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Protistology

ORIGINAL ARTICLE

Host specificity of *Phytomonas serpens* (Kinetoplastea: Trypanosomatida) to experimental vectors from two families of the true bugs, Pentatomidae and Coreidae

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Summary

The possibility of development of dixenous trypanosomatids *Phytomonas serpens* in the southern green shield bug *Nezara viridula* (fam. Pentatomidae) and the dock bug *Coreus marginatus* (fam. Coreidae) was studied experimentally. A complete development of the parasite, resulting in the formation of promastigotes and endomastigotes, was observed only in *C. marginatus*. We also showed that the flagellates developing in the salivary glands of the dock bugs could infect tomato fruits. Our results and the literature data indicate that the bugs from the family Coreidae are the specific vectors of *P. serpens*. The coreid bugs can apparently play a decisive role in the global distribution of these parasites.

Key words: Coreidae, experimental infection, host specificity, Pentatomidae, *Phytomonas serpens*

Introduction

Trypanosomatids of the genus *Phytomonas* have a dixenous (two-host) life cycle and are adapted to parasitism in vascular plants (Camargo, 1999; Jaskowska et al., 2015). It had long been thought that any flagellates found in plants belonged to this genus. However, it was then shown that some monoxenous trypanosomatids such as *Herpetomonas* spp., *Leptomonas* spp. and *Crithidia* spp. could also persist in the juice and, in particular, in the fruits of plants (Conchon et al., 1989; Catarino et al., 2001; Fiorini et al., 2001).

It has been proven experimentally that the vectors of phytomonads are phytophagous bugs (Louise et al., 1986; Jankevicius et al., 1989, 1993; Freymuller et al., 1990). An overwhelming majority of potential vectors belong to three families of true bugs: Coreidae, Pentatomidae and Lygaeidae (Camargo, 1999; Jaskowska et al., 2015).

Phytophagous bugs feeding on a broad range of plants harbour their own rich fauna of monoxenous trypanosomatids (Podlipaev, 1990). Their life cycles have a typical structure, with the phase of endogenous agglomeration in the host midgut and the phase of formation of dispersal stages in

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the hindgut (Frolov et al., 2021). New host individuals are usually infected through the substrate contaminated by the faeces of infected bugs.

In contrast to monoxenous trypanosomatids, the life-cycle phases of the phytomonads are spatially separated within the host. The flagellates reproduce in the gut and then migrate via the hemolymph to the salivary glands. There, they also reproduce forming the stages capable of getting into plant tissues with the saliva during feeding of the hosts on plants (Jankevicius et al., 1989, 1993; Freymuller et al., 1990). Host preferences of the phytomonads are poorly studied and the range of potential vectors of a given parasite species is usually not known at all.

Flagellates *Phytomonas serpens* parasitize tomatoes in Brazil (Jankevicius et al., 1989). This is one of the few species of phytomonads for which not only the host plant, *Solanum lycopersicum*, but also the vector, the bug *Phthia picta* (Hemiptera, Coreidae), were established at the time of the first description (Jankevicius et al., 1989). The authors also reported that, according to their preliminary data, *P. serpens* could successfully develop in bugs *Nezara viridula* from the family Pentatomidae (Jankevicius et al., 1989). This fact is particularly interesting because these bugs are the vectors of parasitic flagellates from tomatoes in South Africa described as *Leptomonas serpens* (Gibbs, 1957). The culture of *P. serpens* is available, but the culture of *L. serpens* is not, and so it is impossible to check whether the tomato parasites from South Africa and from Brazil belong to the same species. However, if *P. serpens* can indeed successfully develop in bugs *N. viridula*, this may be considered as an indirect evidence of the identity of these two species of flagellates.

The question about potential vectors of *P. serpens* has recently arisen in an unusual quarter. This phytomonad was found in the south of Russia in an invasive North American coreid bug, the Western conifer seed bug *Leptoglossus occidentalis* Heidemann, 1910, which feeds on coniferous plants (Ganyukova et al., 2022). The phytomonads were shown to complete their development in *L. occidentalis* forming dispersal stages (endomastigotes) in their salivary glands and retaining the ability to infect tomato fruits. In other words, the parasites apparently "considered" a non-specific facultative vector *L. occidentalis* as a "comfortable" host, even though it is geographically and ecologically distant from their specific vector, the bugs *Phthia picta* from Brazil.

This paper presents the results of experimental study aimed at testing whether the flagellates *P. serpens* could develop in non-specific hosts, the bugs *Coreus marginatus* from the family Coreidae and the bugs *Nezara viridula* from the family Pentatomidae.

Material and methods

Culturing of phytomonads

We used the isolate *P. serpens* Yalt2 from the culture collection of the Zoological Institute of the Russian Academy of Sciences, St. Petersburg. The origin of this culture and the culturing technique were described in a previous study (Ganyukova et al., 2022).

Culturing of insects and their experimental infection with phytomonads

Two species of true bugs were used in the experiments: *Nezara viridula* L. (fam. Pentatomidae) and *Coreus marginatus* L. (fam. Coreidae). The bugs *N. viridula* were reared in the laboratory from eggs laid by the bugs collected in nature, in the Krasnodar Territory of the Russian Federation. Adult individuals of *C. marginatus* were picked by hand from generative parts of the Russian dock *Rumex confertus* and the rosebay willow herb *Chamaenerion angustifolium* in the north of the Leningrad Region of the Russian Federation.

The bugs used in our study were kept in separate cages equipped with drinking troughs under light conditions day16/night8 at a temperature of ~22 °C. Bugs *N. viridula* were fed on tomato fruits, while bugs *C. marginatus* were fed on Russian sorrel seeds. Bugs *N. viridula* were infected either via tomato fruit pre-infected with the isolate *P. serpens* Yalt2 or via the drinking troughs with the culture of these flagellates. The bugs *C. marginatus* were infected via the drinking troughs with the culture of *P. serpens* Yalt2.

Infection of tomato fruit

Tomatoes used in the experiments were bought in a supermarket. A six-day-old culture of *P. serpens* Yalt2 (~10^5 cells/ml) was injected into the tomato flesh to a depth of ~3–5 mm with the help of a capillary. The inoculation volume was 0.01 ml. From four to six injections were made on each tomato from different sides. After infection, the tomatoes were placed into the refrigerator and stored at 10 °C until the beginning of the experiment.

Table 1. Experimental infection of *Coreus marginatus* (n=27) with *Phytomonas serpens* Yalt2.

Time post infection (days) / Localization in the host	10	14	21
Intestine	10/7*	10/9	7/5
Salivary glands	10/0	10/1	7/4

*number of dissected/infected insects

Table 2. Experimental infection of *Nezara viridula* (n=17) with *Phytomonas serpens* Yalt2

Time post infection (days) / Localization in the host	10	14	21
Intestine	5/1*	5/0	7/0
Salivary glands	5/0	5/0	7/0

*number of dissected/infected insects

Light and transmission electron microscopy

The smears prepared from fragments of the infected intestine and salivary glands of insects and from the infected tomato fruit were air-dried, fixed with 96% ethanol for 30 min, and Giemsa-stained for 30 min (pH 6.8). Microphotographs were taken using Leica DM 2500 microscope equipped with UCMOS14000KPA 14-Mpx camera (Toup Tek, Hangzhou, China) at ×1,000 magnification.

The salivary gland samples were prepared for TEM as described earlier (Frolov et al., 2018). Ultrathin sections were examined in Morgagni 268-D microscope (FEI Company/Thermo Fisher Scientific, Hillsboro, OR, USA) with accelerating voltage of 80.00 kV.

Identification of phytomonads in experimentally infected hosts

Phytomonads from the infected hosts were identified according to the following scheme. To begin with, cultures of the flagellates Cor3, Cor5 and Cor8 were isolated from the salivary glands of three experimentally infected individuals of *C. marginatus* as described previously (Ganyukova et al., 2022). Genome DNA was extracted from the sedimented cell cultures with HiPure Universal DNA Kit (Magen, Guangdong, China) according to the manufacturer's instructions. SSU rRNA gene fragment was amplified with the use of S762 and S763 primers according to the protocol described in Maslov and co-authors (1996) and Kostygov and Frolov (2007). A757 primer was used to sequence gene fragments ~880–1000 bp long. The sequences obtained in this study were deposited in GenBank, nos. OQ979216—OQ979218

Results

Experimental infection of *C. marginatus* and *N. viridula* with the isolate of *P. serpens* Yalt2 showed that these flagellates could develop only in bugs *C. marginatus* (Tables 1, 2). In the bug *N. viridula*, the flagellates could only subsist in the intestine for a short time. A few pro- and endomastigotes could be observed there for two weeks. In contrast, in the bug *C. marginatus*, the actively dividing phytomonads formed stable micropopulations in the intestine and the salivary glands. Since the insects used in our study were collected in the nature, the species of the flagellates from the salivary glands of the experimentally infected bugs was confirmed by molecular-genetic diagnostics with the use of BLAST software. Sequences OQ979216—OQ979218 18S of trypanosomatids from the cultures isolated from the infected salivary glands of *C. marginatus* showed a 100 % similarity with the sequence of the original isolate of *P. serpens* Yalt2 deposited in GenBank (Ganyukova et al., 2022).

The development of phytomonads in the bug *C. marginatus* started with the proliferation of flagellates in the midgut. Leaving M1 region of the midgut fairly soon, the flagellates occupied the posterior regions, M2 and M3, after 10 days. Long narrow promastigotes (Fig. 1, A) and actively dividing shorter forms (Fig. 1, B) were present there. At two weeks post infection, the phytomonads were recorded in the salivary glands for the first time (Table 1). At three weeks post infection, the flagellates were found in the salivary glands of 4 out of the 7 experimentally infected insects (Table 1). Promastigotes with a characteristic claviform shape, both dividing and solitary, were very numerous on the smears of the infected salivary glands (Fig. 1, C, D). Small endomastigotes that were often found in groups of three or more individuals were also abundant (Fig. 1, E).

Localization of the parasites in the salivary glands of *C. marginatus* was analyzed in semi-thin and thin sections. Both pro- and endomastigotes could be seen in the lumen of the gland without forming any contacts with its epithelium (Fig. 1, H, I). The lumen of the salivary glands of *C. marginatus* was filled with a dense secreted substance, which had a complex spongy ultrastructure (Fig. 1, I).

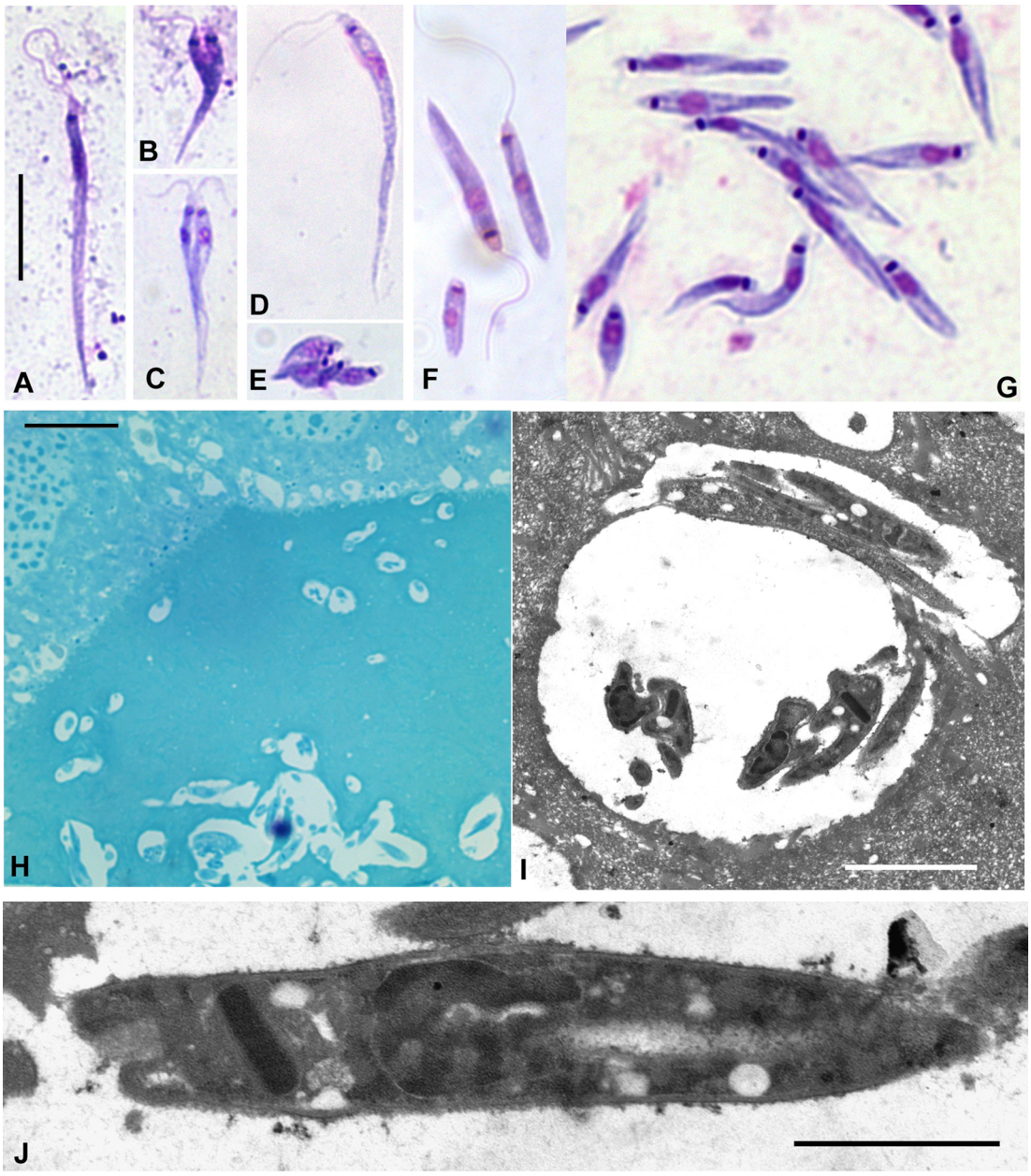

Fig. 1. Development of flagellates *Phytomonas serpens* in experimentally infected bugs *Coreus marginatus*. A-G – Giemsa-stained preparations; H – a semithin section stained after Richardson; I, J – TEM. A, B – Promastigotes from the midgut; C, D – promastigotes, and E – endomastigotes from salivary glands; F – flagellates from the culture Cor3; G – flagellates in the juice of an experimentally infected tomato fruit; H, I – localization of the phytomonads in the secretion of the salivary gland; J – endomastigote from the salivary gland. Scale bars: A-H – 10 μm; I – 2 μm; J – 1 μm.

The flagellates were localized in the lacunae with a transparent content, which were present in this substance (Fig. 1, H-J).

In order to check whether the phytomonads that have completed their development in a non-specific host retained the ability to infect tomato fruit, the culture of flagellates Cor3 was used (see above). The flagellates in this culture were represented by pro- and endomastigotes (Fig. 1, F). A single injection of 0.1 ml of a six-day-old culture of Cor3 into the flesh of a tomato resulted in an intense infection (Fig. 1, G), which persisted until the fruit has dried up or rotted. The experiment was performed in five replications with identical results.

Discussion

Phytomonads capable of implementing a part of their life cycle in tomato fruit have been reported from three continents. *Phytomonas serpens* was described from South America and *Leptomonas serpens* – from South Africa, while *Phytomonas* sp. (isolate TCC 305E) occurs in the south of Europe (Gibbs, 1957; Jankevicius et al., 1989; Sanchez-Moreno et al., 1995). These descriptions were made in the pre-molecular era, and for a long time the comparison of flagellates from different geographic locations was questionable. Besides, laboratory cultures were obtained only for the parasites from the European and the Brazilian tomatoes (Jankevicius et al., 1989; Sanchez-Moreno et al., 1995), while the description of the South African *L. serpens* remained on paper (Gibbs, 1957). Later, it was shown with the use of the available laboratory cultures that flagellates from the European tomatoes (isolate TCC 305E) were not identical to a large group of Brazilian *P. serpens* isolates but formed a separate cluster, which also included phytomonads isolated in Europe from *Annona cherimola, Trifolium* sp. and *Amaranthus retroflexus* (Zanetti et al., 2016).

Since phytomonads have a dixenous life cycle, their association with certain species of vectors may be an important diagnostic character, although less reliable than the barcoding data. The vector of *Phytomonas* sp. from European tomatoes is unknown (Sanchez-Moreno et al., 1995). The vectors of the other two phytomonads considered in our study have been established alongside the original description and confirmed experimentally (Gibbs, 1957; Jankevicius et al., 1989). The vector of *Leptomonas serpens* in South Africa is the southern green shield bug *Nezara viridula* (Hemiptera, Pentatomidae), also known as "southern green stink bug" or "green vegetable bug" (Gibbs, 1957). The vector of *Phytomonas serpens* from Brazil is the coreid bug *Phthia picta* (Hemiptera, Coreidae) (Jankevicius et al., 1989).

It has long been thought that the distribution of *P. serpens* is confined to the Neotropics (Jaskowska et al., 2015). Recently, however, these flagellates were unexpectedly discovered in the south of the European part of Russia in the coreid bugs *Leptoglossus occidentalis*. A facultative (i.e., largely random) character of this infection is fairly evident, considering a unique food specialization of these bugs (coniferous plants only) and their original distribution in North America (Gapon, 2012; Ganyukova et al., 2022). Nevertheless, two facts are noteworthy: firstly, flagellates *P. serpens* are present in the European continent; secondly, they can successfully develop in a facultative host, *L. occidentalis*, in the same manner as in the obligate host *P. picta* (Jankevicius et al., 1989; Freymuller et al., 1990; Ganyukova et al., 2022).

In this study, we experimentally tested the hypotheses that might elucidate the origin of the European isolate of *P. serpens* Yalt2. The authors of the first description of *P. serpens* reported, based on their preliminary experimental data, that *N. viridula* was "...also easily infected with culture forms of the flagellate" (Jankevicius et al., 1989). Unfortunately, they did not examine the nature of this infection in more details (Jankevicius et al., 1989), and this line of study was not continued.

Probably *Nezara viridula* was initially distributed in the Ethiopian zoogeographical region, whence it expanded into Asia, Europe and the Americas. This distribution argues in favor of multiple secondary invasions (Musolin and Saulich, 2012). Should it be proven that flagellates *P. serpens* can infect *N. viridula* and complete their life cycle in them, this discovery would clarify the origin of European and Brazilian isolates of *P. serpens* and their potential relationship with the African *Leptomonas serpens* Gibbs 1957.

Phytomonads are known to have a complex developmental pathway in the vector, infecting successively various regions of the intestine, the hemolymph and the salivary glands, where stages infective for the host plant are formed (Freymuller et al., 1990; Frolov et al., 2021). Our results do not support the idea that flagellates *P. serpens* Yalt2 can develop in the bugs *N. viridula*. After persisting in the intestine for a short time, they were completely eliminated from their alimentary tract. This means that there are currently no reasons to consider *N. viridula* as a potential vector of *P. serpens* and to explain the successful expansion of these parasites into various new territories by their association with this species of bugs.

The second bug species involved in our experiments was the dock bug *Coreus marginatus*. Considering its ecology, distribution and trophic chains, it has not been initially accepted as an obligate host of *P. serpens* (Hruškovšá et al., 2005). We tested the hypothesis about the possible absence of host limitations on horizontal transmission of the flagellates *P. serpens* to the coreid bugs. In our experiments, these flagellates successfully infected the

bug *C. marginatus*, completing the development in the salivary glands and forming invasive endomastigotes. The flagellates developed in the lumen of the glands of *C. marginatus* in the same way as they do in the salivary glands of their obligate host, the bug *P. picta*, that is, without attaching to the apical surface of the epithelial cells. This localization is also characteristic of the flagellates *Phytomonas lipae* that are specific parasites of *C. marginatus*, and of *P. oxycareni* from bugs *Oxycarenus lavaterae* (Lygaeidae) (Seward et al., 2017; Frolov et al., 2019). On the contrary, phytomonads *P. nordicus* from the predatory pentatomid bugs *Troilus luridus* use modified flagella for attaching to the brush border of the cells of the salivary gland of the host (Frolov and Malysheva, 1993; Frolov and Skarlato, 1995; Frolov et al., 2016).

The fact that the flagellates *P. serpens* Yalt2 isolated from the salivary glands of experimentally infected bugs *C. marginatus* successfully infected tomato fruit suggests that the development of *P. serpens* in the facultative vector host did not influence their invasive capacity. Moreover, the isolate Cor3 is represented by flagellates that have successively infected two species of non-specific (facultative) hosts and still retained the ability to infect plants.

In conclusion, we would like to emphasise that only the bugs from the family Coreidae can currently be considered as vectors of the flagellates *P. serpens*. At the same time, the expansion of these phytomonads into new territories may also involve facultative hosts, in which *P. serpens* can develop normally and form stages infective for tomato fruits.

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