually inspected on a fluorescence microscope. Several attempts were made to cultivate *P. oxycareni* in different media with or without antibiotics (Amikacin) including blood agar medium, RPMI, M199, Schneider Medium, BHI Medium supplemented with FCS and Hemin, Warren's Medium, and the mix of these media (RPMI, M199, Schneider Medium and BHI Medium in a 1:1:1:1 ratio, supplemented with FCS). Although the parasites survived for several days in these media, there was no sign of growth and so no culture was obtained. The infected salivary glands were not composed of mixed infections as the same 18S rRNA and SL RNA gene amplicons were cloned from several independent insects and localities (data not shown).

**Transmission and scanning electron microscopy:** Dissected salivary glands were resuspended in 0.1 M phosphate-buffered saline, fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 hr at 4 °C and processed for scanning and transmission electron microscopy as

described previously (Yurchenko et al. 2006). Ultrathin sections were analysed and imaged with a FEI Tecnai 12 microscope at 120 kV.

**PCR amplification, cloning, and sequencing:** Total genomic DNA was isolated from the field samples using a DNA isolation kit for cells and tissues (Roche) according to the manufacturer's protocol. Small subunit rRNA gene (SSU rDNA, ~2100 bp) was PCR amplified using the primers S762 (5′-GAC TTT TGC TTC CTC TAD TG-3′)/S763 (5′-CAT ATG CTT GTT TCA AGG AC-3′) (Maslov et al. 1996). The PCR thermocycler settings for DNA amplification were: denaturing at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 90 s, 72 °C for 90 s, and a final elongation at 72 °C for 5 min. For the second round of PCR, 1 μL of the previous reaction was added to 24 μL of a PCR reaction and amplified using the primers TRnSSU-F2 (5′-GAR TCT GCG CAT GGC TCA TTA CAT CAG A-3′) and TRnSSU-R2 (5′-CRC AGT TTG ATG AGC TGC GCC T-3′). The thermocycler settings were: denaturing at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 64 °C for 90 s, 72 °C for 90 s, and a final elongation at 72 °C for 5 min. The amplified DNA sequences were subject to sequencing and the new sequence deposited in GenBank acc. no. KX257483 (18S rRNA).

The splice leader RNA gene was PCR amplified using the primers M167 (5′-GGG AAG CTT CTG ATT GGT TAC TWT A-3′)/M168 (5′-GGG AAT TCA ATA AAG TAC AGA AAC TG-3′) (Westenberger et al. 2004). Amplicons were cloned into the pGEM-T Easy (Promega, Madison, USA) vector system and subject to sequencing. The new sequence was deposited in GenBank acc. no. KX611848 (Spliced leader, SL).

**Phylogenetic analyses:** The 18S rRNA sequences of seven different isolates of *Phytomonas* spp. and 33 insect trypanosomatid species were retrieved from GenBank (Supplementary Material Table S1) and aligned using MAFFT v7.058b (Katoh and Standley 2013). Default parameters were used and the number of iteration steps capped at 1000. The resulting alignment was refined manually using Cinema5 multiple sequence alignment software (Lord et al. 2002) to trim the alignment file to start and end in line with the two ends of the *P. oxycareni* sequence (ie. the final dataset contained 2287 columns covering the full 1926 nucleotide sequence for *P. oxycareni*). Maximum likelihood-based phylogenetic inference was performed in RAxML version 8.2.4 using default parameters and the GTRGAMMA model with 1000 bootstrap replicates (Stamatakis 2006).

To provide additional phylogenetic support, the same sequences were analysed using Mr Bayes (Ronquist et al. 2012). The evolutionary model used was GTR with gamma-distributed rate variation across sites and a proportion of invariable sites. The covarion-like model was used and two runs, each of four chains were initiated and allowed to run for 500,000 generations sampling every 1,000 generations. Convergence was assessed through visual inspection of log-likelihood traces and through analysis of the standard deviation of split frequencies. The analysis had reached stationary phase after 5,000 generations and so this analysis was run for ample time. All other parameters used were the default.

Finally to support the position of the new species as being a sister to all other *Phytomonas* spp., an approximately unbiased (AU) test was performed. Tree 1 (Supplementary Material Fig. S2) was the RAxML tree described above (Fig. 1). Trees 2-4 were identical to Tree 1 apart from the position of *Phytomonas oxycareni* n. sp. within the *Phytomonas* genus (Supplementary Material Fig. S2). The p-value of the AU test for tree 1 was 0.92 compared to <0.13 for trees 2-4, indicating that tree 1 has the greatest probability of being the true tree (Shimodaira 2002).

Additional spliced leader sequences were downloaded from NCBI and aligned as above. Bootstrap support and posterior probabilities were calculated as above. SH values were calculated using RAxML (as above) on the maximum likelihood tree.

## Acknowledgements

EAS is supported by a BBSRC studentship through BB/J014427/1 and a Junior Research Fellowship from the BSPP. SK is a Royal Society University Research Fellow. This work was partially funded by Czech Grant Agency (14-23986S) to JL and by the Ministry of Culture of the Czech Republic (DKRVO 2016/14, National Museum, 00023272) to PK, and by a project from the Czech Ministry of Education (Czech-BioImaging LM2015062 and COST-CZ LD14076). The authors would like to thank Mr and Mrs Kment for hosting EAS, JV and PK during the field expeditions. Finally we would like to thank the anonymous reviewers for their comments and corrections which have improved the manuscript.

## 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.protis.2016.11.002.

## References

**Camargo EP** (1999) *Phytomonas* and other trypanosomatid parasites of plants and fruit. Adv Parasitol **42**:29–112

**Catarino LM, Serrano MG, Cavazzana M, Almeida ML, Kaneshina EK, Campaner M, Jankevicius JV, Teixeira MMG, Itow-Jankevicius S** (2001) Classification of trypanosomatids from fruits and seeds using morphological, biochemical and molecular markers revealed several genera among fruit isolates. FEMS Microbiol Lett **201**:65–72

**Donovan C** (1909) Kala-azar in Madras, especially with regard to its connexion with the dog and the bug (*Conorrhinus*). The Lancet **174**:1495–1496

**Franchini G** (1922) Sur un flagellé de lygoeide (*Crithidia oxycareni* n. sp.). Bulletin de la Socié té de Pathologie Exotique **15**:113–116

**Freymuller E, Milder R, Jankevicius JV, Jankevicius SI, Camargo EP** (1990) Ultrastructural studies on the trypanosomatid *Phytomonas serpens* in the salivary glands of a phytophagous hemipteran. J Protozool **37**:225–229

**Frolov AO, Malysheva MN, Yurchenko V, Kostygov AY** (2016) Back to monoxeny: *Phytomonas nordicus* descended from dixenous plant parasites. Europ J Protistol **52**:1–10

**Jankevicius JV, Jankevicius SI, Campaner M, Conchon I, Maeda LA, Teixiera MMC, Freymuller E, Camargo EP** (1989)

Life cycle and culturing of *Phytomonas serpens* (Gibbs), a trypanosomatid parasite of tomatoes. J Protozool **36**:265–271

**Jaskowska E, Butler C, Preston G, Kelly S** (2015) *Phytomonas*: Trypanosomatids adapted to plant environments. PLOS Pathog **11**:e1004484

**Kalushkov P, Nedvěd O** (2010) Suitability of food plants for *Oxycarenus lavaterae* (Heteroptera: Lygaeidae), a Mediterranean bug invasive in Central and South-East Europe. Comptes Rendus l Acad Bulg des Sci **63**:271–276

**Katoh K, Standley DM** (2013) MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol Biol Evol **30**:772–780

**Kment P, Vahala O, Hradil K** (2006) First records of *Oxycarenus lavaterae* (Heteroptera: Oxycarenidae) from the Czech Republic, with review of its distribution and biology. Klapalekiana **42**:97–127

**Lord PW, Selley JN, Attwood TK** (2002) CINEMA-MX: a modular multiple alignment editor. Bioinformatics **18**:1402–1403

**Lukeš J, Skalický T, Týč J, Votýpka J, Yurchenko V** (2014) Evolution of parasitism in kinetoplastid flagellates. Mol Biochem Parasitol **195**:115–122

**Maslov D, Lukes J, Jirku M, 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 Parasitol **75**:197–205

**Nedvěd O, Chehlarov E, Kalushkov P** (2014) Life history of the invasive bug *Oxycarenus lavaterae* (Heteroptera: Oxycarenidae) in Bulgaria. Acta Zool Bulg **66**:203–208

**Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP** (2012) Mrbayes 3. 2: Efficient bayesian phylogenetic inference and model choice across a large model space. Syst Biol **61**:539–542

**Shimodaira H** (2002) An approximately unbiased test of phylogenetic tree selection. Syst Biol **51**:492–508

**da Silva RV, Malvezi AD, Augusto LDS, Kian D, Tatakihara VLH, Yamauchi LM, Yamada-Ogatta SF, Rizzo LV, Schenkman S, Pinge-Filho P** (2013) Oral exposure to *Phytomonas serpens* attenuates thrombocytopenia and leukopenia during acute infection with *Trypanosoma cruzi*. PLoS ONE **8**:e68299

**Stamatakis A** (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics **22**:2688–2690

**Vickerman K, Preston TM** (1976) Comparative cell biology of the kinetoplastid flagellates. Biol Kinetoplastida **1**:35–130

**Votýpka J, d'Avila-Levy CM, Grellier P, Maslov DA, Lukeš J, Yurchenko V** (2015) New approaches to systematics of Trypanosomatidae: Criteria for taxonomic (re)description. Trends Parasitol **31**:460–469

**Votýpka J, Maslov DA, Yurchenko V, Jirků M, Kment P, Lun ZR, Lukeš J** (2010) Probing into the diversity of trypanosomatid flagellates parasitizing insect hosts in South-West China reveals both endemism and global dispersal. Mol Phylogenet Evol **54**:243–253

**Westenberger SJ, Sturm NR, Yanega D, Podlipaev SA, Zeledon R, Campbell DA, Maslov DA** (2004) Trypanosomatid biodiversity in Costa Rica: genotyping of parasites from Heteroptera using the spliced leader RNA gene. Parasitology **129**:537–547

**Wheeler RJ, Gluenz E, Gull K** (2011) The cell cycle of *Leishmania*: Morphogenetic events and their implications for parasite biology. Mol Microbiol **79**:647–662

**Yurchenko V, Kostygov A, Havlová J, Grybchuk-Ieremenko A, Ševčíková T, Lukeš J, Ševčík J, Votýpka J** (2015) Diversity of trypanosomatids in cockroaches and the description of *Herpetomonas tarakana* sp. n. J Eukaryot Microbiol **63**:198–209

**Yurchenko VY, Lukes J, Jirku M, Zeledon R, Maslov DA** (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