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Phylogenetic Relationships of a Distinct Species of *Globodera* from Portugal and Two *Punctodera* Species[1]

A. Sabo,[2] L. G. L. Reis,[3] E. Krall[4], M. Mundo-Ocampo[5], and V. R. Ferris[6]

Abstract: Evolutionary relationships based on ribosomal DNA (rDNA) sequence data for a previously unknown species of *Globodera* from Portugal, *Punctodera chalcoensis* from Mexico, and *P. punctata* from Estonia, plus previously published sequences, support the following relationships: (((*Cactodera weissi, G. artemisiae, C. milleri*), ((*G.* sp. Bouro, *G.* sp. Canha, *G.* sp. Ladoeiro), ((*G. pallida, G. rostochiensis*), (*P. chalcoensis, P. punctata*)))), *Heterodera avenae*). *Globodera* sp. from Portugal, which can be confused with potato cyst nematodes by phytosanitary services when the identification is based only on morphological characters, is clearly different based on our molecular data. In addition, the rDNA data show the *Globodera* sp. to be only distantly related to other European *Globodera* species that parasitize Asteraceae. *Punctodera chalcoensis* and *P. punctata* form a sister clade to the *G. pallida* + *G. rostochiensis* clade.

Key words: 5.8S gene, *Globodera,* ITS1, ITS2, nematode, phylogenetic analysis, *Punctodera, Punctodera chalcoensis, Punctodera punctata,* ribosomal DNA.

Introduction

Cysts of *Globodera* sp. that parasitize a member of the Asteraceae (''Compositae'') *Chamaemelum mixtum* were recently discovered in Portugal (Reis, 1997). Previous to this finding, only two species of the cyst nematode genus *Globodera* had been reported in Portugal, *viz.,* the two potato cyst nematodes, *G. pallida* and *G. rostochiensis.* The newly discovered *Globodera* sp., to be described by L.G.L. Reis, has been shown to differ morphologically from the potato cyst nematodes (Reis, 1997). *Globodera millefolii* and *G. artemisiae* also have plant hosts in the Asteraceae and have been shown, based on molecular data, to be very similar to each other and to have closer phylogenetic affinities with *Cactodera* species than with the potato cyst nematodes (Ferris et al., 1999). Recent study of evolutionary relationships among cyst-forming nematodes (Subbotin et al., 2001) strongly supports monophyly of the subfamily Punctoderinae, comprising *Globodera, Punctodera,* and *Cactodera* genera, and placement of the genus *Heterodera* within a sister clade to Punctoderinae. Given the great importance of potato cyst nematodes as parasite and quarantine species, the presence of the third *Globodera* species complicates the work of phytosanitary services in Portugal (Reis, 1997). We used ribosomal DNA (rDNA) sequence data to investigate the evolutionary relationships of the newly discovered *Globodera* sp. to the two potato cyst nematodes, and other nominal *Globodera* species with Asteraceae hosts represented by *G. artemisiae* in this study. New data for rDNA were obtained and analyzed for two species of *Punctodera, P. chalcoensis* Stone, Sosa Moss & Mulvey from Mexico, and *P. punctata* (Thorne) Mulvey & Stone from Estonia. The distribution of *P. chalcoensis* is restricted to Mexico, where it causes important losses to corn (Baldwin and Mundo-Ocampo, 1991). *Punctodera punctata,* which has been reported in Europe, Canada, and the United States, and isolated from wheat and other grasses, does not cause any crop damage (Evans and Rowe, 1998). Previously published sequence data for six cyst nematode species were included in the phylogenetic analyses (Ferris et al., 1994; Ferris et al., 1999; Ferris et al., 1995). These species include *Globodera artemisiae* (Eroshenko & Kasachenko) Behrens, *G. pallida* (Stone) Behrens, *G. rostochiensis* (Wollenweber) Behrens, *Cactodera milleri* Graney & Bird, *C. weissi* (Steiner) Krall & Krall, and *Heterodera avenae* Wollenweber.

Materials and Methods

Cysts of *Globodera* sp. were extracted from roots of chamomile *Chamaemelum mixtum* from three localities (Bouro, Canha, and Ladoeiro in Portugal) by L. G. L. Reis. Specimens of *P. chalcoensis* were collected by M. Mundo-Ocampo in Charapan, Mexico, from corn roots; cysts of *P. punctata* were collected from soil in the root area of *Poa pratensis* in Maeksa, Estonia, by E. Krall. Nematode specimens (Table 1) were preserved in 70% ethanol. For each nematode isolate, DNA preparations were made from single cysts. Nematodes were homogenized in 25 µl TE buffer (pH 7.5), and total genomic DNA was extracted using InstaGene Matrix (Bio-Rad, Hercules, CA). Primers used for amplification of a ribosomal DNA fragment that spanned the two internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene as well as the PCR reaction parameters were as previously described (Ferris et al., 1993). The amplified fragment was cloned in pGEM-T vector (Promega, Madison, WI) and transformed into *Escherichia coli* strain JM109. Plasmid preparations were made using the Wizard plus mini prep system (Promega) from bacterial colonies containing inserts of the expected size as assessed by PCR amplification. Sequencing of the plasmid preparations was carried out using automatic sequencers (ALFexpress, Pharmacia Biotech; and LI-COR) at

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[1] Funded in part by National Science Foundation grant DEB 975740 to V.R. Ferris.
[2] Graduate Assistant and [6] Professor, Department of Entomology, Purdue University, West Lafayette, IN 47907-1158.
[3] Departamento de Protecção das Plantas, Estação Agronómica Nacional, 2784-505 Oeiras, Portugal.
[4] Estonian Agricultural University, 51014 Tartu, Estonia.
[5] Department of Nematology, University of California, Riverside, CA 92521. E-mail: anisabo@purdue.edu
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TABLE 1. Source of isolates and host data for species used in the study.

Species name	Source of isolate	Host
Globodera sp. Reis, 1997	Bouro, Canha, and Ladoeiro, Portugal	*Chamaemelum mixtum* (L.) All.
G. artemisiae (Eroshenko & Kasachenko) Behrens, 1975	Tartu, Estonia	*Artemisia rubripes* Nakai
G. pallida (Stone) Behrens, 1975	Caddishead, UK	Potato (*Solanum tuberosum* L.)
G. rostochiensis (Wollenweber) Behrens, 1975	Feltwell, UK	Potato (*Solanum tuberosum* L.)
Punctodera punctata (Thorne) Mulvey and Stone, 1976	Maeksa, Estonia	*Poa pratensis* L.
P. chalcoensis Stone, Sosa Moss, and Mulvey, 1976	Charapan, Mexico	Corn (*Zea mays* L.)
Cactodera milleri Graney and Bird, 1990	Indiana, US	Lambsquarter (*Chenopodium album* L.)
C. weissi (Steiner) Krall and Krall, 1978	Indiana, US	Smartweed (*Polygonum pennsylvanicum* L.)
Heterodera avenae Wollenweber, 1924	Oregon, US	Oats (*Avena sativa* L.)

the Purdue High Definition Genomics Center. Both strands of DNA from several clones (2-4) were sequenced for each nematode isolate. Sequences from different clones were identical, and the resulting sequences were deposited in GenBank (accession numbers AY090882-AY090886).

Sequences were aligned using the computer program PILEUP in the Sequence Analysis Software package of the Genetics Computer Group (GCG) version 9.1 (Devereaux et al., 1984) with default penalty values (gap weight = 50, gap length = 3), and then manually adjusted using MacClade 4.0 (Maddison and Maddison, 2000). Phylogenetic inference was based on both default and manually adjusted alignments. Phylogenetic analysis was carried out using PAUP* 4.0b8 (Swofford, 1998) under parsimony and maximum likelihood optimality criteria. Maximum parsimony analysis was performed using the branch-and-bound search with gaps treated as missing, and also as the 5th character state.

The model of sequence evolution is a crucial part of maximum likelihood analysis, and accuracy and consistency are reduced when the wrong model is assumed (Bruno and Halpern, 1999). There are several statistical methods developed for selecting a model that best fits the data at hand (Posada and Crandall, 2001). A method that uses log likelihood scores to compare different models is implemented in Modeltest, version 3.06 (Posada and Crandall, 1998), which we used to select a model that best fits our data set. The selected model and its estimated parameters were implemented in the maximum likelihood inference using PAUP* 4.0b8 with a heuristic search and 100 random replicates under the stepwise-addition option. Support for individual branches was evaluated using the bootstrap method with heuristic search and 500 and 100 replicates for parsimony and maximum likelihood analysis, respectively. Previous studies (Ferris et al., 1999; Subbotin et al., 2001) showed that *H. avenae* was an appropriate outgroup for taxa in our study, and therefore inferred trees were rooted using *H. avenae.*

RESULTS

Sequence length of the ribosomal DNA fragments varied among species from 890 bp in *Globodera rostochiensis* and *Cactodera weissi* to 959 bp in *Heterodera avenae.* Multiple-sequence alignment of 971 characters generated by PILEUP revealed 529 characters that were constant, 184 variable parsimony uninformative, and 258 parsimony informative. The manually adjusted alignment had 978 total characters, with 545 constant, 210 parsimony uninformative, and 223 parsimony informative characters. Both alignments, as well as the resulting trees, have been deposited in TreeBASE (http://www.treebase.org) study number 5720.

DNA sequences of *Globodera* sp. from the three populations in Portugal were almost identical. Numbers of base pair differences between populations were one between the Canha and Bouro populations, two between the Ladoeiro and Bouro populations, and three between the Ladoeiro and Canha populations. Sequences of two *Punctodera* species, *P. chalcoensis* and *P. punctata,* differed in 32 base pairs. The numbers of base pair differences among these taxa were the same in both the default and the manually adjusted alignments. Numerous sites at which character states for *Globodera* sp. from Portugal were identical among sequences from the three populations and unique among the taxa under study were observed. Although most comprised only one or two base pairs, there were a few longer ones. One example includes sites 909-913 (default alignment, see TreeBASE study number 5720) which had sequence CACGC in the three *Globodera* sp. populations, and GCACG in both species of potato cyst nematodes. These sites could be used for diagnostic methods using restriction fragment length polymorphisms, and investigators interested in designing such diagnostic methods need to investigate in greater detail the alignments deposited in TreeBASE.

Phylogenetic analyses based on the default and manually adjusted alignments under the parsimony optimality criterion with gaps treated as missing or 5th character states resulted in trees with nearly identical topologies. Inferred trees differed in the relationships of the *Globodera* sp. population from Bouro, which formed a clade with the population from Ladoeiro when gaps were treated as the 5th character state. Relationships among these three nearly identical populations were unresolved on the consensus of MP trees

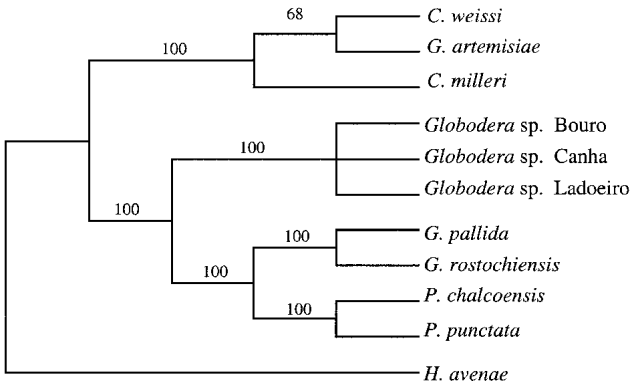

FIG. 1. Consensus of two most parsimonious trees for *Cactodera, Globodera,* and *Heterodera* species with *H. avenae* as the outgroup. Parsimony analysis is based on default alignment with gaps treated as missing. Bootstrap support values for monophyletic groups are indicated above branches.

inferred with gaps treated as missing. Trees also differed in the grouping of *C. weissi* with either *C. milleri* or *Globodera artemisiae.* Most of the clades were supported by bootstrap values of 100 (Fig. 1). The evolutionary relationships among the three nearly identical populations of *Globodera* sp. from Portugal were unresolved on the bootstrap trees with gaps treated as missing, and were weakly (60–65%) supported when gaps were treated as 5th character state.

The sequence evolution model selected by Modeltest software based on the default alignment was K81uf+G, which represents the Kimura81 model (Kimura, 1981) with unequal base frequencies and rate heterogeneity among sites. Empirical base frequencies were A = 0.20, C = 0.24, G = 0.28, and T = 0.28, and estimated gamma distribution shape parameter 0.53. Maximum likelihood analysis with the K81uf+G model of sequence evolution and heuristic search resulted in a tree (Fig. 2) with -ln likelihood of 4,337.46. All 100 random replicates resulted in the same tree. The selected model of sequence evolution based on the manually adjusted alignment was the Transitional model with rate heterogeneity among sites, TIM+G (Rodríguez et al., 1990). The best tree inferred from the manually adjusted alignment under the maximum likelihood criterion with the TIM+G model had -ln likelihood of 4,245.22 and the same topology as the tree inferred from the default alignment. Most of the clades were supported by high bootstrap values of 93% and greater (Fig. 2). Grouping of *Globodera* sp. population from Bouro with

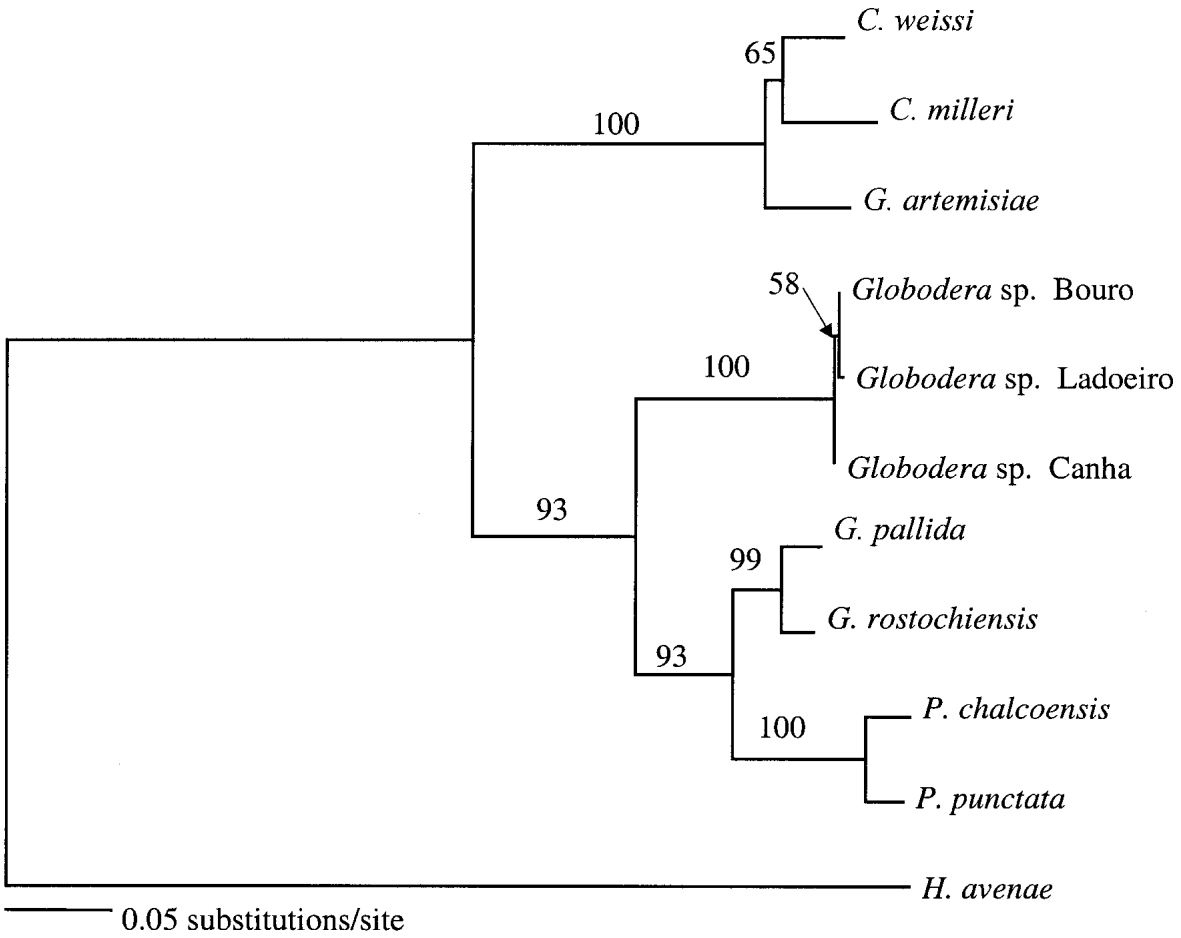

FIG. 2. Maximum likelihood tree inferred from default alignment using the K81uf+G model of sequence evolution for *Cactodera, Globodera,* and *Heterodera* species with *H. avenae* as the outgroup. Analyses based on default, and manually adjusted alignment resulted in trees with the same topology.

the population from Ladoeiro, and *C. weissi* with *C. milleri,* was only weakly supported with bootstrap support of 58% and 65%, respectively.

Topologies of the most parsimonious trees and the best tree under the maximum likelihood criterion were identical except for *C. weissi* that grouped with *C. milleri* in the maximum likelihood tree and with *G. artemisiae* in some of the most parsimonious reconstructions.

Discussion

Manual adjustments of the multiple sequence alignment had a negligible effect on our phylogenetic analysis. The maximum likelihood method based on two different alignments produced trees with the same topology. Trees inferred under parsimony had slightly different topologies, but differences were confined to placement of *C. weissi* and the relationships among the three populations of *Globodera* sp. from Portugal. The latter relationships could not be inferred with confidence because the nearly identical rDNA sequence of specimens from the three geographic localities had pairwise sequence differences of three or fewer nucleotides. Considering the number of base pair differences among other species in this analysis, 29 or more, we concluded that cysts from the three localities in Portugal represent one species. Based on rDNA sequence, the *Globodera* species from Portugal differs markedly from the potato cyst nematodes, *G. pallida* and *G. rostochiensis,* and is only distantly related to *G. artemisiae* and *G. millefolii,* also parasites of Asteraceae from Europe. *Punctodera chalcoensis* from Mexico and *P. punctata* from Estonia form a sister clade to the *G. pallida* + *G. rostochiensis* clade and are more distantly related to the *Globodera* sp. from Portugal. Our data clearly indicate that *Globodera* sp. in Portugal found on *Chamaemelum mixtum* is a species distinct from the two potato cyst nematode species and from other known or nominal species of *Globodera* as well.

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