Integrative diagnosis and parasitic habits of *Cryphodera brinkmani* a non-cyst forming heteroderid nematode intercepted on Japanese white pine bonsai trees imported into Italy

Nicola Vovlas[1], Nicola Trisciuzzi[2], Alberto Troccoli[1], Francesca De Luca[1], Carolina Cantalapiedra-Navarrete[3], Juan E. Palomares-Rius[4], and Pablo Castillo[3]

[1] *Istituto per la Protezione delle Piante (IPP), Consiglio Nazionale delle Ricerche (CNR), U.O.S. di Bari, Via G. Amendola 122/D, 70126 Bari, Italy*

[2] *Centro Ricerca e Sperimentazione in Agricoltura (CRSA) "Basile Caramia", Via Cisternino, 281 Locorotondo (BA), Italy*

[3] *Instituto de Agricultura Sostenible (IAS), Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 4084, 14080 Córdoba, Campus de Excelencia Internacional Agroalimentario, ceiA3, Spain*

[4] *xxxxxxxxxxxxxxxxxx, Japan*

Received: _____/Accepted _____.

*Author for correspondence: P. Castillo

E-mail: p.castillo@csic.es

Fax: +34957499252

Short Title: *New* Heterodera elachista *on corn in northern Italy*

Abstract The non-cyst forming heteroderid nematode *Cryphodera brinkmani* was detected in Italy parasitizing roots of Japanese white pine bonsai (*Pinus parviflora*) trees imported from Japan. Morphology and morphometrical traits of the intercepted population on this new host for *C. brinkmani* were in agreement with the original description, except for some minor differences on male morphology. Integrative molecular data for this species were obtained using D2-D3 expansion regions of 28S rDNA, ITS1-rDNA, the partial 18S rDNA, and the protein-coding mitochondrial gene, cytochrome oxidase c subunit I (*COI*). The phylogenetic relationships of this species with other representatives of non-cyst and cyst-forming Heteroderidae using ITS1 are presented and indicated that *C. brinkmani* clustered together with other *Cryphodera* spp. and with *Meloidodera alni* suggesting a monophyletic origin of non-cyst forming nematodes (Heteroderinae *sensu* Luc et al. 1988), which have been considered close to the ancestor of most species of Heteroderidae. Histological observations of *P. parviflora* feeder roots infected by *C. brinkmani* indicated that nematode females induce similar anatomical alterations to those reported for *C. kalesari* and consisting of formation of a single uninucleate giant cell (nurse cell) with hypertrophied nucleus, prominenet nucleolus, thickened cell wall and expanding into the stele and in contact of xylem, vacuum cambium and phloem. These findings are in agreement with the results of the phylogenetic analysis and indicate a close relationship in the plant responses induced by *Cryphodera* nematode females with those caused by the genetically related *Meloidodera* spp., which also induce formation of a uninucletate giant cell.

Keywords: Alien nematode pest interception, Cytochrome c oxidase subunit 1 (*COI*), Heteroderidae, Histopathology, Japanese white pine nematode, rDNA, Taxonomy.

Introduction

The arrival of alien plant-parasitic nematodes into a new habitat may affect its diversity, productivity and natural biological equilibrium. International trade has long been recognized as a major pathway by which non-indigenous plant pathogens and pests are moved into new areas (McCullough et al. 2006). The use of nematode-infested nursery stock and other plant material intended for propagation or landscape beautification from distant geographical areas facilitates the spread of alien nematode pests into new habitats (McCullough et al. 2006). Imported bonsai plants from Asia have been involved in the spread of alien nematode pests into Europe and the Americas (Roques and Auger-Rozenberg 2006; Quénéhervé et al.1998).

The trade of woody and pine bonsai trees has played an important role in the arrival of *Cryphodera* species into Europe and USA (Wouts 1985) from Japan, where undescribed *Cryphodera* species on pine and black mulberry where reported by Hirata and Yuhara (1986) and Toida (1984), respectively. A new species of this genus *Cryphodera brinkmani* was described by Karssen and Van Aelst (1999) from pine (*Pinus thunbergii* Parlatore) bonsai trees imported into the Netherlands from Japan. Subsequently, this *Cryphodera* was detected in Italy (2010) on another pine species, the Japanese white pine (*Pinus parviflora* Siebold and Zucc.) imported also from Japan.

The genus *Cryphodera*, which was erected by Colbran (1966) with the type species *Cryphodera eucalypti,* includes at present six nominal species, which parasitize forest trees in Australia, India, Japan and New Zealand (Wouts 1973). Other undescribed species were detected in Vietnam (Nguyen et al. 2011) (Table 1). However, little information exists on the host-parasite relationships between these nematodes and their host-plants, except for *Cryphodera kalesari* Bajaj, Walia, Dabur and Bhatti 1989, which parasitizes roots of Indian

laurel (*Terminalia tomentosa* Wight and Arn.), in the Himalayas forests, and induces a unique uninucleate giant cell in the stelar region (Bajaj and Walia 2000).

The description of *C. brinkmani* was based on the results of the analysis of the morphological features of this species, which was illustrated by schematic drawings. Molecular data for this species and undescribed Vietnamese *Cryphodera* were obtained by Subbotin et al. (2001). However, the phylogenetic relationship of *C. brinkmani* with other species of Heteroderidae needs to be supplemented by additional analyses.

The objectives of the present study were: 1) to provide additional morpho-biological information of *C. brinkmani*, by illustrating in more details the features of the life stages and those of the male that were not included in the original description; 2) to integrate the molecular characterization of this nematode species with previous studies; and 3) to confirm the host-parasite relationships of *C. brinkmani* on Japanese white pine plants with those reported in literature for other *Cryphodera* species.

Table 1

Materials and methods

Morphological identification

Specimens of *C. brinkmani* used in this study were isolated from infested soil and root samples collected from Japanese white pine bonsai trees imported from Japan into central Italy by a commercial nursery (Pistoia, Pistoia Province). The population used in this study was extracted by magnesium sulphate ($MgSO_4$) centrifugal flotation method (Coolen 1979). Nematodes were preliminarily identified by morphological features. Specimens for microscopic observation were killed by gentle heat, fixed in a solution of 4% of

formaldehyde + 1% propionic acid, and processed to glycerol according to Seinhorst's method (Hooper, 1986). Photographs were taken with a Leica DFC 425 system mounted on a Leitz Wetzlar optical microscope, on specimens mounted on water agar temporary slides (Troccoli 2002), whereas measurements and drawings were made at the *camera lucida* on glycerine infiltrated specimens. Abbreviations used in Table 2 are defined in Siddiqi (2000).

Molecular identification

For molecular analyses, two live nematodes were temporarily mounted in a drop of 1M NaCl containing glass beads and after taking measurements and photomicrographs of diagnostic characters the slides were dismantled and DNA extracted. Nematode DNA was extracted from single individuals and PCR assays were conducted as described by Castillo et al. (2003). The D2-D3 expansion segments of 28S rDNA was amplified using the D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (Castillo et al. 2003). The ITS1 region was amplified using forward primer TW81 (5′ GTTTCCGTAGGTGAACCTGC-3′) and reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) as described in Subbotin et al. (2001); and the partial 18S was amplified using primers A (5'-AAAGATTAAGCCATGCATG-3'), 13R (5'-GGGCATCACAGACCTGTTA-3') and 18P-SSU_R_81 (5' TGATCCWKCYGCAGGTTCAC-3') (Boutsika et al. 2004). Finally, the portion of the *COI* gene was amplified as described by Derycke et al. (2005) using forward primer JB3 (5'- TTTTTTGGGCATCCTGAGGTTTAT -3') and reverse primer JB4 (5'-TAAAGAAAGAACATAATGAAAATG -3').

PCR products were purified after amplification using ExoSAP-IT (Affymetrix, USB products, High Wycombe, UK), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct sequencing in both directions

using the primers referred above. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyser; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA), at the SCAI, University of Córdoba sequencing facilities (Córdoba, Spain). The newly obtained sequences were submitted to the GenBank database under accession numbers JQ965677, JQ965678, JQ965679, JQ965680.

Phylogenetic analysis

ITS1 rDNA sequences of different Heteroderidae including selected cyst and non-cyst forming species from GenBank (two sequences for each genus) were used for phylogenetic reconstruction, and *Xiphinema index* (JF437918) as outgroup taxa. The newly obtained and published sequences for each gene were aligned using Clustal W (Thompson et al. 1994) with default parameters. Sequence alignments were manually edited using BioEdit (Hall et al. 1999). Phylogenetic analyses of the sequence data sets were performed with Maximum Likelihood (ML) using PAUP * 4b10 (Swofford 2003) and Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). The best fitted model of DNA evolution was obtained using jModelTest v. 0.1.1 (Posada 2008) with the Akaike Information Criterion (AIC). The Akaike-supported model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then used in phylogenetic analyses. BI analysis for ITS1 region under the TVM + I + G model was initiated with a random starting tree and run with the four Metropolis-coupled Markov chain Monte Carlo (MCMC) for 4 x 10^6 generations. The MCMC were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority rule

consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees were visualised using TreeView (Page 1996). In ML analysis the estimation of the support for each node was obtained by bootstrap analysis with 100 heuristic replicates.

Histopathology

For histopathological observations infected roots were gently washed free of adhering soil and debris. Individual infected and healthy root pieces were fixed in FAA (formaldehyde-acetic acid- alcohol) for a minimum of 48 h, dehydrated in tertiary butyl alcohol series (70-85-90-100%) and embedded in Histosec® embedding paraffin melting point 56-58°C (Merck, Darmstadt, Germany). Embedded tissues were sectioned transversely and longitudinally at 10-12 µm with a rotary microtome, mounted on glass slides, stained with safranin and fast green (Johansen 1940), mounted permanently and observed with an optical microscope. Images were taken with a Leica DFC 425 system.

Results and discussion

Morphological observations of the intercepted *Cryphodera brinkmani* population

Morphology and morphometrics of female, male and second-stage juveniles fit closely with those of the original description of the species (Karssen and Van Aelst 1999). Additional morphological and morphometric data of males of the present population are illustrated in Figs. 1-3 and Table 2. Nearly 50% of second-stage juvenile specimens of the present population exhibited a small mucro at the end of tail hyaline portion, as illustrated in Fig. 2F. Males were rare in our population (8-10/500 cm^3 of soil) as reported in the original description. They were described as having two lip annuli in lateral view, however

specimens of our population showed, in lateral view, either three lip annuli on both sides, or two on a side (basal one larger, slightly indented) and three on the other side, as illustrated in Fig. 3 A-C. Lateral fields, in males and second-stage juveniles, with three incisures, delimiting two frequently areolated bands (Fig. 3D). Vulva-anus profiles in swollen females slightly concave (Fig. 2A, M) to almost flat. Vulva barely protruding (Fig. 2J).

Figs. 1-3

Molecular characterization and phylogenetic relationships of *Cryphodera brinkmani*

The amplification of D2-D3 expansion segments of 28S rDNA, ITS1, partial 18S, and the partial *COI* regions yielded a single fragment of approximately 700 bp, 750 bp, 1600 bp, and 400 bp, respectively, based on gel electrophoresis. D2-D3 expansion segments of 28S rDNA of *C. brinkmani* (JQ965677) from commercial nurseries of Pistoia matched well (99% similarity) with the D2-D3 sequence of the *C. brinkmani* deposited in GenBank (DQ328705), differing only by 8 nucleotides (657/665 identities) and presented 2 gaps (2/665); the next close Heteroderidae was *Meloidodera alni* (DQ328706) differing by 74 nucleotides (89% similarity, 601/675 identities), and presented 31gaps (5%, 31/675). Similarly, ITS1 of *Pistoia* specimens (JQ965678) matched well (96% similarity) with ITS1 of *C. brinkmani* deposited in GenBank (AF274418), differing by 32 nucleotides (96% similarity, 701/733 identities), and presented 5 gaps (1%, 5/733); and 86% similarity (376/436 identities) with *Cryphodera* sp. C SAS-2011 (JF894398) from natural forests of Vietnam. The partial 18S and partial *COI* sequences were obtained for the first time for this species in this study. The partial 18S (JQ965679) was 96% similar to *Globodera tabacum* (FJ040401), 1592/1666 identities and 18 gaps (1%, 18/1666); and 95% similar to *Globodera artemisiae* (FJ040400), 1591/1666 identities, and 18 gaps (1%, 18/1666). Finally, the partial *COI* was 86% similar to *Heterodera cardiolata* (HM640929), 370/432 identities and 1 gap

(0.23%, 1/432); and 83% similar to *Punctodera chalcoensis* (HM640928), 362/436 identities and 5 gaps (1%, 5/436). Consequently, species identification based on sequencing of rDNA regions (D2-D3, ITS1, and 18S) and mtDNA (partial *COI*) was congruent with morphological and morphometrical data, and showed useful for species identification.

Phylogenetic relationships among heteroderid nematodes were inferred from analyses of ITS1-rRNA gene of a multiple edited alignment including 22 sequences and 879 total characters using Bayesian inference and maximum likelihood as given in Figure 5. BI and ML trees were congruent and only differed in the positions of poorly supported clades. Phylogenetic analysis (BI and ML) showed two well to moderately supported major clades: I) with 15 sequences including 5 subclades (a = *Punctodera* spp., *Paradolichodera tenuissima*, *Cactodera* spp., *Globodera* spp., and *Betulodera betae*; b = *Heterodera* spp.; c = *Atalodera crassicristata* and *Rhizonema sequoiae*; d = *Vittatidera zeaphila;* and e = *Bilobodera flexa* and *Ekphymatodera thomasoni*); and II) with 6 sequences including *Cryphodera* spp. in one subclade (a), and *Meloidodera alni* in a separate subclade (b) (Fig. 5). These results suggest a polyphyletic origin of heteroderids (including cyst-forming and non-cyst forming species), and agrees with previous results (Subbotin et al. 2001; 2011).

Fig. 5

Phylogenetic analysis of ITS1 of this and previous studies also demonstrate that *C. brinkmani* clustered together with other *Cryphodera* spp. as well as with *Meloidodera alni* (Subbotin et al. 2001). These results suggest a monophyletic origin of non-cyst forming nematodes (Meloidoderinae), and both of them have been considered close to the ancestor of most species of the Heteroderidae (Krall and Krall 1978; Baldwin 1992; Subbotin et al. 2001). In fact, the ability of some species of *Meloidodera*, *Cryphodera* (Meloidoderinae) and *R. sequoiae* (Ataloderinae) to parasitize plants from *Pinus* and *Sequoia*, respectively, is consistent with the argument of Krall and Krall (1978) that suggests an ancient origin for these genera. Also, the phylogenetic relationships inferred in this study based on the ITS1 of

rRNA gene mostly agrees with results by Subbotin *et al*. (2001, 2011) that showed the clustering of *Punctodera*, *Globodera*, *Cactodera*, *Paradolichodera*, and *Betulodera* (Fig. 5). Similarly, *Atalodera*, *Rhizonema* and *Vittatidera* clustered outside *Heterodera* and *Globodera* groups as previously reported by Bernard et al. (2010) and Subbotin et al. (2011).

Histopathology

Japanese white pine infected roots showed the posterior portion of nematode females protruding from the root surface (Fig. 4A). Histological observations of cross and longitudinal root sections revealed the nematode body embedded in the cortex obliterating portion of the cortical parenchyma (Fig. 4 C). After penetrating the root cortex, the nematode established a permanent feeding site in an endodermal or pericyclic cell inducing the formation of an uninucleate giant cell that expanded into the vascular tissues of the stele (Fig. 4 C,F). The giant cell was surrounded by mature females and frequently enclosed into the root stele and contact xylem, vacuum cambium and phloem elements (Figs. 4 C,E,F). Giant cell elliptical in shape 80-220 µm long and 50-120 µm wide and contained dense and granulated cytoplasm (resulting from intensive metabolic activity). The nucleus appeared also hypertrophic (25-30 µm in diameter), and with several organelles. Giant cells wall was thickened especially in correspondence of the feeding site where the stelar tissues surrounding the nematode lip region were compressed and darkened. No hyperplasia of tissues adjacent to giant cell was observed.

The histological changes induced by *C. brinkmani* on *P. parviflora* are quite similar to those induced by *C. kalesari* on Indian laurel (*T. tomentosa*) (Bajaj and Walia 2000). The formation of an uninucleate giant cell is a host response shared by *Cryphodera* spp. with other non-cyst forming Heteroderids (i.e. *Bellodera*, *Meloidodera*, *Sarisodera* and *Hylonema*), and especially with *Meloidodera floridensis* and *M. charis*, which both induce a

single uninucleate giant cell extending in some cases in more than 50% of the stele (Inserra and Vovlas 1986; Mundo-Ocampo and Baldwin 1983). These findings are in agreement with the results of the phylogenetic analysis confirming the close genetic and behavioural relationships between *Cryphodera* spp. and *Meloidodera* spp.

As concluding remarks we would like to point out that in spite of the damage that *C. brinkmani* induces in the tissues of the infected roots by disrupting the vascular system of the stele and normal flow of nutrients and water in the root system, the effect of its infestation on pine growth remains unclear. The potential suppressing effect of the nematode on plant growth may be desirable for bonsai growers who want to limit the growth of the plants used for bonsai production. However, the spread of this nematode pest in pine forests should be avoided and may constitute a serious potential risk to the fragile pine ecosystem of Europe and other areas where the nematode has been accidently imported. The interception of *C. brinkmani* in plant shipments destined to the Italian market confirms further the need for the implementation of exclusion and certification programs aiming to the production of nematode-free bonsai plants for export to national and international markets.

Furthermore, the knowledge of some of the genes (i. e. endoglucanase, chorismate dismutase) involved in the molecular basis of nematode host responses (cyst nematodes) will lead to a better understanding of the regulatory mechanisms affecting plant parasitism by these nematodes and should allow the development of target-specific strategies to limit crop damage by these pathogens (Baum et al. 2007).

Fig. 4

In summary, the present study enlarges the molecular characterization of the non-cyst forming nematode *C. brinkmani*, and provides additional molecular markers for precise and unequivocal diagnosis of this species (partial 18S and partial *COI*), which may help for effective quarantine inspection and appropriate application of exclusion principles.

Acknowledgements

Authors thank Dr. G. Vettori of phytosanitary control service of Tuscany, Italy; and Dr. B.B. Landa and J.A. Navas-Cortés (IAS-CSIC) for critical reading of the manuscript before submission. The present work was supported with funds provided by the Italian Ministry of Economy and Finance to the National Research Council for the project "Innovazione e Sviluppo del Mezzogiorno-Conoscenze Integrate per Sostenibilità ed Innovazione del Made in Italy Agroalimentare-Legge n. 191/2009.

References

Bajaj, H.K., Walia, R.K, Dabur, K.R., and Bhatti, D.S. (1989). *Cryphodera kalesari*, a new heteroderid nematode species from Haryana, India. *Systematic Parasitology*, 14, 113-116.

Bajaj, H.K., and Walia, R.K (2000). Host response to *Cryphodera kalesari* (Heteroderidae: Nematoda). *Nematologia mediterranea*, *28*, 197-199.

Baldwin, J.G. (1992). Evolution of cyst and noncyst-forming Heteroderidae. *Annual Review of Phytopathology*, *30*, 271-290.

Baum, T.J., Hussey, R.S., and Davis, E.L. (2007). Root-knot and cyst nematode parasitism genes: the molecular basis of plant parasitism. In: J.K. Setlow (Ed.), *Genetic Engineering* (pp. 17-43). Springer Science, London, UK.

Bernard, E.C., Handoo, Z.A., Powers, T.O., Donald, P.A., and 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*, 139-150.

Boutsika, K., Brown, D.J.F., Phillips, M.S., and Blok, V.C. (2004). Molecular characterisation of the ribosomal DNA of *Paratrichodorus macrostylus*, *P. pachydermus*, *Trichodorus primitivus* and *T. similis* (Nematoda: Trichodoridae). *Nematology*, *6*, 641-654.

Castillo, P., Vovlas, N., Subbotin, S., and Troccoli, A. (2003). A new root-knot nematode, *Meloidogyne baetica* n. sp. (Nematoda: Heteroderidae), parasitizing wild olive in Southern Spain. *Phytopathology,* 93, 1093-1102.

Colbran, R.C. (1966). Studies of plant and soil nematodes. 12. The eucalypt cystoid nematode *Cryphodera eucalypti* n. g., n. sp. (Nematoda: Heteroderidae) a parasite of eucalypts in Queensland. *Queensland Journal of Agricultural and Animal Sciences, 23*, 41-47.

Coolen, W.A. (1979). Methods for extraction of *Meloidogyne* spp. and other nematodes from roots and soil. In: F. Lamberti and C.E. Taylor (Eds.), *Root-knot nematodes (Meloidogyne species). Systematics, Biology and Control* (pp. 317-329). Academic Press, New York, NY, USA.

Derycke, S., Remerie, T., Vierstraete, A., Backeljau, T., Vanfleteren, J., Vincx, M., and Moens, T. (2005). Mitochondrial DNA variation and cryptic speciation within the free-living marine nematode *Pellioditis marina*. *Marine Ecology Progress Series*, *300*, 91-103.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98NT. *Nucleic Acids Symposium Serial*, *41*, 95-98.

Hirata, K., and Yuhara, I. (1986). Plant parasitic nematodes detected from Bonsai trees and Bonsai nurseries. *Research Bulletin of the Plant Protection Service*, *Japan*, *22*, 111-117.

Hooper, D.J. (1986). Handling, fixing, staining and mounting nematodes. In: Southey, J. (Ed). *Laboratory Methods for Work with Plant and Soil Nematodes.* (6th ed.). Ministry

of Agriculture, Fisheries and Food. London, U. K.: Her Majesty's Stationery Office, pp. 59-80.

Huelsenbeck, J.P., and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, *17*, 754-755.

Inserra, R.N., and Vovlas, N. (1986). Cystoid nematodes and their economic importance. Nematology Circular No. 128. Florida Department of Agriculture and Consumer Services, DPI, Gainesville, Florida, pp.4.

Johansen, D.A. (1940). *Plant Microtechnique*. New York, USA: McGraw-Hill Book Co.

Karssen G., and Van Aelst, A. (1999). Description of *Cryphodera brinkmani* n. sp. (Nematoda: Heteroderidae), a parasite of *Pinus thunbergii* Parlatore from Japan, including a key to the species of the genus *Cryphodera* Colbran, 1966. *Nematology*, *1*, 121-130.

Krall, E.L. and 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.

Luc, M., Taylor, D.P., and Cadet, P. (1978). Description of a new tropical Heteroderidae, *Hylonema ivorense* n. gen., n. sp., and a new outlook on the family Heteroderidae (Nematoda: Tylenchida). *Revue de Nématologie, 1*, 73-86.

McCullough, D.G., Work, T.T., Cavey, J.F., Liebhold, A.M., and Marshall, D. (2006). Interceptions of nonindigenous plant pests at US ports of entry and border crossings over a 17-year period. *Biological Invasions, 8*, 611-630.

Mundo-Ocampo, M., and Baldwin, J.G. (1983). Host response of to *Meloidodera* spp. (Heteroderidae). *Journal of Nematology 15,* 544-554.

Nguyen, C.N., Sturhan, D., and Subbotin, S.A. (2011). Studies on the occurrence abd diversity of Heteroderidae and Meloidogynidae (Nematoda:Tylenchida) in natural forests of Vietnam. *Russian Journal of Nematology 19*, 159-172.

Page, R.D. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Computer Applications in the Bioscience, 12*, 357-358.

Posada, D. (2008). jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution, 25*, 1253-1256.

Quénéhervé, P., Topart, P., and Poliakoff, F. (1998). Interception of nematodes on imported bonsai in Martinique. *Nematropica 28*, 101-105

Roques, A., and Auger-Rozenberg, M.A. (2006). Tentative analysis of the interceptions of non-indigenous organisms in Europe during 1995-2004. *Bulletin OEPP/EPPO Bulletin*, *36*, 490-496.

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

Subbotin, S.A., Vierstraete, A., De Ley, P., Rowe, J., Waeyenberge, L., Moens, M., and 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., Del Prado Vera, I.C., Mundo-Ocampo, M., and Baldwin, J.G. (2011). Identification, phylogeny and phylogeography of circumfenestrate cyst nematodes (Nematoda: Heteroderidae) as inferred from analysis of ITS-rDNA. *Nematology*, *13*, 805-824.

Swofford, D.L. (2003). PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods) 4.0 Beta. (Florida State University).

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting,

positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*, 4673-4680.

Toida, Y. (1984). Nematode species from mulberry fields and their geographical distribution in Japan. *Japanese Journal of Nematology*, *14*, 20-27.

Troccoli A. (2002). Aspetti pratici e comparativi di alcune metodologie tradizionali applicate all'identificazione dei nematodi da quarantena. *Nematologia mediterranea, 30*, 107-110.

Wouts, W.M. (1973). A revision of the family Heteroderidae (Nematoda: Tylenchoidea) II. The subfamily Meloidoderinae. *Nematologica, 19*, 218-235.

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

Figure legend

Fig. 1 *Camera lucida* drawings of life stages of *Cryphodera brinkmani*. A-E: Male. F, G: Female (anterior body). H, I: Second stage juvenile. A-H: Entire body of male and second-stage juvenile, respectively; B, C: Anterior body portion of male; I, Anterior body of second stage juvenile; D: Tail of male; E: Detail of lateral field of male.

Fig. 2 Light microscopy micrographs of *Cryphodera brinkmani* detected in Italy. A: Post-embryonic life stages (second-stage juveniles, young female and male). B; C: Anterior body portion of second-stage juvenile and male respectively. D: Male pharyngeal body portion showing the excretory pore (arrowed). E: and I: Female anterior body portion, showing in detailed view of the basal head annulus (inset), the stylet and the excretory pore (arrowed), respectively. F: Second-stage juvenile's tail. Note (inset) the tail end with a small terminal mucro. G; H: Male posterior body portions at two different foci. J: Entire body shape of young female. K: Adult females body shape variation. L: Perineal pattern, showing the vulval slit and the anus (arrowed). M: Vulval region (anus arrowed) in lateral view of mature female containing embrionated eggs. Scale bars: A, J = 100 μm; K = 1 mm; all others = 20 μm).

Fig. 3 Light micrographs of male morphological features. A-C: lip region showing two or three lip annuli (arrowed). D: lateral field with two areolate bands (Scale bar = 20 μm).

Fig. 4 Parasitc habits and anatomical alterations induced by *Cryhodera brikmani* in *Pinus parviflora* roots. A: Root segment of with the posterior portion of the body of a white female (arrowed) protruding from the root surface. B, C: Cross sections of female body (n) embedded in the cortical parenchyma. D, E: Root cross sections showing a nematode (n)

embedded in the cortical parenchyma and an uninucleate giant cell (ugc) in the stele and surrounded by vascular elements, F: Root cross section showing a nematode (n) with the head surrounded by compressed endodermal and peryciclic cells with thick walls in correspondence of uninucleate giant cell (not visible). G, H: Root cross section showing the thick wall (tw) at the nematode feeding point on the uninucleate giant cell rich in densely stained cytoplasm. I: Root longitudinal section showing a giant cell with hypertrophied nucleus and prominent nucleolus. (Scale bars = A: 500 µm; B: 250 µm; C-F: 100 µm).

Fig. 5 Phylogenetic relationships within Heteroderidae. Bayesian 50% majority rule consensus trees as inferred from ITS1 rRNA gene sequence alignment under the TVM + I + G model. Posterior probabilities more than 65% are given for appropriate clades (in bold letters); bootstrap values greater than 50% are given on appropriate clades in ML analysis. Newly obtained sequence in this study is underlined.

Table 1. Species and host of the genus *Cryphodera.*

Species	Host-plant roots or soil around roots (s)	References
C. brinkmani	*Pinus thunbergii*	Karsen and Van Aelst, 1999
	Pinus pentaphylla	present study
C. coxi	*Metrosideros robusta*	Wouts, 1973
	Eucalyptus major	
C. eucalyptii	*E. sideroxylon, E. crebra*	Colbran 1966
	E. punctata, E. andrewsi	
C. kalesari	*Terminalia tomentosa*	Bajaj and Walia, 2000
C. nothophagi	*Nothophagus solandri*	Wouts, 1973
C. podocarpi	*Podocarpus totara*	Wouts, 1973
Cryphodera sp. A	*Musa acuminate* (s)	Nguyen et al., 2011
Cryphodera sp. B	*Fuchsia* sp. (s), *Sapindus* sp. (s) *Polypodium vulgare* (s)	Nguyen et al., 2011
Cryphodera sp. C	natural forests Vietnam (s)	Nguyen et al., 2011

Table 2. Morphometrics of males of *Cryphodera brinkmani* (all measurements in µm).

Characters	Male
N	6
L	906 ± 63.7 (812-1004)
a	36.3 ± 3.2 (31.2-40.2)
b	7.6 ± 0.5 (6.9-8.2)
b'	5.0 ± 0.4 (4.5-5.5)
c	118.6 ± 31.1 (88.6-173.9)
c'	0.5 ± 0.1 (0.4-0.7)
head width	9.5 ± 0.4 (9.0-10.0)
head height	5.2 ± 0.3 (4.7-5.7)
Stylet	34.6 ± 1.8 (32.7-38.0)
Conus	17.4 ± 1.5 (14.7-18.7)
Knobs width	5.2 ± 0.8 (4.7-6.7)
D.G.O.	3.2 ± 0.7 (2.7-4.0)
O (%)	9.7 ± 2.0 (7.7-11.9)
Metacorpus height	12.4 ± 1.4 (10.7-14.0)
Metacorpus diam.	9.7 ± 1.0 (8.7-11.3)
Anterior end to centre of metacorpus	83.6 ± 8.4 (68.7-92.7)
Cardia	120 ± 13.8 (101-136)
end of pharyngeal gland lobe	182.5 ± 18.4 (149-200)
secretory/excretory pore	128.5 ± 10.9 (119-143)
Isthmus	28.4 ± 7.1 (20.7-40.0)
Length of pharyngeal overlap	62.5 ± 10.4 (48.5-78.5)
Maximum body diam.	25.0 ± 1.3 (22.7-26.0)
Annuli width	2.4 ± 0.1 (2.2-2.5)
Testis length	492 ± 101 (331-579)
T (%)	54.7 ± 11.8 (33.0-64.4)
Tail length	8.1 ± 2.2 (4.7-11.3)
Anal body diam.	14.8 ± 1.7 (12.0-17.3)
Spicule length (n = 3)	27.7 ± 4.7 (23.3-32.7)
Gubernaculum (n = 3)	8.0 ± 0.7 (7.3-8.7)