Biological insights into *Globodera ellingtonae*

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Summary

In 2012, a new cyst nematode species, *Globodera ellingtonae*, was described from Oregon and Idaho, USA. Initial studies indicated a close phylogenetic relationship of this nematode to the potato cyst nematodes (*G. pallida* and *G. rostochiensis*) and demonstrated that *G. ellingtonae* reproduces readily on potato (*Solanum tuberosum*). Over the past 5 years we have conducted research to gain biological insights into *G. ellingtonae*. Combined data from hatching assays, host range studies, and a multigene phylogenetic analysis point to the fact that *G. ellingtonae* is more similar to *G. rostochiensis* than *G. pallida*. Key biological features that support this conclusion is the rapid hatch of *G. ellingtonae* in potato root diffusate, inability to reproduce on potato varieties with the gene for resistance to *G. rostochiensis* Ro1, and a highly supported phylogenetic analysis based upon 6,933 bp encompassing 11 genes grouping *G. ellingtonae* with *G. rostochiensis* and *G. tabacum* separate from *G. pallida*.

Key words: *Globodera ellingtonae*, root diffusate, resistance, phylogeny

Introduction

The pale potato cyst nematode *Globodera pallida* (Stone, 1973) Behrens, 1975 and the golden potato cyst nematode *G. rostochiensis* (Wollenweber, 1923) Behrens, 1975 are regulated pathogens of potato. Collectively, these species are referred to as potato cyst nematodes (PCN). Within the United States, *G. rostochiensis* was first found in New York in 1941 and has been the subject of quarantine restriction for decades (Brodie & Mai, 1989). *Globodera pallida* was first discovered in Idaho in 2006 (Hafez et al., 2007). This discovery had a tremendous impact on the US potato industry, valued at c. $3.9 billion (Agricultural Statistics Board, 2014). In response to the detection of *G. pallida*, Canada, Mexico, and Korea suspended importation of potatoes from Idaho, while Japan cut off importation of potatoes from the US. All of these export markets have been re-opened, with the exception of the Japanese market which remains closed to Idaho potatoes. Another outcome that arose from the discovery of *G. pallida* in Idaho was that an extensive survey of the major potato growing acreage was undertaken to determine the extent of PCN distribution in the United States. In 2008, unusual populations of *Globodera* were found in one field in Powell Butte, Oregon and two fields in Teton County, Idaho. All three locations had a history of potato production. In the United States, material suspected to contain PCN cysts is analyzed at the United States Department of Agriculture-Agriculture Research Service Nematology Laboratory in Beltsville, Maryland (Skantar et al., 2011). Based on morphological and molecular data, this nematode was described as
a new species, *Globodera elli
genae* Handoo, Carta, Skantar, and Chitwood, 2012 (Handoo et al., 2012). This cyst nematode is most similar to atypical *Globodera* populations from Argentina and Chile, and together these populations are distinct from *G. rostochiensis*, *G. tabacum*, *G. mexicana*, and *G. pallida*.

Research was undertaken in 2010 to provide data about the biology of *G. elli
genae* to assist regulatory agencies in decision making regarding this new species. All of this research has been conducted in Oregon using the population that was originally found at Powell Butte. Prior to the discovery of *G. elli
genae*, this site served as the home for the tri-state (Idaho, Oregon, and Washington) potato breeding program that commenced in 1983. Potatoes were grown on a four-year rotation at this farm with the field used for this research last cropped to potato in 2008. During the initial survey for *Globodera* spp. conducted in 2008, *G. elli
genae* was found at very low densities (one to seven cysts 2.3 kg\(^{-1}\) soil) across this field, with one hotspot being identified with 24 cysts per 2.3 kg\(^{-1}\) soil. Over the past five years, not only have biological insights into *G. elli
genae* been revealed but much of the data has also enabled comparisons regarding the relationship of *G. elli
genae* to *G. pallida* and *G. rostochiensis*.

**Materials and Methods**

For all experiments, cysts of *G. elli
genae* were reared in the field at Powell Butte, Oregon. Soil was collected from the site and air-dried prior to extraction of cysts for inoculum or for use in experiments. Cysts were extracted from soil using a USDA cyst extractor (Ayoub, 1980).

**Hatching assays**

To produce root diffusates, potato (*Solanum tuberosum*) (PRD) and tomato (*Solanum lycopersicum*) (TRD) plants were grown in 15 cm pots containing potting soil in a greenhouse. Plants were fertilized and grown for 1 to 2 weeks until the plants had sufficient root mass. At this time, the soil was saturated with deionized water and then another 50 to 100 mL of deionized water was added to the saturated soil and the resulting leachate collected. Root diffusates were kept at -20°C until used. A 96-well plate assay system modified from Byrne et al. (2001) and Twomey et al. (1995) was used to evaluate effects of diffusates on *G. elli
genae* egg hatch. To each well, a 100 µL aliquot of a 1:5 to 1:100 v v\(^{-1}\) water to diffusate (PRD or TRD) solution was added followed by a single *G. elli
genae* cyst. A water control was included and each treatment was replicated five times and the experiment repeated. The assay plates were sealed with parafilm, covered with aluminum foil, and incubated at room temperature (~22°C). Cysts were incubated in test solutions for 24 days. During this period of time, the number of second-stage juveniles (J2) emerging from eggs in each well was enumerated daily up to 15 days and then at 17 and 24 days thereafter using an inverted compound microscope (Magnification = ×40). After enumeration on days 3, 10, and 17, the cysts were moved to new wells containing 100 µL aliquots of fresh solutions. Any J2 inadvertently transferred with the cyst were noted at the time of transfer. Data were analyzed by analysis of variance (ANOVA) means were compared using Tukey’s Honestly Significant Difference (HSD) test (\(P<0.05\)).

**Greenhouse host assays**

The host status of the nine diverse potato cvs Atlantic, Russet Burbank, Desirée, Maris Piper, Modoc, Norland, Satina, Umatilla, and Yukon Gold were evaluated. Atlantic is a chipper; Russet Burbank and Umatilla are russeted varieties used in processing and table stock; Desirée (PCN susceptible standard), Modoc, and Norland are red table stock varieties; Satina and Yukon Gold are white and yellow table stock cvs, respectively; and Maris Piper (PCN resistant standard) produces small white tubers used in specialty processing. Additionally, the host status of commonly grown rotation crops at Powell Butte, OR, barley, oats, and alfalfa, to *G. elli
genae* was also considered; tomato was included in this trial as well All plants were pre-sprouted and transplanted into 9.6 L
potato and tomato) or 3.7 L (rotation crops) pots containing a 1:1 washed sand and Willamette loam mix. Plants were inoculated with soil from Powell Butte containing on average across two trials of 60 cysts (potato and tomato) or 30 cysts (rotation crops/tomato) with 193 eggs cyst\(^{-1}\). Plants were arranged in a randomized complete block design with five replications in the greenhouse; two trials were conducted for each set of plants. Plants were grown for 12 weeks or until they senesced. At harvest, the above ground portion of the plant was removed and discarded. The contents of the pot were emptied into a bin and air dried. To determine final nematode populations, two 500 g soil subsamples were collected and cysts were extracted using a USDA cyst extractor. Cysts were handpicked from washed samples and counted. The number of eggs cyst\(^{-1}\) was determined by crushing all collected cysts within a subsample with a rubber stopper on a 60- over a 500-mesh sieve. Eggs retained on the 500-mesh sieve were collected and enumerated at ×40 using an inverted microscope. Reproductive factors (RF = final egg density/initial egg density) were determine for each pot. Data were analyzed with Kruskal-Wallis and Duncan’s multiple range test was used to separate means only when Kruskal-Wallis was significant (P<0.05).

Multigene Globodera species phylogeny

A multigene phylogeny was conducted to allow inference of the evolutionary history of PCN and *G. tabaccum* (tobacco cyst nematode; TCN). We used published *G. pallida* (Cotton *et al.*, 2014) and publically available *Heterodera glycines* (Genbank GCA_000150805.1) and *G. rostochiensis* genome assemblies (http://globodera.bio.ed.ac.uk/) and our own draft assemblies of *G. elli+gtonae* and *G. tabacum* genomes for the analysis. To generate a gene set for the phylogeny, predicted protein sequences from a set of 248 highly conserved core eukaryotic genes (CEGS) were first identified in *G. elli+gtonae* (Parra *et al.*, 2007). Those 151 predicted protein sequences were then used as queries in tblastn searches to identify genes that each were nearly fully assembled on single contigs in the genome assemblies of all species. From those searches, 11 genes were identified and their sequences extracted from all genomes: KOGS 25, 142, 180, 290, 279, 376, 400, 424, 477, 567, 650, and 4655 (Tatusov *et al.*, 2003). The sequences for each gene were aligned using ClustalW, with hand alignment sometimes required for the *H. glycines* sequences due to extensive divergence in introns. Following removal of ambiguous alignment regions and gaps, gene sequences were concatenated, yielding a total of 6,933 positions used in phylogenetic analysis. A Maximum Likelihood (ML) method based on the Kimura 2-parameter model (Kimura, 1980) using a discrete Gamma distribution model of evolutionary rate differences among sites (four categories) was implemented using MEGA6 (Tamura *et al.*, 2013) and 1,000 bootstrap replicates were conducted for support evaluation.

**Results**

**Hatching assays**

No difference in hatching dynamics of *G. ellingtonae* among the different concentrations of PRD and TRD were detected (P=0.32); therefore, the data was combined within each diffusate. The hatching dynamics of *G. ellingtonae* over time in PRD and TRD were for the most part similar (Fig. 1). The only point in time where percentage egg hatch (J2 emerging each day/total J2 emerging over 24 days) differed was at day 2 where 34% more J2 hatched in TRD compared to PRD (P<0.05). Egg hatch plateaued at day 4 in TRD and at day 10 in PRD. In corresponding water controls very few J2 emerged from cysts (13±6) over the course of the experiment compared to the average number emerging in PRD and TRD (167±19).

**Greenhouse host assays**

Data from repeated trials demonstrated that Désirée, Russet Burbank, Umatilla, Modoc, Norland, and Yukon Gold were all very good hosts for *G. ellingtonae* (Table 1). Russet Burbank was the best host for *G. ellingtonae* supporting higher reproduction factor values. Three of the varieties
Fig. 1. Cumulative percentage egg hatch (number of second stage juveniles (J2) emerging/total number of J2 emerging after 24 days of *Globodera ellingtonae* in potato (PRD) and tomato (TRD) root diffusates averaged over concentrations ranging from 1:5 to 1:100 (v v⁻¹) diffusate to water. * above values on a given day indicates that there was a significant difference (*P* <0.05). Each data point represents *n*=50. From Zasada *et al.* (2013).

evaluated, Maris Piper, Atlantic, and Satina were poor hosts (Rf values <1) for *G. ellingtonae*. None of the non-solanaceous plants (barley, oats, and alfalfa) were hosts for *G. ellingtonae*, with a mean Rf value across plant types of 0.97 (±0.05). This is in comparison to tomato which in the same experimental venue was a host for *G. ellingtonae* with a mean RF value of 7.1 (±0.6). These data also indicate that none of these non-solanaceous plants stimulated egg hatch of *G. ellingtonae*.

Table 1. *Reproduction factors (Rf) of Globodera ellingtonae on potato varieties*

<table>
<thead>
<tr>
<th>Potato variety</th>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desirée</td>
<td>29.8 b</td>
<td>36.4 b</td>
</tr>
<tr>
<td>Russet Burbank</td>
<td>50.0 a</td>
<td>57.9 a</td>
</tr>
<tr>
<td>Umatilla</td>
<td>25.5 b</td>
<td>29.1 c</td>
</tr>
<tr>
<td>Modoc</td>
<td>21.8 bc</td>
<td>38.7 b</td>
</tr>
<tr>
<td>Norland</td>
<td>18.1 c</td>
<td>27.7 c</td>
</tr>
<tr>
<td>Yukon Bold</td>
<td>14.6 c</td>
<td>14.9 d</td>
</tr>
<tr>
<td>Maris Piper</td>
<td>0.4 d</td>
<td>0.7 e</td>
</tr>
<tr>
<td>Atlantic</td>
<td>0.6 d</td>
<td>0.5 e</td>
</tr>
<tr>
<td>Satina</td>
<td>0.4 d</td>
<td>0.5 e</td>
</tr>
</tbody>
</table>

*From Zasada *et al.* (2013)

| Rf = number of eggs at harvest/egg inoculum. |
| Means within the same column under the same trial that are followed by the same letter are not significantly different according to Duncan’s multiple range test (*P*<0.05). |
Multigene Globodera species phylogeny

The multigene phylogeny is presented in Fig. 2. Sequence divergence between *H. glycines* and the *Globodera* species was 17.8%. Of the 6,933 bp analyzed, *G. pallida* exhibited a 2.8 to 2.9% divergence with the clade containing *G. ellingtonae*, *G. rostochiensis*, and *G. tabacum*. Within this clade, sequence divergence ranged from 1.6% between *G. rostochiensis* and *G. tabacum* to 1.9 to 2.0% between *G. ellingtonae* and *G. rostochiensis/G. tabacum*.

![Fig. 2. Maximum likelihood phylogeny of 11 genes extracted from all genomes: KOGS 25, 142, 180, 290, 279, 376, 400, 424, 477, 567, 650 and 4655 (Tatusov et al., 2003), yielding 6993 positions from Globodera species with outgroup Heterodera glycines, and with bootstrap values from 1000 replicates as implemented in MEGA6 (Tamura et al., 2013). Bootstrap values are shown above branches.](image)

**Discussion**

*Globodera ellingtonae* is a new member of the genus which has been shown to reproduce on potato (Lax *et al.*, 2014; Zasada *et al.*, 2013). Because of this plant-parasitic nematodes ability to parasitize potato and the close morphological and molecular relationship to *G. rostochiensis* and *G. pallida*, information on the biology of this nematode is needed to guide regulatory decisions and to determine the potential risk of this nematode to US potato production. It is important to note that *G. ellingtonae* has not been detected in any other locations in the US beyond the initial detections. The USDA-Animal and Plant Health Inspection Service and individual states have collected and analyzed over 600,000 soil samples in Idaho and approximately 225,000 soil samples from other states for *Globodera* since 2006 (Jonathan Jones, personal communication).

In hatching assays, *G. ellingtonae* hatched very rapidly, within 3 days, after exposure to PRD and TRD. The reported hatch of PCN in PRD has varied across experiments. Hatch of *G. pallida* after exposure to PRD was reported to range from 4 days (Forrest & Farrer, 1983) to 10 to 14 days (Arntzen *et al.*, 1993). *Globodera rostochiensis* has also been shown to hatch 3 days after exposure to PRD (Doncaster & Shepherd, 1967). Variability among and within experiments may be attributed to quality of root diffusate, diapause in the nematode, and experimental conditions (i.e. temperature).

The potato varieties included in this study containing the gene for resistance to *G. rostochiensis* Ro1 were also resistant to *G. ellingtonae*. This finding leads to an immediate and meaningful strategy to manage *G. ellingtonae*, plant resistance. Only three varieties (Maris Piper, Atlantic, Satina) with *G. rostochiensis* Ro1 resistance were evaluated; additional varieties varying in resistance to PCN should be evaluated to further confirm this finding. All of the other potato varieties evaluated are commonly grown in the region and were hosts for *G. ellingtonae*. Similar to *G. rostochiensis* and *G. pallida*, non-solanceous plants appear not to be hosts for *G. ellingtonae*, however, this observation needs to be greatly expanded. The only other report of host status of additional plants to *G. ellingtonae* is that of hairy nightshade as a very good host (W S Phillips, personal communication) and tobacco as a non host for this nematode (Lax *et al.*, 2014).

The multigene phylogeny including 11 highly conserved eukaryotic genes supports previously published single-gene phylogenies of *Globodera* grouping *G. ellingtonae* with *G. rostochiensis* and *G. tabacum*, and all of these *Globodera* being more distantly related to *G. pallida*. Phylogenetic
analysis based on ITS1 and -2 showed a well-supported monophyletic clade containing *G. tabacum*, *G. rostochiensis*, *G. ellingtonae*, *G. mexicana*, and *G. pallida* (Skantar et al., 2011). Within this clade, two subclades were present, one containing *G. pallida* and *G. mexicana* and the other containing *G. tabacum*, *G. rostochiensis*, and *G. ellingtonae*; however, these subclades were not as well supported (bootstrap values <68). Another phylogenetic analysis based on a 213–250 bp sequence subset of HSP90 revealed similar tree topography with much higher support (bootstrap values >99) for the two previously mentioned subclades (Lax et al., 2014). The multigene phylogeny presented here greatly expands the genetic information upon which to infer relationships, both by including a diversity of genes and by increasing the number of nucleotides used in the analysis.

Our data indicates that *G. ellingtonae* is more similar to *G. rostochiensis* than *G. pallida*. While hatching data can be variable within and across experiments, our laboratory and field observations (unpublished data) point to a hatching dynamic more similar to *G. rostochiensis* (greater percentage hatch, shorter duration) than *G. pallida* (lower percentage hatch, longer duration) (Lane & Trudgill, 1999). To date, we have only identified solanaceous plants as hosts for *G. ellingtonae*. When potato varieties were challenged with *G. ellingtonae*, this nematode behaved much like *G. rostochiensis* in that varieties with the gene for resistance to *G. rostochiensis* Ro1 were also resistant to *G. ellingtonae*. Finally, the multigene phylogeny supports the grouping of *G. ellingtonae* with *G. rostochiensis*, and both being more distantly related to *G. pallida*. Additional information on developmental biology, host range, and pathogenicity are still required to facilitate management and regulatory decisions.

References


