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Identification, phylogeny and phylogeography of circumfenestrate cyst nematodes (Nematoda: Heteroderidae) as inferred from analysis of ITS-rDNA

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Summary – Some 134 ITS rRNA gene sequences for circumfenestrate cyst nematodes and two sequences for non-cyst nematodes of the family Heteroderidae, of which 46 were newly obtained, were analysed by phylogenetic and phylogeographic methods. Sequence and phylogenetic analysis combined with known morphological, biological and geographical data allowed the identification, amongst samples original to this study, of several belonging to known valid species as well as others that might be new species. The phylogenetic analysis revealed six major clades for circumfenestrate cyst nematodes: *i*) *Globodera* from South and North America; *ii*) *Globodera* from Europe, Asia, Africa and Oceania; *iii*) *Paradolichodera*; *iv*) *Punctodera*; *v*) *Cactodera*; and *vi*) *Betulodera*. Monophylies of *Punctodera*, *Cactodera* and *Betulodera* were highly supported. The *Betulodera* clade occupied a basal position on all trees. Phylogeographic analysis suggested a North American origin of Punctoderinae with possible further long distance dispersal to South America, Africa and other regions. Molecular data supported synonymisation of *G. achilleae* with *G. millefolii* and of *G. hypolysi* with *G. artemisiae*. PCR-RFLP diagnostic profiles for some *Globodera* and *Cactodera* species are given. Problems of diagnostics for *Globodera* species using PCR with specific primers are discussed.

Keywords – *Betulodera*, *Cactodera*, diagnostics, DIVA, *Globodera*, *Globodera artemisiae*, *Globodera mexicana*, *Globodera millefolii*, *Globodera pallida*, PCR-ITS-RFLP.

Species belonging to six genera, *Betulodera*, *Cactodera*, *Dolichodera*, *Globodera*, *Paradolichodera* and *Punctodera*, share the character of possessing a vulval circumfenestra, which is a cuticular opening that develops in the terminal region of mature cysts. For circumfenestrate cyst nematodes, Krall and Krall (1978) established the Punctoderinae within the Heteroderidae. Although Siddiqi (1986, 2000) and Luc *et al.* (1988) did not accept the subdivision of cyst nematodes into two subfamilies, the Heteroderinae and Punctoderinae, the phylogenetic analyses of rRNA gene sequences gave clear evidence that circumfenestrate nematodes represent one of two separate major lineages, within cyst nematodes (Subbotin *et al.*, 2001, 2010; Tanha Maafi *et al.*, 2003). Among circumfenestrate cyst nematodes, 30 species are presently considered valid (Subbotin *et al.*, 2010). Several species, including the potato cyst nematodes (PCN) *Globodera pallida* and *G. rostochiensis*, are of special scientific and practical interest due to their great agricultural importance. Several others are pests of tobacco (tobacco cyst nematode (TCN) *G. tabacum*), maize (*Punctodera chalcoensis*), cereals (*Cactodera rosae*), grasses (*P. punctata*, *P. matadorensis*, *P. stonei*) and cacti (*C. cacti*). Identification of the species using morphological and morphometric characters requires taxonomic skills and time-consuming procedures. Analysis using the ITS-rRNA gene, for which

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sequences are now available for 14 valid species and several unidentified species, provides an attractive solution for diagnostics of circumfenestrate nematodes. PCR-ITS-RFLP, conventional PCR and Real-Time PCR with species-specific primers have been developed and are now becoming useful tools for identification of *Globodera* species (Subbotin *et al.*, 2010). However, recent sequence analyses of South American populations of *G. pallida* revealed high levels of genetic diversity among them (Picard *et al.*, 2007, 2008). Consequently, Grenier *et al*. (2010) noted the importance of evaluating available molecular diagnostic tools in relation to the new understanding of the wider diversity in South American populations. It is likely that some available tools, with their design based on limited sampling and knowledge of the species, will not remain reliable for species diagnosis in the face of new awareness of the high level of intraspecific genetic diversity. As for other circumfenestrate genera, PCR-RFLP profiles are available only for *C. cacti* and *P. punctata*; thus, the need remains for development of molecular diagnostic tools for additional circumfenestrate cyst nematodes.

Inter- and intraspecific genetic variability of species provides proven tools for analysis of phylogeny and biogeography of nematodes. These analyses are demonstrated not only to be of theoretical interest, but to assist in understanding aspects of the biology and ecology of these pests. Phylogenetic relationships within circumfenestrate cyst nematodes were analysed using morphological datasets with a few characters traditionally used in genus identification and using biological datasets including taxon-specific nematode/plant-host ranges (Krall & Krall, 1978; Ferris, 1979; Stone, 1979; Wouts, 1985). Applications of molecular approaches with analysis of ribosomal RNA gene sequences have added new reliability to understanding relationships within circumfenestrate cyst nematodes (Ferris *et al.*, 1999, 2004; Subbotin *et al*., 2000, 2001; Sabo *et al.*, 2002; Skantar *et al.*, 2007; Bernard *et al.*, 2010; Madani *et al.*, 2010). These studies, however, were mainly limited to a few representative taxa or they were restricted to studying interspecific relationships within a single genus. Moreover, reconstructed relationships between some genera still remain rather confusing and not yet fully resolved (Ferris *et al.*, 1999; Sabo *et al.*, 2002).

Two hypotheses of the origin and dispersal of circumfenestrate cyst nematodes have been proposed considering models of plate tectonics. The first was developed by Ferris (1979) and later supported by Picard *et al*. (2008), who believed that the Punctoderinae clade originated in Laurasia as a population ancestral to the South American *Globodera* species dispersed from North America to South America during a period when both continents were connected, but after America had completely separated from Africa. The second hypothesis was first proposed by Stone (1979), who suggested that *Globodera* might have originated in Gondwana and remained on the part of the supercontinent that later became South America, while the ancestors of European *Globodera* species were carried northwards when fragments of Gondwana encountered Laurasia. After analysis of host-plant ranges and distribution of cyst nematodes, Sturhan (2007) also suggested the Gondwana hypothesis of origin for Punctoderinae. Since the proposal of these hypotheses, newly developed phylogenetic methods now provide powerful tools for assessing the effect of past and current events on the geographic distribution of species and thereby allow testing such hypotheses on the dispersal of circumfenestrate cyst nematodes.

The main objectives of the present study are therefore to: *i*) identity species for several samples of cyst nematodes; *ii*) present PCR-ITS-RFLP and predict, *in silico*, diagnostic profiles for *Globodera* species; *iii*) estimate, *in silico*, specificity of presently used specific primers for PCN diagnostics using available sequence data; *iv*) estimate ITS-rRNA sequence divergences for some taxa within the clade of circumfenestrate cyst nematodes; *v*) reconstruct the phylogenetic relationships between species and genera of circumfenestrate cyst nematodes based on analysis of ITS-rRNA sequences using Bayesian inference and maximum parsimony; and *vi*) test hypotheses of the origin and dispersal of circumfenestrate cyst nematodes.

Materials and methods

Nematode populations

Nematode isolates were obtained from several sampling trips conducted by Cid Del Prado Vera and Mundo-Ocampo in Mexico and Sturhan in New Zealand. DNA was obtained from non-viable cysts of *G. pallida* and several *Globodera* species parasitising Solanaceae kept in nematode collections at BBA, Münster, Germany and UCRNC, Riverside, CA, USA, respectively. Species and populations from different hosts and localities used in this study are listed in Table 1. Cysts were extracted from soil samples using standard flotation and sieving tech-

Table 1. *Cyst-forming nematodes originally sequenced in the present study.*

Identification based on morphology and ITS-rRNA gene sequence	Original morphological identification	Locality	Host plant	Nematode collection, DNA code	GenBank accession number	Collector or/and identifier
Betulodera betulae	*B. betulae*	USA, Arkansas	*Betula nigra* L.	–	HQ260385	Riggs, R.
Betulodera sp.	Unidentified cysts	Merced River, Mariposa County, Sierra National Forest, California, USA	Unknown	CD164, CD267	HQ260383, HQ260384	Subbotin, S.A.
Cactodera cacti	*C. cacti*	Germany, Münster, BBA glasshouse	Unknown	572	HQ260422	Sturhan, D.
C. galinsogae	*C. galinsogae*	Mexico, La Raya Municipio de Singuilucan, Estado de Hidalgo	*Galinsoga parviflora* Cav.	CD330	HQ260418, HQ260419	Cid Del Prado, I.
C. rosae	*C. rosae*	Mexico, San Juan Ixtilmaco, Municipio de Apan, Hidalgo State	*Hordeum vulgare* L.	CD329	HQ260415, HQ260416	Cid Del Prado, I.
C. rosae	*Cactodera* sp.	Mexico, Morelos, I	Unknown	Sample 2, CD516	HQ260412, HQ260413	Mundo-Ocampo, M.
C. rosae	*Cactodera* sp.	Mexico, Colibri Morelos, II	Unknown	Sample 3, CD517	HQ260414, HQ260417	Mundo-Ocampo, M.
Cactodera sp. 1	*Cactodera* sp.	Mexico	Unknown	Sample 4, CD518	HQ260420, HQ260421	Mundo-Ocampo, M.
Cactodera sp. 2	*Cactodera* sp.	Mexico, Cumuatillo	Unknown	Sample 6, CD520	HQ260423	Mundo-Ocampo, M.
Globodera mexicana	*Globodera* sp.	Mexico, San Pedro Huehuecalco, Municipio de Amecameca Estado de Mexico	*Solanum* sp.	CD514	HQ260405, HQ260406	Cid Del Prado, I.
G. millefolii	*G. millefolii*	Estonia	*Achillea millefolium* L.	571	HQ260407	Krall, E., Sturhan, D.
G. pallida	*G. pallida*	Peru, Capachica	Unknown	–	HQ260426	Rumpenhorst, H.J.
G. pallida	*G. pallida*	Peru, Huamachuco	Unknown	–	HQ260428	Rumpenhorst, H.J.
G. pallida	*G. pallida*	Peru, Otuzco	Unknown	–	HQ260427	Rumpenhorst, H.J.
G. tabacum	*G. tabacum*	Europe	Unknown	GA	HQ260395, HQ260396	Madani, M.
G. tabacum	*G. tabacum solanacearum*	Italy	Unknown	CD523	HQ260403, HQ260404	Mundo Ocampo, M.
G. tabacum	*G. tabacum virginiae*	USA, Virginia, Crutchlow Farm	*Solanum carolinense* L.	CA144	HQ260398, HQ260400	Miller, L., Baldwin, J.G.
G. tabacum	*Globodera* sp.	Venezuela	Unknown	V105, CA139	HQ260386, HQ260399	Miller, L., Baldwin, J.G.
G. tabacum	*G. tabacum virginiae*	USA, Virginia, Horton	Unknown	CA140	HQ260387, HQ260394	Miller, L., Baldwin, J.G.
G. tabacum	*Globodera* sp.	Bolivia	Unknown	BolI, CA142	HQ260390, HQ260391	Miller, L., Baldwin, J.G.
G. tabacum	*G. tabacum solanacearum*	USA, Virginia, Watkins Farm	*Solanum carolinense* L.	CA145	HQ260388, HQ260392	Miller, L., Baldwin, J.G.
G. tabacum	*G. tabacum solanacearum*	USA, Virginia	Unknown	CA141	HQ260389, HQ260393	Miller, L., Baldwin, J.G.
G. tabacum	*G. mexicana*	Mexico	Unknown	CA143	HQ260397, HQ260401, HQ260402	Miller, L., Baldwin, J.G.
G. zelandica	*G. zealandica*	New Zealand, Banks Peninsula, South Island	*Plagianthus regius* (Poit) Hochr	Z794, NZ26, CA116	HQ260410	Sturhan, D.
G. zelandica	*G. zelandica*	New Zealand, Niagara Falls, South Island	*Sophora microphylla* Ait.	608	HQ260411	Sturhan, D.
Globodera sp. 1	*Globodera* sp.	New Zealand, Desert Road, North Island	Subalpine vegetation	Z745, 582	HQ260408	Sturhan, D.
Globodera sp. 2	*Globodera* sp.	New Zealand, Lake Lyndon area, Canterbury, South Island	Subalpine scrub vegetation	NZ20, CA114	HQ260409	Sturhan, D.
Atalodera crassicrustata	*A. crassicrustata*	Russia, Kamchatka	*Mertensia maritima* (L.) Gray.	–	HQ260425	Eroshenko, A.S., Subbotin *et al.* (2001)
Rhizonema sequoiae	*R. sequoiae*	USA, California	*Sequoia sempervirens* (D. Don) Endl.	–	HQ260424	Cid Del Prado, I.

niques. For morphological identification, slides of permanent mounts of the vulval cone regions of cysts and of second-stage juveniles (J2) were prepared using standard methods (Southey, 1986). Species delimiting of the studied populations was accomplished by integrating results of morphological and morphometric studies, phylogenetic and sequence analysis, as well as analysis of nematode host-plant specificity and geographic distribution of studied samples (Subbotin *et al.*, 2010).

DNA EXTRACTION, PCR AND SEQUENCING

DNA was extracted from a single or several cysts filled with desiccated J2 and eggs using the proteinase K protocol. Crushed J2 and eggs were transferred to an Eppendorf tube containing 16 μl double distilled water, 2 μl 10× PCR buffer and 2 μl proteinase K (600 μg ml^{-1}) (Promega). The tubes were incubated at 65°C (1 h) and then at 95°C (15 min). Detailed protocols for PCR, cloning and sequencing all nematode samples, except for *G. pallida*, were as described by Tanha Maafi *et al.* (2003). Extracted DNA (2 μl) was transferred to an Eppendorf tube containing 2.5 μl 10× *Taq* incubation buffer, 5 μl Q solution, 0.5 μl dNTPs mixture (*Taq* PCR Core Kit, Qiagen), 0.15 μl of each primer (1.0 μg μl^{-1}), 0.2 μl *Taq* polymerase and double distilled water to a final volume of 25 ml. The PCR amplification profile consisted of 4 min at 94°C; 35 cycles of 1 min at 94°C, 1.5 min at 55°C and 2 min at 72°C, followed by a final step of 10 min at 72°C. PCR protocol described by Wouts *et al.* (2001) was used for three *G. pallida* samples. The following primers were used for amplification of the ITS-rRNA gene for all samples, except for *G. pallida*: TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Subbotin *et al.*, 2001) and for South American *G. pallida* – F194 (5′-CGTAACAAGGTAGCTGTAG-3′) and F195 (5′-TCCTCCGCTAAATGATATG-3′) (Ferris *et al.*, 1993). The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen) and used for direct sequencing or cloning and RFLP. Usually two clones were sequenced from each sample. The resulting products were purified and run on a DNA sequencer at the University of California, Riverside, CA, USA sequencing facility. Newly obtained sequences were submitted to the GenBank database under the accession numbers HQ260383-HQ260428 as indicated in Table 1.

RFLP-ITS-RRNA

This analysis was made for those samples from which a sufficient amount of PCR product had been obtained. From 2-5 μl of purified product was digested by one of the following restriction enzymes: *Bsh*1236I, *Hin*fI, *Hpa*I, *Pst*I or *Rsa*I in the buffer stipulated by the manufacturer. The digested DNA was run on a 1.5% TAE buffered agarose gel, stained with ethidium bromide, visualised on a UV transilluminator and photographed. The exact length of each restriction fragment from the PCR products was obtained by a virtual digestion of the sequences using WebCutter 2.0 (www.firstmarket.com/cutter/cut2.html).

SEQUENCE AND PHYLOGENETIC ANALYSES

Using default parameters the newly obtained sequences were aligned using ClustalX 1.83 (Thompson *et al.*, 1997) with other sequences of species from *Globodera*, *Cactodera*, *Punctodera*, *Betulodera* and *Paradolichodera* (Ferris *et al.*, 1999; Subbotin *et al.*, 2001; Sabo *et al.*, 2002; Tanha Maafi *et al.*, 2003; Ferris *et al.*, 2004; Manduric & Andersson, 2004; Sirca & Urek, 2004; Sturhan *et al*., 2007; Bernard *et al*., 2010; Grenier *et al.*, 2010; Lax *et al*., unpubl.; Moreland & Fleming, unpubl.; Nowaczyk *et al*., unpubl.; Peng *et al*., unpubl.) and *Atalodera crassicrustata* and *Rhizonema sequoiae* used as outgroup taxa (Subbotin *et al.*, 2001, 2006). Only two sequences of *G. rostochiensis* were taken for the present analysis because, in the study by Madani *et al.* (2010) that included all available sequences for this species, no groupings were observed for *G. rostochiensis*. Sequence alignment was manually edited using GenDoc 2.5.0. Pairwise divergence between taxa was calculated as the absolute distance value and the percent of mean distance, with adjustment for missing data, using PAUP* 4b10 (Swofford, 2003). Only the ITS1-5.8S-ITS2 region, for which boundaries were estimated according to Subbotin *et al.* (2000), was used for analysis. The sequence dataset was analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) and the equally weighted maximum parsimony (MP) method using PAUP* 4b10. The best fit model of DNA evolution for BI was obtained using the program MrModeltest 2.2 (Nylander, 2002) with the Akaike Information Criterion in conjunction with PAUP*. BI analysis under the GTR + I + G model was initiated with a random starting tree and run with the four Metropolis-coupled Markov chain Monte Carlo (MCMC) for 10^6 generations. The MCMC were sampled at intervals of 100 generations. The log-likelihood values of the

sample points stabilised after approximately 10^3 generations. After discarding burn-in samples and evaluating convergence the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. For MP we used a heuristic search setting with ten replicates of random taxon addition with MaxTrees = 1000 and tree bisection-reconnection branch swapping to seek the most parsimonious trees. Gaps were treated as missing data. To obtain an estimate of the support for each node, a bootstrap analysis using 1000 replicates with MaxTrees = 100 was performed as was a heuristic search and simple addition of sequence. Posterior probabilities (PP) and bootstrap support (BS) are given on appropriate clades for BI and MP tree, respectively. Trees were visualised with the TreeView program and drawn with Adobe Illustrator v.10.

Phylogeographic analysis

The reduced DNA dataset for phylogeographic analysis comprised one sequence from each species of circumfenestrate nematode, including representatives of the main *G. pallida* clades as well as four sequences of non-cyst nematodes used as the outgroup. Sequences were aligned with Clustal X and trees for phylogeographic analysis were obtained using MP in PAUP*. Dispersal-vicariance analysis was used to estimate the ancestral locations of various clades in 28 MP trees using S-DIVA version 1.5 (Yu *et al.*, 2010) with up to eight unit areas. The S-DIVA program is a tool that complements DIVA 1.2 (Ronquist, 1997, 2001). DIVA reconstructs the ancestral distribution in a phylogeny by optimising distributions, allowing vicariance events and minimising the number of assumed extinctions and dispersals. By contrast, S-DIVA uses a statistical dispersal-vicariance analysis to statistically evaluate the alternative ancestral ranges at each node in a tree accounting for phylogenetic uncertainty and uncertainty in DIVA optimisation. For our study, six areas of distribution were used for taxon coding: North America, South America, Africa, New Zealand, Europe and Asia.

Results

Unidentified and putative new species of Punctoderinae

Sequence and phylogenetic analysis combined with known morphological, biological and geographical data allowed us to identify, among samples original to this study, several belonging to known valid species, as well as others that might be new species. Among putative new species revealed in the present study were two *Globodera* species from New Zealand. These were found by D. Sturhan from samples collected from subalpine vegetation, Desert Road, North Island, and subalpine scrub vegetation of the Lake Lyndon area, Canterbury, South Island, respectively. Another putative new species was a *Betulodera* species collected by S.A. Subbotin from unidentified trees along the south bank of the Merced River, Mariposa County, Sierra National Forest, CA, USA. Two unidentified and possibly new species were found among *Cactodera* samples collected in Mexico by M. Mundo-Ocampo. Additional detailed morphological and molecular analysis is still required to confirm the unique species status of some samples included in this study.

PCR-ITS-RFLP diagnostics

Amplification of the ITS-rRNA gene region with TW81 and AB28 primers yielded single fragments ranging from *ca* 976 to 1013 bp depending on the samples. Diagnostic PCR-ITS-RFLP profiles for *Globodera* and *Cactodera* species are given in Figures 1 and 2, respectively. The approximate sizes of restriction fragments for species from these genera are presented in Tables 2 and 3 based on generating the fragments using four diagnostic enzymes following digestion of PCR products. Digestion of PCR-ITS-rRNA products by one of four restriction enzymes (*Bsh*1236I, *Hin*fI, *Pst*I or *Rsa*I) enabled discrimination among most valid, and several still undescribed, species of *Globodera*. For example, enzyme *Rsa*I might distinguish a group of *G. pallida* populations (subclade 1) presently distributed in Europe, Asia, North America and Oceania from the South American groups of *G. pallida* (Clades 3, 5, 6) (Table 2). Nevertheless, none of these used enzymes can reliably differentiate *G. pallida* populations of subclade 1 from *G. mexicana*. However, virtual digestion shows that the enzyme *Sfa*NI can distinguish between them, generating two bands (690 and 290 bp) for *G. mexicana*, whereas the PCR product for *G. pallida* populations (subclade 1) remains unrestricted.

Four restriction enzymes (*Bsh*1236I, *Hin*fI, *Hpa*II and *Pst*I) differentiated several *Cactodera* species from one another, although *C. rosae*, *C. salina* and *C. weissi* were not distinguishable by any of the enzymes employed (Fig. 2; Table 3).

Because of a lack of sufficient nematode materials for some samples and the absence of sufficient amounts of

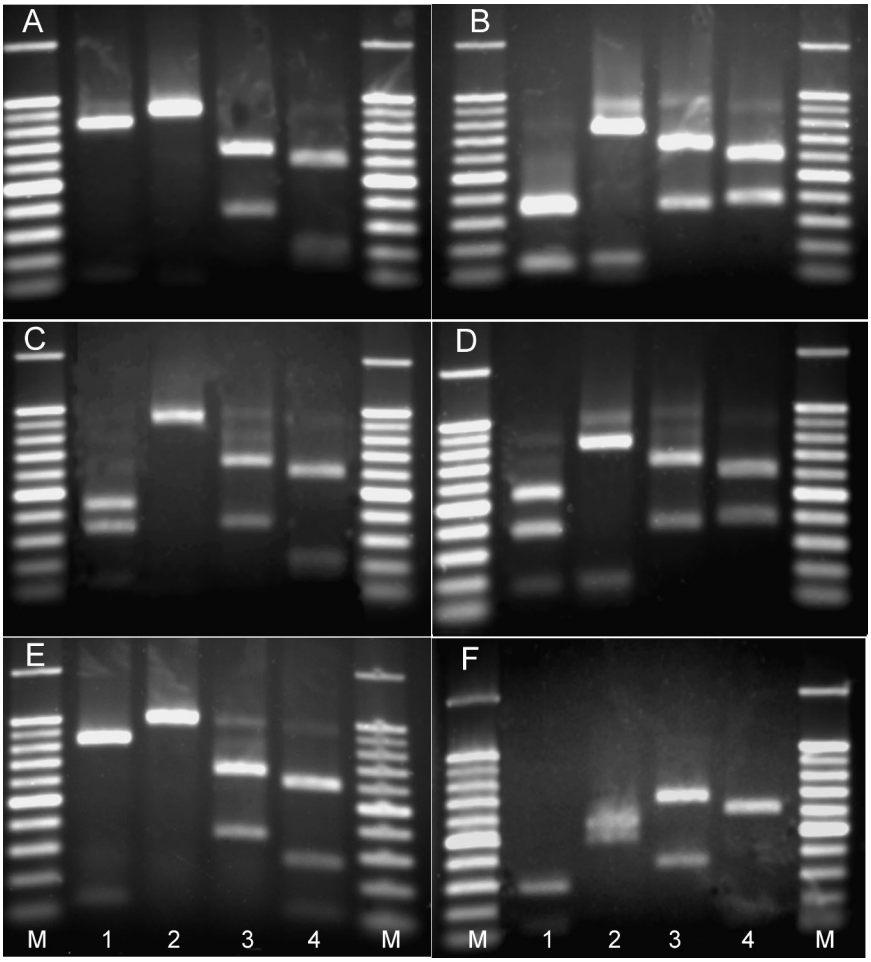


Fig. 1. *PCR-ITS-RFLP profiles for* Globodera *species. A:* G. rostochiensis*; B:* G. pallida *(subclade 1); C:* G. tabacum*; D:* G. mexicana*; E:* G. artemisiae*; F:* G. zelandica*. Lanes: M = 100 bp DNA ladder (Promega); 1 =* Bsh*1236I; 2 =* Hin*fI; 3 =* Pst*I; 4 =* Rsa*I*.

obtained PCR products, we were not able to make a digestion for some samples. In such cases, their diagnostic profiles are presented as results based on *in silico* prediction. Such results should be regarded as tentative pending verification by PCR-RFLP technique.

Sequence analysis

Sequence alignment included 134 sequences, 46 of which were newly obtained in this study. The alignment of the ITS1-5.8S-ITS2 contained 1098 sites. Intraspecific variations for some taxa were as follows: *G. tabacum* (30 sequences), 0-9 nucleotides (nt) or 0-1.0%; *G. mexicana* (5), 1-5 nt or 0.1-0.5%; *G. pallida* (42), 0-19 nt or 0-2.5%; *G. artemisiae* (5), 0-5 nt or 0-0.5%; *G. millefolii* (8), 0-4 nt or 0-0.4%; *Globodera* sp. from Argentina (3), 8-15 nt or 1-2.4%; *Globodera* sp. from Portugal (3), 1-3 nt or 0.1-0.3%; *C. rosae* (6), 1-7 nt or 0.1-0.8%; and *C. salina* (three sequences, with two of them identified as *Globodera* representatives), 0-4 nt or 0-0.4%. Interspecific sequence variation was resolved for some taxa including for two species of *Betulodera*, 21-22 nt or 2.9-3.0%; and two species of *Globodera* sp. 1 and *Globodera* sp. 2 from New Zealand, 28 nt or 3.1%.

Verification of primer specificity for PCN using *in silico* approach

Fragments of sequence alignment for *Globodera* species, with marked positions for specific primers for *G. pallida* (PITSp4), *G. rostochiensis* (PITSr3) and *G. tabacum* (PITSt4) developed by Bulman and Marshall

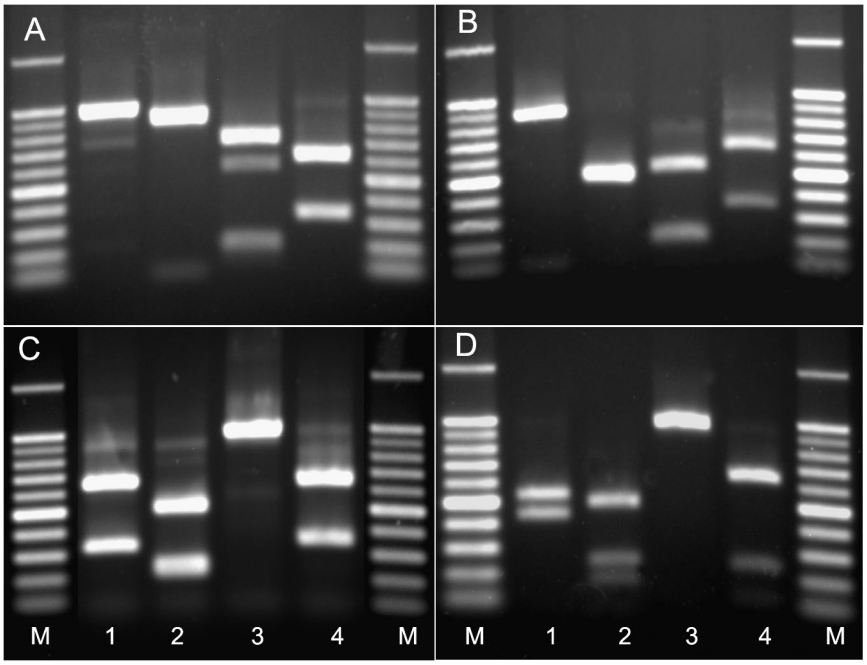


Fig. 2. *PCR-ITS-RFLP profiles for* Cactodera *species. A:* C. galinsogae*; B:* C. rosae*; C:* C. cacti*; D:* Cactodera *sp. 1. Lanes: M = 100 bp DNA ladder (Promega); 1 =* Bsh*1236I; 2 =* Hin*fI; 3 =* Hpa*I; 4 =* Pst*I*.

(1997) and Skantar *et al.* (2007), are presented in Figure 3. Our *in silico* analysis showed that the sequence of the *G. pallida* specific primer PITSp4 was well matched with the corresponding sequence region for the *G. mexicana* ITS and differed from it only by two nucleotides (Fig. 3A). Thus, false positive results could be obtained if this primer is used in PCR with a *G. mexicana* sample. The *G. rostochiensis* specific primer (PITSr3) was well matched with the corresponding sequence regions for the ITS of *G. artemisiae*, *G. millefolii* and *Globodera* sp. from Portugal, as well as a *Globodera* sp. from South Africa, differing only in two or three nucleotides (Fig. 3B) and potentially giving false-positive results with these samples. Skantar *et al.* (2007) proposed including an additional primer, PITSt4, in multiplex PCR together with PITSr3 and ITS5 for diagnostic techniques targeting *G. tabacum*. Our *in silico* analysis showed the *G. tabacum* specific primer PITSt4 (Fig. 3A) to be well matched with the corresponding rRNA genes of *G. artemisiae* and *G. millefolii*, and *Globodera* spp. from Portugal and South Africa, so that it might potentially lead to false-positive results by PCR. However, primer PITSt3 (Skantar *et al.*, 2007) and its modification PITSt3mr (Nakhla *et al.*, 2010) (Fig. 3B) were specific for *G. tabacum.*

Phylogenetic analysis

Phylogenetic relationships among circumfenestrate cyst nematodes were inferred from analyses of ITS-rRNA gene sequences using Bayesian inference and maximum parsimony as given in Figures 4 and 5, respectively. BI and MP trees were congruent and differed mainly in the positions of poorly supported clades. The 50% majority rule consensus BI tree and the strict consensus MP tree consisted of six major clades: *i*) *Globodera* from South and North America (PP = 100%; BS = 92%); *ii*) *Globodera* from Europe, Asia, Africa and Oceania (96; 62); *iii*) *Paradolichodera*; *iv*) *Punctodera* (100; 100); *v*) *Cactodera* (PP = 100; BS = 100); and *vi*) *Betulodera* (100; 100). Monophylies of *Punctodera*, *Cactodera* and *Betulodera* were highly supported, whereas monophyly of *Globodera* was not evident from the present analysis. The *Betulodera* clade occupied a basal position on all trees. Relationships of *Paradolichodera* and *Punctodera* with other genera were not well resolved and varied among BI and MP trees.

Globodera was represented by two main lineages. The first included species collected mainly in South and North America and parasitising Solanaceae; the second contained species collected from Africa, Europe, Asia and

Table 2. *Approximate sizes (in bp)* of restriction fragments generated by four diagnostic enzymes after digestion of PCR products of the ITS-rRNA regions amplified by TW81 and AB28 primers for* Globodera *spp.*

Species	Unrestricted PCR product	*Bsh*1236I	*Hin*fI	*Pst*I	*Rsa*I
G. rostochiensis	**976**	**826, 130, 20**	**902, 74**	**642, 334**	**584, 221, 162, 9**
G. pallida (subclade 1)	**979**	**484, 342, 130, 20 or 352, 342, 135, 130, 20**	**753, 152, 74**	**643, 336 or** 504, 336, 139	**586, 384, 9**
G. pallida (subclade 3)	978	486, 342, 130, 20	752, 152, 74	642, 336	585, 222, 162, 9
G. pallida (subclade 5)	979	829, 130, 20	753, 152, 74	640, 339	586, 222, 162, 9
G. pallida (subclade 6)	979	487, 342, 130, 20	753, 152, 74	643, 336	586, 222, 162, 9
Globodera sp. (Argentina)	977	*900*, *500*, 486, 341, 130, 20	*900*, 749, *620*, 151, 73	641, 336	586, 221, 161, 9
G. mexicana	**980**	**488, 342, 130, 20**	**754, 152, 74**	**644, 336**	**587, 384, 9**
G. tabacum	**980**	**431, 342, 130, 57, 20**	**906, 74**	**643, 337**	**587, 222, 162, 9**
G. millefolii	990	581, 258, 131, 20	990	649, 331	595, 274, 112, 5, 4
G. artemisiae	**989**	**839, 130, 20**	**989**	**649, 330**	**594, 274, 115, 5, 4**
G. zelandica	**1013**	**292, 282, 160, 113, 110, 25, 20, 11**	**543, 470**	**664, 349**	**605, 168, 116, 115, 5, 4**
Globodera sp. (Portugal)	986	836, 130, 20	986	646, 340	593, 384, 5, 4
Globodera sp. 1 (New Zealand)	1002	514, 335, 132, 21	535, 467	658, 344	597, 284, 112, 5, 4
Globodera sp. 2 (New Zealand)	1004	518, 332, 131, 12, 11	533, 471	662, 342	595, 167, 121, 112, 5, 4

* Fragments obtained in results of virtual sequence digestion using Webcutter 2. Bold font, verified by PCR-RFLP study; normal font, without RFLP verification; italic font, additional fragments, sometimes poorly visualised on a gel.

Table 3. *Approximate sizes (in bp)* of restriction fragments generated by four diagnostic enzymes after digestion of PCR products of the ITS-rRNA regions amplified by TW81 and AB28 primers for* Cactodera *cyst nematodes.*

Species	Unrestricted PCR product	*Bsh*1236I	*Hin*fI	*Hpa*II	*Pst*I
C. cacti	**979**	**642, 337**	**497, 258, 224**	**979**	**643, 336**
C. rosae	**979**	**875, 104**	**495, 484**	**536, 234, 209**	**642, 337**
C. galinsogae	**976**	**976**	**902, 74**	**750, 226**	**639, 337**
C. salina	971	866, 105	494, 477	534, 228, 209	634, 337
C. weissi	968	863, 105	493, 475	534, 225, 209	631, 337
C. milleri	963	963	494, 469	736, 227	626, 337
Cactodera sp. 1	**975**	**553, 442**	**494, 256, 184, 41**	**975**	**639, 238, 98**

* Fragments obtained in results of virtual sequence digestion using Webcutter 2. Bold font, verified by PCR-RFLP study; normal font, without RFLP verification.

New Zealand and parasitising non-Solanaceous plants. Samples identified as *G. pallida* were distributed into six subclades. *Globodera mexicana* clustered with subclade 1 of *G. pallida*. A *Globodera* sample from South Africa occupied a basal position within the second lineage with a following position for samples from New Zealand. *Globodera millefolii* clustered with samples originally identified as *G. achilleae* and *G. artemisiae* samples formed a clade with *G. hypolysi*. The Argentinean sample originally identified as *G. pallida* formed a clade with two unidentified *Globodera* samples from Chile, and this clade was apart from all other populations of *G. pallida*.

```
                                240         *       260         *
G. pallida subclade 1 : TGGTGGCCCAATGGGTGACTC--GACGATTGCTGTTGTCGTC :  249
G. pallida subclade 3 : ....................--.................... :  249
G. pallida subclade 5 : ....................--.................... :  249
G. pallida subclade 6 : ....................--....M.............. :  249
Globodera sp. Arg     : .........G...K.C..G..--...A.......C...T... :  249
G. rostochiensis      : .........T........GC.--...........C........ :  249
G. mexicana           : ....................--........T..C....... :  249
G. tabacum            : .........T........GC.--...........C.R..... :  249
G. artemisiae         : ..................GC.--............C....G. :  249
G. millefolii         : ..................GC.--............CA...G. :  249
Globodera sp. Port    : ...C..A...........GC.--............C....G. :  247
Globodera sp. 1 NZ    : .........G........GC.AC........T..C........ :  256
Globodera sp. 2 NZ    : .........G........GC.AC...........C........ :  260
G. zelandica          : .........G........GC.--...........C......G :  257
Globodera sp. SA      : ......T..G........GC.--........T...CA..... :  256
```

A

```
                              420         *       440         *
G. pallida subclade 1 : TCGTACGTGCCGTACCCAGCGGCATGTCTGCGCTTGTGTGCT :  422
G. pallida subclade 3 : .T..............G..............Y........ :  422
G. pallida subclade 5 : .T..............G.......A.............. :  422
G. pallida subclade 6 : .T..............G.......A.....A.......... :  422
Globodera sp. Arg     : .T.......................................... :  421
G. rostochiensis      : .T............TT.......................... :  421
G. mexicana           : .......................................... :  422
G. tabacum            : .T............C.......A.................. :  422
G. artemisiae         : ........A.......TG..........G...T......... :  424
G. millefolii         : ........A.......TG..........G...T......... :  424
Globodera sp. Port    : ........A.......TG..........G.............. :  422
Globodera sp. 1 NZ    : .T......A...................G.............. :  434
Globodera sp. 2 NZ    : .T......A...................G.............. :  438
G. zelandica          : .T......A........T..........G.............. :  437
Globodera sp. SA      : .T....A.A.......T..........G.............. :  431
```

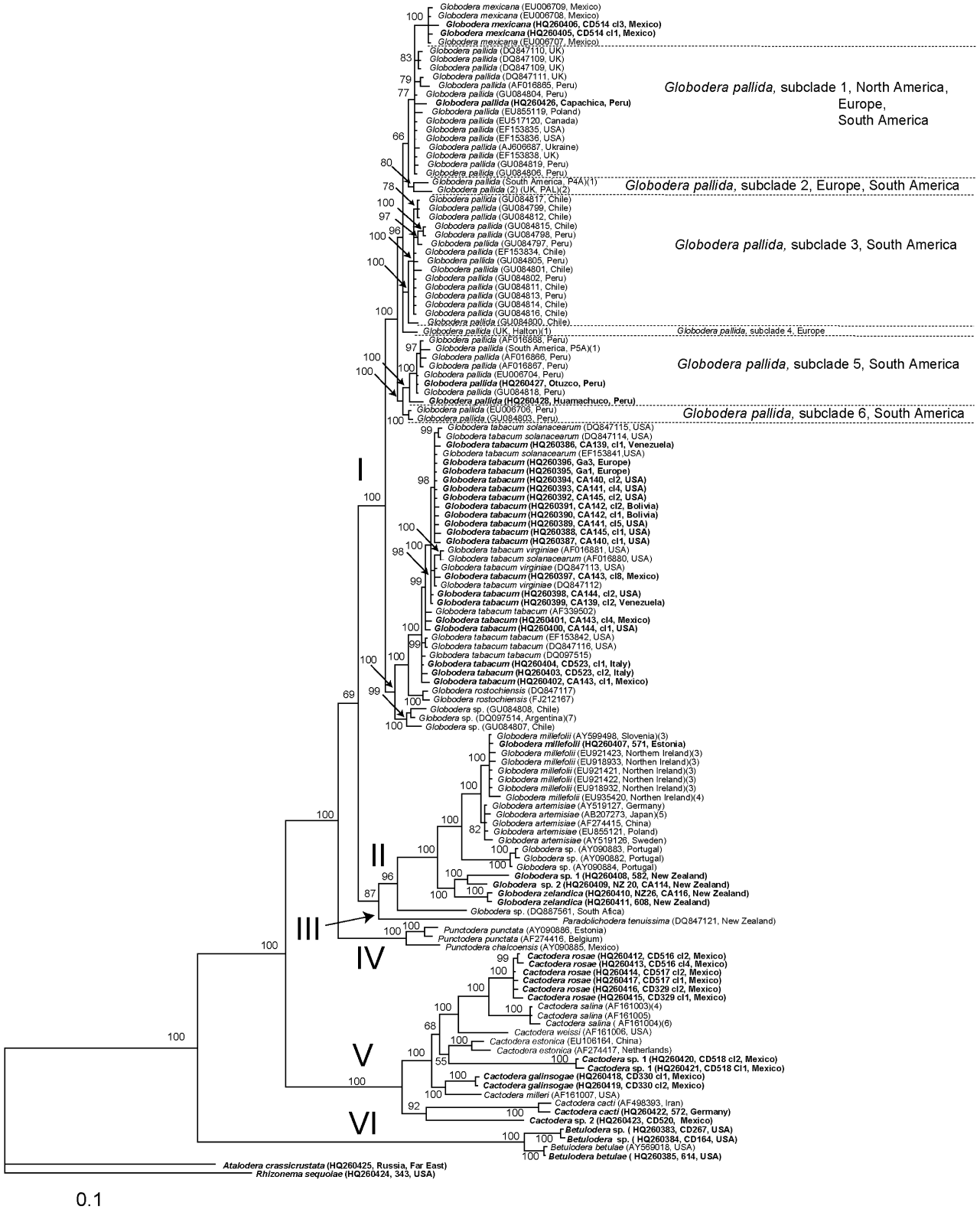
B

Fig. 3. *Fragments of alignment of the ITS-rRNA gene sequences for the* Globodera *species. Underlined sequences indicated the positions for PITSp4 (AGCGCAGACATGCCGCAA) (A) and PITSr3 (ACAACAGCAATCGTCGAG) primers (B) by Bulman and Marshall (1997) used for diagnostics of* G. pallida *and* G. rostochiensis*, respectively; grey areas are possible matching these primers with sequences of other species; black areas indicated the primer positions for PITSt4 (ACAGCAGCAATCGTCGGC) (A) by Skantar* et al. *(2007) and PITSt3 (AGCGCAGATATGCCGCGG) (B) by Skantar* et al. *(2007) and PITSt3mr (AGCGCAGATATGCCGCG) (B) by Nakhla* et al. *(2010) used for diagnostics of* G. tabacum*. GenBank accession numbers for* Globodera *sp. Port (Portugal) – AY090882-AY090884,* Globodera *sp. Arg (Argentina) – DQ097514,* Globodera *sp. SA (South Africa) – DQ887561.*

Cactodera was resolved as monophyletic in the BI and MP trees. *Cactodera cacti*, together with an unidentified *Cactodera* species from Mexico, occupied a basal position within the *Cactodera* clade, although with weak support. Sequences originally identified as *Globodera* species: *G. millefolii* (AF161004) and *G. artemisiae* (AF161003) clustered with *C. salina*.

Ancestral areas

The strict consensus of MP trees with ancestral area codes and probabilities, as identified by S-DIVA, are given in Figure 6. Dispersal-vicariance analysis identified the ancestral area of the Punctoderinae as having the highest probability in North America. For many nodes, the


Globodera pallida, subclade 1, North America, Europe, South America
Globodera pallida, subclade 2, Europe, South America
Globodera pallida, subclade 3, South America
Globodera pallida, subclade 4, Europe
Globodera pallida, subclade 5, South America
Globodera pallida, subclade 6, South America
I
II
III
IV
V
VI
Atalodera crassicrustata (HQ260425, Russia, Far East)
Rhizonema sequoiae (HQ260424, 343, USA)
0.1

probabilities for a particular area were maximum, whereas for six nodes multiple ancestral areas with various probabilities were reconstructed. The analysis suggested that extant *Cactodera* and *Punctodera* diversified in North America and may have then dispersed to other continents from this area. The clade containing *Globodera*, *Punctodera* and *Paradolichodera* appeared particularly complex with respect to reconstructing ancestral areas. The ancestral reconstruction for the *Globodera* clade was ambiguous, and this node was not well supported, although the most favoured reconstructions included South America (B) and Africa (C).

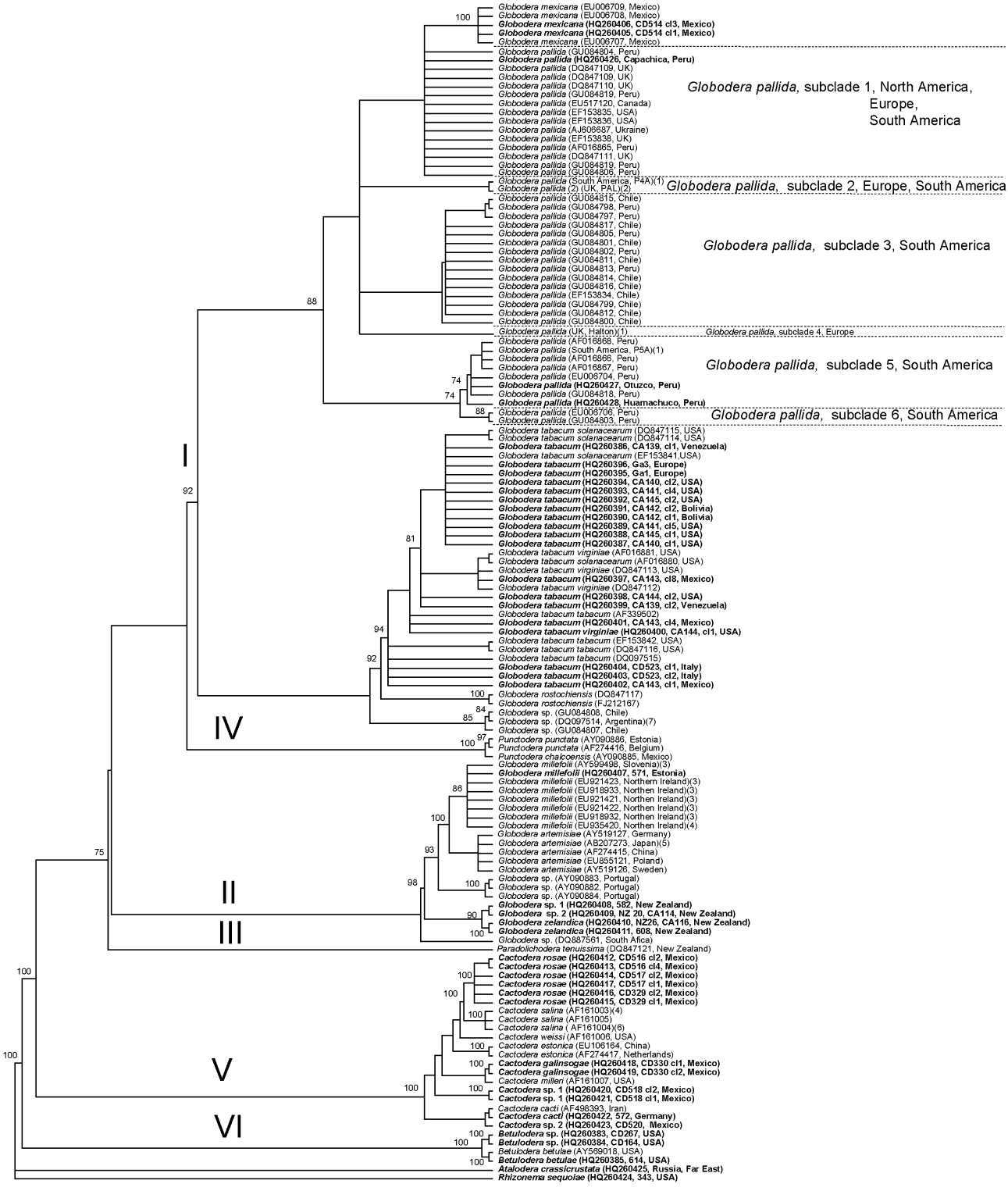
Discussion

Identification of cyst nematodes using PCR-ITS-RFLP and PCR with species-specific primers

PCR-ITS-RFLP and PCR with specific primer techniques are currently applied in many laboratories for identification of cyst nematodes, including *Globodera*, and these techniques were recently reviewed by Subbotin *et al.* (2010). Our results for PCR-RFLP and *in silico* analysis indicated that the PCR-RFLP technique could be used for diagnostics of a group of *G. pallida* populations, presently widespread in Europe, Asia, North America and Oceania, relative to other groups of *G. pallida* presently known in only in South America. PCR-RFLP clearly allows distinguishing PCN from other species, which are not agriculturally important but potentially might be found in field soil samples. *In silico* analysis also predicted that profiles generated by some enzymes for PCN are more diverse than has been previously reported (Thiéry & Mugniéry, 1996; Subbotin *et al.*, 2010). PCR-RFLP for *G. pallida* samples belonging to subclade 1 with *Bsh*1236I and *Pst*I showed the presence of several ITS haplotypes and might lead to complex patterns that could confound identification. The complex patterns and presence of additional bands in some RFLP profiles may also result from a higher level of rRNA gene heterogeneity for some *Globodera* isolates or from the presence of a natural hybrid between species within a single sample (Thiéry *et al.*, 1996; Reid & Pickup, 2005). The diagnostic profiles, as presented in Tables 2 and 3 for some species, should be tested with additional samples. In addition, *in silico* predicted profiles should be verified by the RFLP method. Using the data obtained solely from *in silico* analysis in these tables may potentially face some problems for diagnostics by generating different profiles on RFLP gels from those obtained from a virtual sequence digestion.

Results from the analysis of ITS-rRNA gene sequences have been widely used by many researchers to design species-specific primers and probes for conventional PCR and RT-PCR to identify *Globodera* species (*e.g.*, Bulman & Marshall, 1997; Knoetze *et al.*, 2006; Skantar *et al.*, 2007; Nakhla *et al.*, 2010). However, in some cases, ITS-rRNA sequence data for non-agriculturally important *Globodera* species have not been included or taken into consideration for diagnostic designs. Application of such methods that are not fully verified does not exclude the chance of false positive results when applying such diagnostic techniques to samples from unknown locations. Bulman and Marshall (1997) designed specific primers for *G. pallida* (PITSp4) and *G. rostochiensis* (PITSr3) which have two specific nucleotides at the 3′ end corresponding to ITS-rRNA genes for each species and they successfully tested these primers against two PCN species. Knoetze *et al.* (2006), using slightly modified specific primers for multiplex PCR developed by Bulman and Marshall (1997), reported that the specific primer for *G. rostochiensis* generated a band for an unidentified South African *Globodera* on some occasions, indicating a possible false positive reaction. Based on our *in silico* analysis, it can not be excluded that false positives with the primer PITSr3 might also be obtained with *G. artemisiae*, *G. millefolii* and unidentified Portuguese *Globodera* samples. The primer PITSp4 can also give false positive reactions with *G. mexicana* samples. Skantar *et al.* (2007) proposed the inclusion of an additional primer, PITSt4, in multiplex PCR together with PITSr3 and ITS5 for diagnostics that included *G. tabacum*. According to Skantar *et al.* (2007), the appearance of two bands is diagnostic for *G. rostochiensis*, one band indicated *G. tabacum*

Fig. 4. *Phylogenetic relationships within populations and species of Punctoderinae* sensu *Krall and Krall, 1978. The 50% majority rule consensus trees from Bayesian analysis generated from two runs as inferred from the analysis of the ITS-rRNA gene sequences under the GTR+G+I model. PP values are given in appropriate clades. Newly sequenced samples are indicated by bold font. (1) = Sequence from Blok* et al. *(1998); (2) = sequence from Ferris* et al. *(1995); (3) = originally identified as* Globodera achilleae*; (4) = originally identified as* G. artemisiae*; (5) = originally identified as* G. hypolysi*; (6) = originally identified as* G. millefolii*; (7) = originally identified as* G. pallida*.*


Globodera pallida, subclade 1, North America, Europe, South America
Globodera pallida, subclade 2, Europe, South America
Globodera pallida, subclade 3, South America
Globodera pallida, subclade 4, Europe
Globodera pallida, subclade 5, South America
Globodera pallida, subclade 6, South America
I
II
III
IV
V
VI

and the absence of product was consistent, but not definitive, for *G. pallida*. Our *in silico* analysis showed that samples of *G. artemisiae*, *G. millefolii*, unidentified Portuguese *Globodera*, *G. zelandica* and unidentified South African *Globodera* might also generate one band in such multiplex PCR and thus disrupt diagnostics of *G. tabacum* using this method. Thus, our analysis emphasises the need for expanded practical testing of all these methods across a range of *Globodera* species in order to confirm the reliability of presently proposed diagnostic approaches for PCN and TCN.

Globodera pallida species complex

Distinct genetic differences among different *G. pallida* populations spanning Europe and those found in South America were revealed by sequence and phylogenetic analyses of the ITS-rRNA (Blok *et al.*, 1998; Subbotin *et al.*, 2000; Madani *et al*., 2010), cytb (Picard *et al.*, 2007; Plantard *et al.*, 2008; Pylypenko *et al*., 2008; Madani *et al*., 2010), PCR-ITS-RFLP (Ayub & Rumpenhorst, 2000; Grenier *et al.*, 2001), simple sequence repeat primer analysis (Blok & Phillips, 1995), PCR-RAPD (Bendezu *et al.*, 1988; Blok *et al.*, 1997; Grenier *et al.*, 2001; Rumpenhorst & Ayub, 2001), IEF of proteins (Rumpenhorst & Ayub, 2001), 2-DGE of proteins (Grenier *et al.*, 2001), and satellite DNA analysis (Grenier *et al.*, 2001; Plantard *et al.*, 2008). The results of phylogenetic analysis of the ITS-rRNA gene sequences of *Globodera* species parasitising solanaceous plants suggested the presence of a separate group of populations closely related to *G. pallida*, which might represent an undescribed *Globodera* species (Subbotin *et al.*, 2000). After PCR-RAPD, IEF and ITS-rRNA RFLP and sequence analyses of *Globodera* populations collected in South America and Europe, Rumpenhorst and Ayub (2001) also came to the conclusion that a third species might exist within PCN. Analysing the ITS-rRNA and cytb gene sequences, Madani *et al.* (2010) confirmed the possible representation of a third PCN species, and, moreover, suggested additional putative undescribed species for consideration. In our ITS-rRNA trees, these possible candidates are represented by the subclades 3, 5 and 6 of *G. pallida* from South America. Thus, the pale potato cyst nematode, *G. pallida*, may indeed represent a species complex, although we agree with Grenier *et al.* (2001) who emphasised the need to augment molecular data with biological and morphological studies before addressing the exact taxonomic positions of the South American populations.

Comparing the present study with that of Madani *et al.* (2010), we are able more precisely to reconstruct the position of a *Globodera* sample from Argentina previously identified as *G. pallida* by Lax *et al.* (unpubl.). The sequence of the Argentinean sample clustered with those of two unidentified *Globodera* from Chile in our phylogenetic trees. It has been also reported that an unidentified population of *Globodera* recently found in an experimental potato farm in Oregon, USA, had a high degree of similarity in ITS sequences with Argentinean *Globodera* (Nakhla *et al.*, 2010). Thus, we cannot exclude the possibility that the samples from Argentina, Chile and Oregon might be conspecific and represent a new species of *Globodera* parasitising potato. It is interesting that this *Globodera* seems to be more closely related to *G. rostochiensis* and *G. tabacum* rather than to *G. pallida* and that it cannot be formally considered as a member of the *G. pallida* species complex discussed above.

On the validity of *Globodera mexicana*

The Mexican cyst nematode, *Heterodera mexicana*, was described by Campos-Vela (1967) from *Solanum rostratum* Dunal. in Huamantla Valley, Tlaxcala State, Mexico. This cyst nematode does not complete its life cycle on potato, *S. tuberosum*, or *Nicotiana* species. Several years later, however, Golden and Ellington (1972) considered *H. mexicana* as a *nomen nudum* because the description of this nematode was presented in a Ph.D. thesis, which could not be considered as a valid publication for the purpose of species nomenclature. Moreover, after examination of the material of the Mexican cyst nematode, Golden and Ellington (1972) concluded that it was conspecific with *H. tabacum virginiae*. Greet (1972) also agreed with this synonymy and showed that protein pattern profiles obtained from electrophoresis were almost identical between *H. tabacum* subspecies and the Mexican cyst ne-

Fig. 5. *Phylogenetic relationships within populations and species of Punctoderinae* sensu *Krall and Krall, 1978. Strict consensus of 1000 maximum parsimony trees as inferred from the analysis of the ITS-rRNA gene sequences. BS values more than 70% are given in appropriate clades. Newly sequenced samples are indicated by bold font. (1) = sequence from Blok* et al. *(1998); (2) = sequence obtained Ferris* et al. *(1995); (3) = originally identified as* Globodera achilleae*; (4) = originally identified as* G. artemisiae*; (5) = originally identified as* G. hypolysi*; (6) = originally identified as* G. millefolii*; (7) = originally identified as* G. pallida.

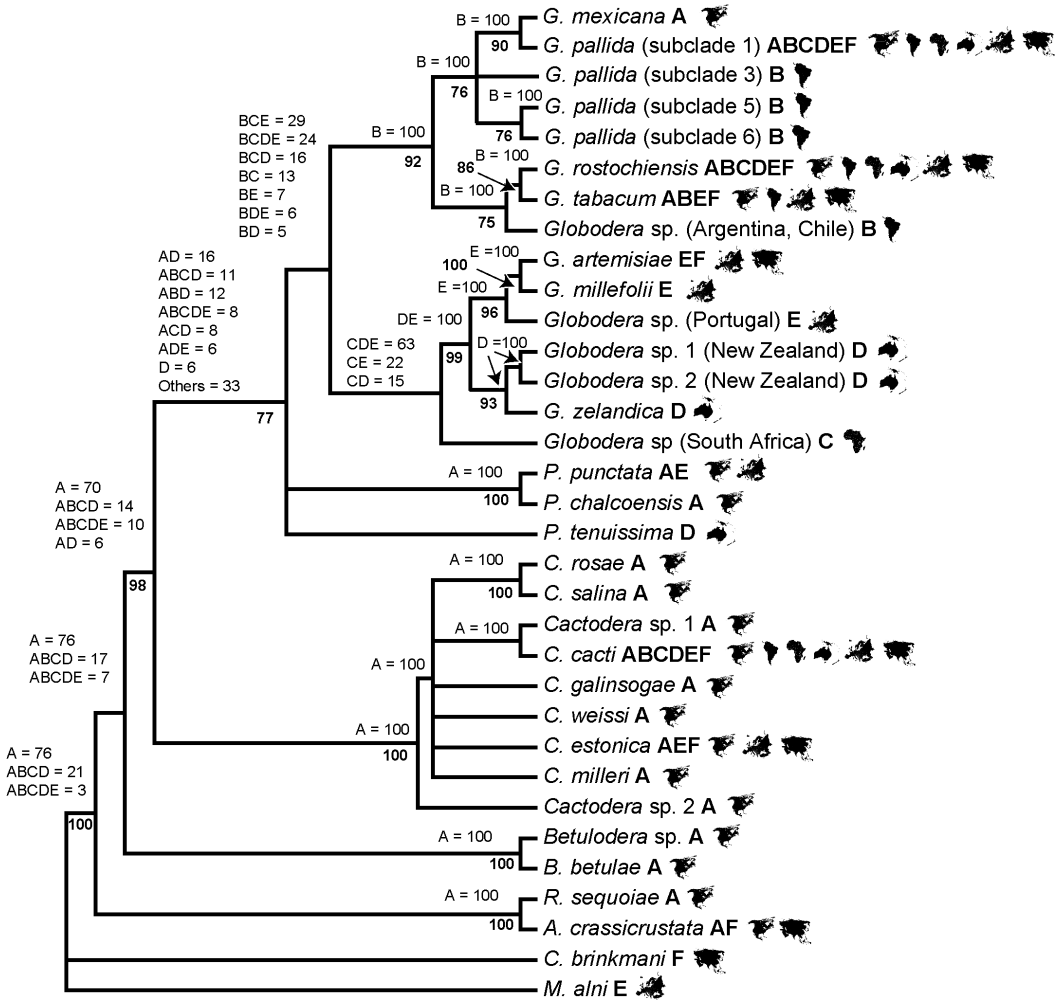


Fig. 6. *Strict consensus of 28 maximum parsimony trees (trees = 1837 steps long) obtained from analysis of the ITS-rRNA gene sequences for species of Punctoderinae* sensu *Krall and Krall, 1978. Letters at nodes indicates putative ancestral areas with frequency of occurrence of the node: A =, North America; B =, South America; C =, Africa; D =, New Zealand (Oceania); E =, Europe; F =, Asia. Letters to the right of taxon name indicate present distribution and thus coding for the ancestral area analysis. BS values more than 70% from 1000 pseudoreplicates are given below branches.*

matode. Stone (1983) suggested that the Mexican cyst nematode should not be given any nomenclatural status, especially in view of the results of comparative analysis of morphometrics and morphology of *G. tabacum* and several Mexican populations, where these species were indistinguishable. A contrasting point of view on relationships of the Mexican cyst nematode with other *Globodera* was presented in several publications by French nematologists (Mugniéry *et al.*, 1992; Bossis & Mugniéry, 1993; Thiéry & Mugniéry, 1996; Thiéry *et al.*, 1997a, b; Marché *et al.*, 2001). Results of two dimensional gel electrophoresis of total proteins (Bossis & Mugniéry, 1993), PCR-ITS-RFLP (Thiéry *et al.*, 1997a; Grenier *et al.*, 2001), PCR-RAPD (Thiéry *et al.*, 1997a) and satellite DNA sequences (Grenier *et al.*, 2001) showed that the Mexican cyst nematode was clearly different from all other *Globodera.* These biochemical and molecular studies showed that *G. mexicana* was more closely related to *G. pallida* than to *G. tabacum*. Moreover, experimental biological studies revealed that *G. mexicana* did not hybridise with *G. tabacum* (Mugniéry *et al.*, 1992), although its males produced viable hybrids when mated with *G. pallida* females

(Thiéry *et al.*, 1997b). Marché *et al.* (2001) also found differences in AFLP profiles between *G. tabacum* and *G. mexicana* and showed that the mean of the genetic distance between *G. mexicana* and *G. tabacum* was greater than the mean genetic distance between the *G. tabacum* subspecies. Such a contradiction between the results given by Golden and Ellington (1972), Greet (1972) and Stone (1983) when compared with those obtained by French nematologists might be explained by the possibility that the conflicting comparisons were actually based on two different species, *i.e.*, *G. tabacum* and the 'true' Mexican cyst nematode (Subbotin *et al.*, 2010). Indirect confirmation of this possibility could be inferred from the result of our molecular characterisation of a *Globodera* sample, labelled as *G. mexicana* from the type locality, and provided to the UCR by L.I. Miller in 1989. ITS-rRNA gene sequences obtained from this sample were similar to those for *G. tabacum*. Because in publications providing lists of species of cyst nematodes the Mexican cyst nematode either was omitted or listed as a *nomen nudum* (Siddiqi, 2000), and considering the fact that the 'true' Mexican cyst nematode is biochemically and molecularly different from other *Globodera*, Subbotin *et al.* (2010) formally described and established this species.

ITS-rRNA gene sequences obtained from a cyst nematode sample found from a wild *Solanum* plant in San Pedro Huehuecalco, Municipio de Amecameca, Estado de Mexico, Mexico, were similar to, and clustered with, those of *G. mexicana*. Thus, this cyst nematode was considered to be conspecific with *G. mexicana*. In our ITS-rRNA phylogenetic trees, *G. mexicana* formed a separate clade related to the subclade I of *G. pallida*. However, the relationships of the Mexican cyst nematode with other species should be confirmed and further studied using more informative gene fragments or genes.

ITS-rRNA gene sequence data support synonymy of some nominal *Globodera* species

Globodera millefolii was described on the basis of a single female collected in 1956 from roots of *Achillea millefolium* L. in Tallinn, Estonia. Some authors (Wouts, 1984; Stone, 1986) considered it as *species inquirenda*, because it was poorly described. Subsequently, re-examination of the holotype by Krall (1977) revealed an error in the description and also led to the conclusion that *G. millefolii* was, in fact, close to *G. achilleae*, a species which had been described from the same host in the former Yugoslavia by Golden and Klindic (1973). After comparison of several descriptions of *G. achilleae* and *G. millefolii*, Subbotin *et al.* (2010) did not find any significant differences in morphology and morphometrics and synonymised *G. achilleae* with *G. millefolii*. Our phylogenetic and sequence analysis of the ITS-rRNA gene sequences of an Estonian population of *G. millefolii* collected close to the type locality with those identified as *G. achilleae* showed that the former clustered within *G. achilleae*. In addition, no significant differences in sequences were resolved between these species. Thus, the ITS-rRNA gene sequence dataset supports synonymy of *G. achilleae* with *G. millefolii*.

Globodera artemisiae isolated from roots of *Artemisia rubripes* Nakai in the Primorsky Territory of the Russian Far East was described by Eroshenko and Kazachenko (1972). In 1976, a similar cyst nematode was found on the roots of *A. princeps* Pamp. in the Shimabara Peninsula, Nagasaki Prefecture, Japan, and described by Ogawa *et al.* (1983) as *G. hypolysi*. Ogawa *et al.* (1983) reported that there were differences between *G. hypolysi* and *G. artemisiae*. However, comparison of the morphometrics of different populations from different locations published by several authors did not support this claim and Subbotin *et al.* (2010) synonymised *G. hypolysi* with *G. artemisiae*. Phylogenetic and sequence analysis of the ITS-rRNA gene sequences of these species did not reveal any significant differences between them and thus supported the synonymisation of *G. hypolysi* with *G. artemisiae*.

ITS-rRNA gene sequence dataset allows correction of species identification from previously published sequences

After studying phylogenetic relationships within some *Globodera* and *Cactodera* species, Ferris *et al.* (1999) found that the ITS-rRNA gene sequences for *G. millefolii*, *G. artemisiae* and *C. salina* were nearly identical and concluded that *G. millefolii* and *G. artemisiae* are more closely related phylogenetically to *Cactodera* than to other nominal *Globodera* species. In our trees the sequences of *G. millefolii* (AF161004) and *G. artemisiae* (AF161003) submitted to Genbank by Ferris *et al.* (1999) clustered within *C. salina*, but our newly obtained ITS sequence of *G. millefolii* obtained from a sample provided by E. Krall from Estonia clustered with other samples of this species and *G. artemisiae*. On this basis we concluded that sequences previously named as *G. millefolii* (AF161004) and *G. artemisiae* (AF161003) were misidentified and likely belonged to *C. salina*.

Sequence and phylogenetic analysis allows the correction of erroneous species identifications for two other

samples. The results of a Blast search of a sequence under the name of '*Cactodera estonica*' (AY692355) across the GenBank database revealed that it is very similar to that of *H. glycines* and is considered here as belonging to that species. In addition, a sequence identified as '*G. artemisiae*' from Northern Ireland (EU935420) by Moreland and Fleming (unpubl.) clustered with those for *G. millefolii* in our trees and likely belongs to this latter species.

Phylogenetic relationships within genera of circumfenestrate cyst nematodes

Our analyses support monophyly of the genera *Betulodera*, *Cactodera* and *Punctodera.* According to results of the ITS (present study) and D2-D3 of 28S rRNA (Subbotin *et al.*, 2010) gene sequence analysis, *Betulodera* occupies a basal position within Punctoderinae which is further nested within the *Cactodera* clade, thus confirming Ferris's (1979) hypothesis of cyst nematode evolution reconstructed using a cladistic morphological approach. Relationships between other genera are still not well resolved by the ITS sequence data. Recognition of *Globodera* as a monophyletic genus is not clear from our BI and MP analyses of the full dataset (Figs 4, 5), although this taxon is monophyletic in MP trees obtained from the reduced dataset (Fig. 6). It has been previously remarked that the observed paraphyly of *Globodera* may simply reflect the general tendency of phylogenetic algorithms to produce unbalanced trees rather than to define any true evolutionary history of a group with high rates of evolution. Genetic divergence within this genus is reflected by the species groupings based on geographical origin and host plant specialisation (Subbotin *et al*., 2001).

Phylogeography and origin of Punctoderinae

The phylogeographic analysis using ITS-rRNA gene sequences provided new insight into the pattern and origin of distribution of circumfenestrate cyst nematodes. Our results show that North America appears to be a centre of early evolution and the area from which subsequent dispersal of Punctoderinae occurred. Indeed, in North America the greatest number of related circumfenestrate cyst nematode species and genera occur: 12 of the 13 known *Cactodera* species, three of four known *Punctodera*, all known *Betulodera* and *Dolichodera* species, a recently described new cyst nematode, *Vittatidera zeaphila* (Bernard *et al*., 2010), and several *Globodera* species. Of six valid Punctoderinae genera, only *Paradolichodera* was described from New Zealand and has never been recorded in North America or in any other region of the world.

Another proposed approach to determine a centre of origin is to identify the area in which the most evolutionarily primitive representatives, closest in form to the supposed ancestral group, occur, with the assumption that they are not likely to have dispersed far from the centre of origin. Phylogenetic analyses using morphological (Krall & Krall, 1978; Wouts, 1985; Baldwin & Schouest, 1990) and molecular (Subbotin *et al*., 2006, 2010) datasets revealed that extant Ataloderinae and Meloidoderinae share common ancestors with cyst nematodes. The species of these subfamilies parasitise a rather wide spectrum of plants, including gymnosperms and angiosperms. The ability of some species of *Meloidodera*, *Cryphodera* (Meloidoderinae) and *Rhizonema sequoiae* (Ataloderinae) to parasitise plants from *Pinus* and *Sequoia*, respectively, is consistent with the argument of Krall and Krall (1978) that suggests an ancient origin for these genera. The distribution of extant Meloidoderinae includes North America, Europe, Asia and Oceania, whilst most representatives of Ataloderinae are found in North America (*Ekphymatodera*, *Sarisodera*, *Rhizonema*, *Bellodera*, *Atalodera*). However, some species of *Atalodera* are not restricted to North America and have been described from South America and Asia. In addition, only two Ataloderinae genera, *Hylonema* and *Camelodera*, are reported from Africa and Asia, respectively. Thus, the general pattern of modern distribution of non-cyst nematodes sharing a common ancestor with Punctoderinae favours the hypothesis of a North American origin of circumfenestrate cyst nematodes.

Although *Cactodera* and *Punctodera* putatively originated, and began to diversify, in North America, results of our phylogeographical analysis, in which we included several new findings of undescribed species of *Globodera* from New Zealand and Africa, revealed that South America or Africa appears to be a centre of origin of *Globodera.* Thus, Stone's (1979) hypothesis of an 'out-of-the-west' Gondwana origin of *Globodera* with subsequent dispersal of the species of this genus to Europe, North America, Asia and Oceania found some support from the ITS-rRNA dataset.

Absence of any fossil records and unequal rates of rRNA gene evolution do not allow reliable application of molecular clock approaches to estimate divergence time for different cyst nematode lineages. It has been assumed that the present biogeographical patterns for cyst nematodes can be explained mainly on the basis

of a vicariance model considering the geological history of earth with continental drift. The ages of cyst nematode divergences have also been estimated using this approach. Picard *et al.* (2008) considered the age of the *Cactodera*-(*Punctodera* + *Globodera*) divergence to be close to the Heteroderinae-Punctoderinae divergence, which might have occurred after separation of Laurasia and Gondwana, *i.e*., 173-130 million years ago. The divergence of the South American and Laurasian *Globodera* lineages was considered to have occurred between 80 and 60 million years ago after the break of a temporary connection between North and South America in the Palaeocene. However, if we consider the estimated ages of host plants presently associated with non-cyst and cyst nematodes, slightly younger dates of divergence between nematode lineages could be suggested. For example, the age of Meloidoderinae associated with *Pinus* could be proposed as the time when *Pinus* diverged from the other genera, *i.e*., 140 million years ago. (Wang *et al*., 2000). For Ataloderinae parasitising *Sequoia*, it might be the late Cretaceous (99-65 million years ago) and Tertiary, periods from which *Sequoia* fossil records are primarily known (Page, 1990). The late Cretaceous is also considered as a possible time period for origin, diversification and spread of various Poaceae (Prasad *et al.*, 2005), which are reported to be hosts for some Ataloderinae. From this line of reasoning one could speculate on the Heteroderinae-Punctoderinae divergence occurring between 90 and 70 million years ago. The divergence of the two main *Globodera* lineages is associated with the time of origin for the Solanaceae, *i.e*., 65-51 million years ago (Wikström *et al.*, 2001; Paape *et al*., 2008). Thus, the split of the two *Globodera* lineages might have occurred subsequent to the break up of Africa and South America in the Mid-Cretaceous, and it seems that the evolution of Punctoderinae cannot be explained solely by the separation of the continents and diffusion expansion. Recent advances in phylogenetics and, in particular, molecular dating, indicate that transoceanic dispersal has played an important role in shaping plant and animal distributions, thereby obscuring any effect of tectonic history (Vanderpoorten *et al.*, 2010). Our results also suggest that 'jump dispersal' might play a significant role in the biogeographic history of cyst nematodes. The presence of almost identical ITS-rRNA sequences among geographically disjunct populations of *Heterodera mani* and *H. avenae* from North America and Europe (Subbotin, unpubl.) or *H. aucklandica* (Subbotin *et al.*, 2003) from Europe and New Zealand might be explained by dispersal events. Thus, we hypothesise that long distance dispersal might even have occurred repeatedly during cyst nematode evolution, including within the Punctoderinae, giving rise to new species with their subsequent migration. We suggest a scenario whereby the ancestral Punctoderinae gave rise to a modern *Globodera* lineage that was introduced to South America or Africa *via* long distance dispersal from North America. Potential dispersal of cyst nematodes by wind has been noted and discussed by several authors (see: Lehman, 1994), although more precise research is required to further test this hypothesis.

In the present study, we made several assumptions considering that: *i*) the analysed dataset for heteroderids reflects actual distribution rather than differences in worldwide sampling; *ii*) the phylogenetic ITS-rRNA gene tree reflects species and genus evolution; and *iii*) the applied dispersal-vicariance analysis with DIVA accurately reconstructs ancestral areas and the history of the circumfenestrate cyst nematodes. Alternative datasets and methods should be used to increase the robustness of biogeographic hypotheses for cyst nematodes and further test the findings made in the present study.

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References

AYUB, M. & RUMPENHORST, H.J. (2000). Genetic variation in potato cyst nematode (*Globodera pallida*) from Europe and South America as revealed by PCR-RFLP of ITS regions. In: *Abstracts der 28 Tagung des Arbeitskreises Nematologie, 1-2 März 2000, Vietshöchheim*, pp. 36-37.

BALDWIN, J.G. & SCHOUEST JR, L.P. (1990). Comparative detailed morphology of the Heteroderinae Filipjev & Schuurmans Stekhoven, 1941, *sensu* Luc *et al.* (1988): phylogenetic systematics and revised classification. *Systematic Parasitology* 15, 81-106.

BENDEZU, I.F., EVANS, K., BURROWS, P.R., DE POMERAI, D. & CANTO-SAENZ, M. (1998). Inter- and intra-specific variability of the potato cyst nematodes *Globodera pallida* and *G. rostochiensis* from Europe and S. America using RAPD-PCR. *Nematologica* 44, 49-61.

BERNARD, E.C., HANDOO, Z.A., POWERS, T.O., DONALD, P.A. & HEINZ, R.D. (2010). *Vittatidera zeaphila* (Nematoda: Heteroderidae). A new genus and species of cyst nematode parasitic on corn (*Zea mays*). *Journal of Nematology* 42, in press.

BLOK, V.C. & PHILLIPS, M.S. (1995). The use of repeat sequence primers for investigating genetic diversity between populations of potato cyst nematodes with differing virulence. *Fundamental and Applied Nematology* 18, 575-582.

BLOK, V.C., PHILLIPS, M.S. & HARROWER, B.E. (1997). Comparison of British populations of potato cyst nematodes with populations from continental Europe and South America using RAPDs. *Genome* 40, 286-293.

BLOK, V.C., MALLOCH, G., HARROWER, B., PHILLIPS, M.S. & VRAIN, T.C. (1998). Intraspecific variation in ribosomal DNA in populations of the potato cyst nematode *Globodera pallida*. *Journal of Nematology* 30, 262-274.

BOSSIS, M. & MUGNIÉRY, D. (1993). Specific status of six *Globodera* parasites of solanaceous plants studied by means of two-dimensional gel electrophoresis with a comparison of gel patterns by a computed system. *Fundamental and Applied Nematology* 16, 47-56.

BULMAN, S.R. & MARSHALL, J.W. (1997). Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New Zealand Journal of Crop and Horticultural Science* 25, 123-129.

CAMPOS-VELA, A. (1967). *Taxonomy*, *life cycle and host range of* Heterodera mexicana *n. sp. (Nematoda: Heteroderidae).* Ph.D. Thesis, University of Wisconsin, 70 pp.

EROSHENKO, A.S. & KAZACHENKO, I.P. (1972). [*Heterodera artemisiae* sp. n. (Nematoda: Heteroderidae) – a new cyst forming nematode species from Primorsk Territory.] *Parazitologiya* 6, 166-170.

FERRIS, V.R. (1979). Cladistic approaches in the study of soil and plant parasitic nematodes. *American Zoologist* 19, 1195-1215.

FERRIS, V.R., FERRIS, J.M. & FAGHIHI, J. (1993). Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. *Fundamental and Applied Nematology* 16, 177-184.

FERRIS, V.R., MILLER, L.I., FAGHIHI, J. & FERRIS, J.M. (1995). Ribosomal DNA comparisons of *Globodera* from two continents. *Journal of Nematology* 27, 273-283.

FERRIS, V.R., KRALL, E., FAGHIHI, J. & FERRIS, J.M. (1999). Phylogenetic relationships of *Globodera millefolii*, *G. artemisiae*, and *Cactodera salina* based on ITS region of ribosomal DNA. *Journal of Nematology* 31, 498-507.

FERRIS, V.R., SABO, A., BALDWIN, J.G., MUNDO-OCAMPO, M., INSERRA, R.N. & SHARMA, S. (2004). Phylogenetic relationships among selected Heteroderoidea based on 18S and ITS ribosomal DNA. *Journal of Nematology* 36, 202-206.

GOLDEN, A.M. & ELLINGTON, D.M.S. (1972). Redescription of *Heterodera rostochiensis* (Nematoda: Heteroderidae) with a key and notes on closely related species. *Proceedings of the Helminthological Society of Washington* 39, 64-77.

GOLDEN, A.M. & KLINDIC, O. (1973). *Heterodera achilleae* n. sp. (Nematoda, Heteroderidae) from yarrow in Yugoslavia. *Journal of Nematology* 5, 196-201.

GREET, D.N. (1972). Electrophoresis and morphometrics of round-cyst nematodes. *Annals of Applied Biology* 71, 283-286.

GRENIER, E., BOSSIS, M., FOUVILLE, D., RENAULT, L. & MUGNIÉRY, D. (2001). Molecular approaches to the taxonomic position of Peruvian potato cyst nematodes and gene pool similarities in indigenous and imported populations of *Globodera*. *Heredity* 86, 277-290.

GRENIER, E., FOURNET, S., PETIT, E. & ANTHOINE, G. (2010). A cyst nematode 'species factory' called the Andes. *Nematology* 12, 163-169.

HUELSENBECK, J.P. & RONQUIST, F. (2001). MrBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754-755.

KNOETZE, R., MALAN, A.P. & MOUTON, C. (2006). Differentiation of South African potato cyst nematodes (PCN) by analysis of the rDNA internal transcribed spacer region. *African Plant Protection* 12, 103-110.

KRALL, E. (1977). Compendium of cyst nematodes in the USSR. *Nematologica* 23, 311-332.

KRALL, E.L. & KRALL, H.A. (1978). [Revision of the plant nematodes of the family Heteroderidae on the basis of trophic specialization of these parasites and their co-evolution with their host plants.] In: *Fitogel'mintologicheskie Issledovaniya.* Moscow, USSR, Nauka, pp. 39-56.

LEHMAN, P.S. (1994). *Dissemination of phytoparasitic nematodes*. Nematology Circular No. 208, Florida Department of Agriculture and Consumer Services, Division of Plant Industry, 4 pp.

LUC, M., MAGGENTI, A.R. & FORTUNER, R. (1988). A reappraisal of Tylenchina (Nemata). 9. The family Heteroderidae Filipjev & Schuurmans Stekhoven, 1941. *Revue de Nématologie* 11, 159-176.

MADANI, M., SUBBOTIN, S.A., WARD, L.J. & DE BOER, S.H. (2010). Molecular characterization of Canadian populations of potato cyst nematodes, *Globodera rostochiensis* and *G. pallida* using ribosomal nuclear RNA and cytochrome b genes. *Canadian Journal of Plant Pathology* 32, 252-263.

MANDURIC, S. & ANDERSSON, S. (2004). The identity of a Swedish *Globodera* (Nematoda: Heteroderidae) population, following comparisons with known populations of *G. artemisiae* (Eroshenko & Kazachenko, 1972) Behrens, 1975. *Russian Journal of Nematology* 12, 39-44.

MARCHÉ, L., VALETTE, S., GRENIER, E. & MUGNIÉRY, D. (2001). Intraspecies DNA polymorphism in the tobacco cyst nematode complex (*Globodera tabacum*) using AFLP. *Genome* 44, 941-946.

MUGNIÉRY, D., BOSSIS, M. & PIERRE, J.-S. (1992). Hybridations entre *Globodera rostochiensis* (Wollenweber), *G. pal-*

lida (Stone), *G. virginiae* (Miller and Gray), *G. solanacearum* (Miller and Gray) et *G. "mexicana"* (Campos-Vela). Description et devenir des hybrides. *Fundamental and Applied Nematology* 15, 375-382.

NAKHLA, M.K., OWENS, H.J., LI, W., WEI, G., SKANTAR, A.M. & LEVY, L. (2010). Multiplex Real-Time PCR assays for the identification of the potato cyst and tobacco cyst nematodes. *Plant Disease* 94, 959-965.

NYLANDER, J.A.A. (2002). *MrModeltest v1.0b*. Dept of Systematic Zoology, Uppsala University. Available online at http://www.ebc.uu.se/systzoo/staff/nylander.html

OGAWA, Y., OHSHIMA, Y. & ICHINOHE, M. (1983). *Globodera hypolysi* n. sp., a mugwort cyst nematode from Japan. *Japanese Journal of Nematology* 12, 41-46.

PAAPE, T., IGIC, B., SMITH, S.D., OLMSTEAD, R., BOHS, L. & KOHN, J.R. (2008). A 15-myr old genetic bottleneck. *Molecular Biology and Evolution* 25, 655-663.

PAGE, C.N. (1990). Coniferophytina. In: Kabitzki, K., Rohwer, J.C. & Bittrich, V. (Eds). *The families and genera of vascular plants*, Vol. 1. Berlin, Germany, Springer, pp. 282-361.

PICARD, D., SEMPERE, T. & PLANTARD, O. (2007). A northward colonisation of the Andes by the potato cyst nematode during geological times suggests multiple host-shifts from wild to cultivated potatoes. *Molecular Phylogenetics and Evolution* 42, 308-316.

PICARD, D., SEMPERE, T. & PLANTARD, O. (2008). Direction and timing of uplift propagation in the Peruvian Andes deduced from molecular phylogenetics of highland biotaxa. *Earth and Planetary Science Letters* 271, 326-336.

PLANTARD, O., PICARD, D., VALETTE, S., SCURRAH, M., GRENIER, E. & MUGNIÉRY, D. (2008). Origin and genetic diversity of Western European populations of the potato cyst nematode *Globodera pallida* inferred from mitochondrial sequences and microsatellite loci. *Molecular Ecology* 17, 2208-2218.

PRASAD, V., STRÖMBERG, C.A.E., ALIMOHAMMADIAN, H. & SAHNI, A. (2005). Dinosaur coprolites and the early evolution of grasses and grazers. *Science* 310, 1177-1180.

PYLYPENKO, L.A., PHILLIPS, M.S. & BLOK, V.C. (2008). Characterisation of two Ukrainian populations of *Globodera pallida* in terms of their virulence and mtDNA, and the biological assessment of a new resistant cultivar Vales Everest. *Nematology* 10, 585-590.

REID, A. & PICKUP, J. (2005). Molecular characterization of a morphologically unusual potato cyst nematode. *EPPO Bulletin* 35, 69-70.

RONQUIST, F. (1997). Dispersal-vicariance analysis: a new approach to the quantification of historical biogeography. *Systematic Biology* 46, 195-203.

RONQUIST, F. (2001). DIVA version 1.2. Computer program for MacOS and Win32. Evolutionary Biology Centre, Uppsala University. Available online at http://www.ebc.uu.se/systzoo/research/diva/diva.html

RUMPENHORST, H.J. & AYUB, M. (2001). Gibt es eine dritte Art beim Kartoffelnematoden? *Die 29 Tagung des Arbeitskreises Nematologie*, Monheim, 14-15, März, 2001.

SABO, A., REIS, L.G.L., KRALL, E., MUNDO-OCAMPO, M. & FERRIS, V.R. (2002). Phylogenetic relationships of a distinct species of *Globodera* from Portugal and two *Punctodera* species. *Journal of Nematology* 34, 263-266.

SIDDIQI, M.R. (1986). *Tylenchida: parasites of plants and insects.* Farnham Royal, UK, Commonwealth Agricultural Bureaux, 645 pp.

SIDDIQI, M.R. (2000). *Tylenchida: parasites of plants and insects*, 2nd edition. Wallingford, UK, CABI Publishing, 833 pp.

SIRCA, S. & UREK, G. (2004). Morphometrical and ribosomal DNA sequence analysis of *Globodera rostochiensis* and *Globodera achilleae* from Slovenia. *Russian Journal of Nematology* 12, 161-168.

SKANTAR, A.M., HANDOO, Z.A., CARTA, L.K. & CHITWOOD, D.J. (2007). Morphological and molecular identification of *Globodera pallida* associated with potato in Idaho. *Journal of Nematology* 39, 133-144.

SOUTHEY, J.F. (ED.) (1986). *Laboratory methods for work with plant and soil nematodes*. Reference Book 402, London, UK, Ministry of Agriculture Fisheries and Food, Her Majesty's Stationery Office, 202 pp.

STONE, A.R. (1979). Co-evolution of nematodes and plants. *Symbolae Botanicae Upsaliensis* 22, 46-61.

STONE, A.R. (1983). Three approaches to the status of a species complex, with a revision of some species of *Globodera*. In: Stone, A.R., Platt, H.M. & Khalil, L.F. (Eds). *Concepts in nematode systematics*. London, UK, Academic Press, pp. 221-223.

STONE, A.R. (1986). Taxonomy and phylogeny of cyst nematodes. In: Lamberti, F. & Taylor, C.E. (Eds). *Cyst nematodes*. London, UK, Plenum Press, pp. 1-21.

STURHAN, D. (2007). Wirte, Verbreitung and Verwandtschaftsbeziehungen von *Globodera* und verwandten Zystennematoden. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 59, 16.

STURHAN, D., WOUTS, W.M. & SUBBOTIN, S.A. (2007). An unusual cyst nematode from New Zealand, *Paradolichodera tenuissima* gen. n., sp n. (Tylenchida: Heteroderidae). *Nematology* 9, 561-571.

SUBBOTIN, S.A., HALFORD, P.D., WARRY, A. & PERRY, R.N. (2000). Variations in ribosomal DNA sequences and phylogeny of *Globodera* parasitising Solanaceae. *Nematology* 2, 591-604.

SUBBOTIN, S.A., VIERSTRAETE, A., DE LEY, P., ROWE, J., WAEYENBERGE, L., MOENS, M. & VANFLETEREN, J.R. (2001). Phylogenetic relationships within the cyst-forming nematodes (Nematoda, Heteroderidae) based on analysis of sequences from the ITS regions of ribosomal DNA. *Molecular Phylogenetics and Evolution* 21, 1-16.

SUBBOTIN, S.A., STURHAN, D., RUMPENHORST, H.J. & MOENS, M. (2003). Molecular and morphological characterisation of the *Heterodera avenae* complex species (Tylenchida: Heteroderidae). *Nematology* 5, 515-538.

SUBBOTIN, S.A., STURHAN, D., CHIZHOV, V.N., VOVLAS, N. & BALDWIN, J.G. (2006). Phylogenetic analysis of Tylenchida Thorne, 1949 as inferred from D2 and D3 expansion fragments of the 28S rRNA gene sequences. *Nematology* 8, 455-474.

SUBBOTIN, S.A., MUNDO-OCAMPO, M. & BALDWIN, J.G. (2010). *Systematics of cyst nematodes (Nematoda: Heteroderinae). Nematology Monographs and Perspectives 8A* (Series Editors: Hunt, D.J. & Perry, R.N.). Leiden, The Netherlands, Brill, 351 pp.

SWOFFORD, D.L. (2003). *PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b 10.* Sunderland, Massachusetts, USA, Sinauer Associates.

TANHA MAAFI, Z., SUBBOTIN, S.A. & MOENS, M. (2003). Molecular identification of cyst-forming nematodes (Heteroderidae) from Iran and a phylogeny based on ITS-rDNA sequences. *Nematology* 5, 99-111.

THIÉRY, M. & MUGNIÉRY, D. (1996). Interspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of Solanaceous plants. *Fundamental and Applied Nematology* 19, 471-479.

THIÉRY, M., MUGNIÉRY, D., FOUVILLE, D. & SCHOTS, A. (1996). Natural hybridization between *Globodera rostochiensis* and *G. pallida*. *Fundamental and Applied Nematology* 19, 437-442.

THIÉRY, M., FOUVILLE, D. & MUGNIÉRY, D. (1997a). Intra- and interspecific variability in *Globodera*, parasites of Solanaceous plants, revealed by Random Amplified Polymorphic DNA (RAPD) and correlation with biological features. *Fundamental and Applied Nematology* 20, 495-504.

THIÉRY, M., MUGNIÉRY, D., BOSSIS, M. & SOSA-MOSS, C. (1997b). Résultats de croisements entre *Globodera pallida* Stone et *G. "mexicana"* Campos-Vela: héritabilité du développement sur pomme de terre et noyion d'espèce. *Fundamental and Applied Nematology* 20, 551-556.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. (1997). The CLUSTAL X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876-4882.

VANDERPOORTEN, A., GRADSTEIN, S.R., CARINE, M.A. & DEVOS, N. (2010). The ghosts of Gondwana and Laurasia in modern liverwort distributions. *Biological Reviews of the Cambridge Philosophical Society* 85, 471-487.

WANG, X.-Q., TANK, D.C. & SANG, T. (2000). Phylogeny and divergence times in Pinaceae: evidence from three genomes. *Molecular Biology and Evolution* 17, 773-781.

WIKSTRÖM, N., SAVOLAINEN, V. & CHASE, M.W. (2001). Evolution of the angiosperms: calibrating the family tree. *Proceedings of the Royal Society of London, Series B, Biological Sciences* 268, 2211-2220.

WOUTS, W.M. (1984). *Globodera zelandica* n. sp. (Nematoda: Heteroderidae) from New Zealand, with a key to the species of the genus *Globodera*. *New Zealand Journal of Zoology* 11, 129-135.

WOUTS, W.M. (1985). Phylogenetic classification of the family Heteroderidae (Nematoda: Tylenchida). *Systematic Parasitology* 7, 295-328.

WOUTS, W.M., RUMPENHORST, H.J. & STURHAN, D. (2001). *Heterodera betae* sp. n., the yellow beet cyst nematode (Nematoda: Heteroderidae). *Russian Journal of Nematology* 9, 33-42.

YU, Y., HARRIS, A.J. & HE, X. (2010). S-DIVA (Statistical Dispersal-Vicariance Analysis): a tool for inferring biogeographic histories. *Molecular Phylogenetics and Evolution* 56, 848-850.