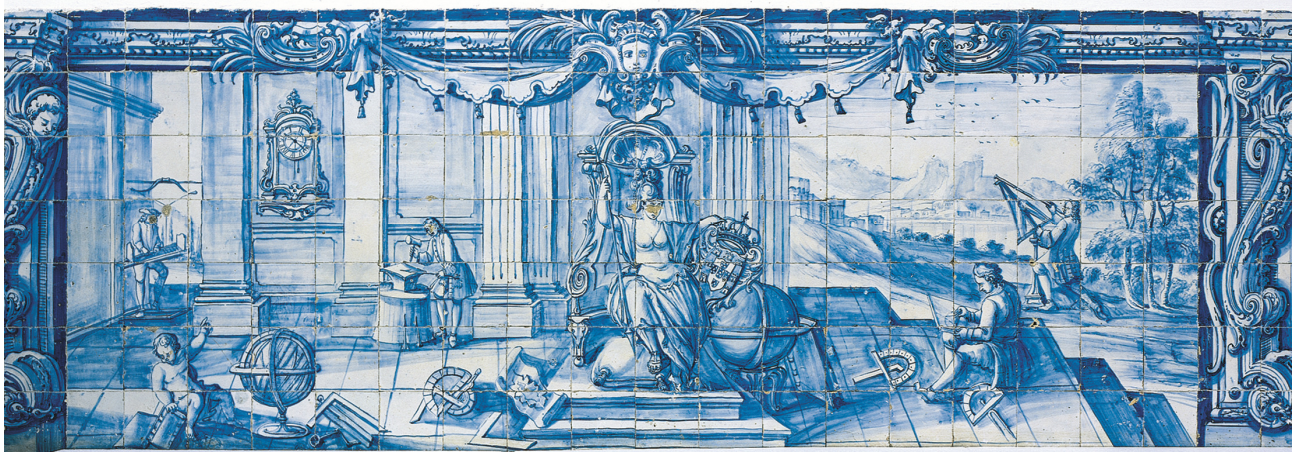
Genetic diversity of entomopathogenic nematodes (Nematoda: Steinernematidae and Heterorhabditidae) and the nematode *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) from continental Portugal

Vera Mónica Piegas Valadas

Thesis presented to obtain the PhD degree
in Biology by the University of Évora

SUPERVISOR: *Professor Manuel Galvão de Melo e Mota*
CO-SUPERVISOR: *Professora Solange Oliveira*

ÉVORA, NOVEMBRO DE 2012



INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

Genetic diversity of entomopathogenic nematodes (Nematoda: Steinernematidae and Heterorhabditidae) and the nematode *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) from continental Portugal

Vera Mónica Piegas Valadas

Thesis presented to obtain the PhD degree
in Biology by the University of Évora

SUPERVISOR: *Professor Manuel Galvão de Melo e Mota*
CO-SUPERVISOR: *Professora Solange Oliveira*

ÉVORA, NOVEMBRO DE 2012

Acknowledgments

The present thesis would not have been possible without the important collaboration of several institutions and people to whom I would like to thank:

- To the Évora University for providing the necessary conditions to carry out my work, namely to ICAAM (Instituto de Ciências Agrárias e Ambientais Mediterrânicas) for supporting the investigation carried out in the "Laboratório de Nematologia" and also for funding my participation in courses and conferences;

- To "Fundação para a Ciência e a Tecnologia" (FCT) for the PhD fellowship (SFRH/BD/22086/2005)

- This thesis was also supported by the EC 7th 324 Framework project REPHRAME (KBBE.2010.1.4-09, "Analysis of the potential of the pine wood nematode (*Bursaphelenchus xylophilus*) to spread, survive and cause pine wilt in European coniferous forests in support of EU plant health policy") and by a national project from the Portuguese National Forestry Authority (AFN, Autoridade Florestal Nacional), "O nemátode da madeira do pinheiro (NMP), *Bursaphelenchus xylophilus*".

- The present study was carried out under collaboration with Soil Microbiology Laboratory from the "Instituto de Ciências Agrárias e Ambientais Mediterrânicas" (ICAAM) and the Department of Biology of the University of Évora, to which I want to thank for providing the necessary conditions to perform this work.

I would like to express my gratitude to the persons who, in one way or another, helped me during the elaboration and conclusion of this work, namely:

Prof. Manuel Mota (Évora University) for accepting to be my thesis supervisor and for always have being present since my graduate degree in Biology in the Évora University. I want to thank you for the support and knowledge transmitted during all these years. Thank you also for the encouragement to visit and experience work in other foreign laboratories.

Prof. Solange Oliveira (Évora University) for accepting to be my co- supervisor during my thesis. Thank you for all the support and knowledge in molecular biology and genetics. I also want to thank you for your friendship and encouragement during some difficult moments.

To **Dr Marta Laranjo**, my colleague, for all the support and knowledge about phylogenetic analysis, molecular biology, genetics and statistic analysis. I also want to thank you for your friendship and encouragement during some difficult moments.

I want to thank **Prof. Thomáe Kakouli-Duarte** for allowing me to visit and work in her lab at the Institute of Technology Carlow, Ireland and to **Dr. Stephen Boyle**, with whom I worked directly. Thank you both very much.

I want to thank to **Prof. Nelson Simões** for allowing me to visit and work in his lab in Ponta Delgada, Azores University, and for all the knowledge.

I also thank **Dr Zdenek Mráček,** Laboratory of Insect Pathology, Czech Academy of Sciences, Czech Republic for receiving me in his lab. I also thank very much to **Dr Vladimir Püza**, for all the support, friendship and knowledge.

I also thank **Dr Alexander Schoüten** and **Professor Richard Sikora** from Bonn University in Germany, for the stay in his lab. A big thank to Dr Alexander Schoüten for all the friendship and support during my stay in the lab and to all the precious knowledge.

I would like to thank **Prof. Isabel Abrantes** (Coimbra University, Portugal) for providing nematode cultures of *B. xylophilus* and to the possibility of sharing knowledgments about *B. xylophilus* during my collaboration in the national project from the Portuguese National Forestry Authority (AFN, Autoridade Florestal Nacional), "O nemátode da madeira do pinheiro (NMP), *Bursaphelenchus xylophilus*". I also want to thank to Dr. Luis Fonseca, for all the friendship and sharing knowledgement.

I would like to thank to Dr Filipe Pereira, Dr Cláudia Moreira, Dr Barbara van Asch and Dr António Amorim for providing sequence data of *B. xylophilus* cytochrome b and for the sharing knowledgment during the national project from

the Portuguese National Forestry Authority (AFN, Autoridade Florestal Nacional), "O nemátode da madeira do pinheiro (NMP), *Bursaphelenchus xylophilus*".

Prof. Helena Adão (Évora University) for helping with the statistical analysis, with her precious knowledge and for all the friendship that helped me to deal with the pressure in the final phase of my thesis.

Prof. Elsa Sampaio (Évora University) for giving me acess to information on soil cartography of continental Portugal which was needed to complete the analysis of my data.

To my lab colleague **Pedro Barbosa** for all the friendship, support knowledge about nematodes, especially bio-ecology, morphology and sample processing. Thank you also for all the good moments in the lab and the supportive words that helped me during the most difficult moments.

To my lab colleague **Dr Paulo Vieira**, for all his help during my work. His encouragement, scientific support and precious advice were crucial during the elaboration of my thesis.

To my good friend and former lab technician of the Nematology Lab, **D. Francisca Figo**, for all the friendship during the good and especially the bad moments of my life during these six years. It was you who first showed me that nematodes can be good agents too and for all the knowledge during the initial phase of my thesis.

I want to thank **José Carlos Garcia dos Santos** for the precious help with the analysis of my data. Thank you for all the help and for the friendship. I will never forget.

To **Dr Ana Catarina Penas** for the friendship and scientific support. Thank you for the possibility of publishing together.

I want to thank **Ana Alves** for her friendship and to all the support that she gave me in the good and especially the bad moments. Thank you also to help me with statistical analysis.

To my colleagues **Clarisse Brígido, Ana Alexandre, Claúdia Vicente, Margarida Espada,** who were present during this difficult journey, my thesis. I specially thank **Clarisse Brígido** for your friendship.

I also want to thank **Inácia Ferreia** the technician of the Nematology Laboratory/ ICAAM for all the help during my work and to all the friendship.

Finally I want to thank my **family**, my sister and niece, to my mother and father. I´m sorry for the bad times and thank you for always being next to me during this period. I want to give a big thank to my **father**, the person that helped me and that was always there for me and that help me during all our trips in continental Portugal for sample collection. THANK YOU very much, dad.

I would like to thank a very special person that helped me during these last four years. I will never forget the time that we spend together and your encouragment words. THANK YOU very much, **João** for always believe me.

Thesis publications

From the present thesis, the following manuscripts have been published, or are in the process of publication:

1. **Valadas, V.,** Boyle, S., Vieira, P., Kakouli-Duarte, T. and Mota, M. (2007). First report of an entomopathogenic nematode from continental Portugal. Helminthologia 44 (4): 226-229.

2. **Valadas, V.,** Vieira, P., Oliveira., S. and Mota, M. (2009). First report of the genus *Heterorhabditis* (Nematoda: Heterorhabditidae) from continental Portugal. Helminthologia 46 (1): 45–48.

3. **Valadas, V.,** Mráček, Z., Oliveira, S. and Mota, M. (2011). Three species of entomopathogenic nematodes of the family Steinernematidae (Nematoda: Rhabditida) new to continental Portugal. Nematologia Mediterranea 39 (2): 169-178.

4**. Valadas, V.,** Laranjo, M., Barbosa, P., Espada, M., Mota, M. and Oliveira, S. (2012). The pinewood nematode, *Bursaphelenchus xylophilus*, in Portugal: possible introductions and spread routes of a serious biological invasion revealed by molecular methods (*accepted in Nematology*: DOI:10.1163/156854112X632673).

5. **Valadas, V.,** Laranjo, M., Mota, M. and Oliveira, S. (2012). Molecular characterization of Portuguese populations of the pinewood nematode *Bursaphelenchus xylophilus* using cytochrome b and cellulase genes (*accepted in Journal of Helminthology:* DOI:10.1017/S0022149X12000673).

6. **Valadas, V.**, Laranjo, M., Mota, M. and Oliveira, S.. A survey of entomopathogenic nematodes species in continental Portugal reveals predominance of *Steinernema feltiae* and *Heterorhabditis bacteriophora (submitted).*

Other publications:

1. Akbulut, S., Vieira, P., Ryss, A., Yuksel, B., Keten, A., Mota, M. and **Valadas, V.** (2006). Preliminary survey of the pinewood nematode in Turkey. EPPO Bulletin 36: 538-542.

2. Penas, A.C., Metge, K., Mota, M. and **Valadas, V.** (2006). *Bursaphelenchus antoniae* sp. n. (Nematoda: Parasitaphelenchidae) associated with *Hylobius* sp. from *Pinus pinaster* in Portugal. Nematology 8: 659-669.

3. Akbulut, S., Vieira, P., Ryss, A., **Valadas, V.,** Keten, A. and Mota, M. (2008). *Bursaphlenchus* Fuchs, 1937 (Nematoda: Parasitaphelenchidae) species associated with *Pinus* species in northern Turkey. Helminthologia 45: 89-95.

4. Penas, A.C., Bravo, M.A., **Valadas, V.** and Mota, M. (2008). Detailed morphobiometric studies of *Bursaphelenchus xylophilus* and characterization of other *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) associated with *Pinus pinaster* in Portugal. Journal of Nematode Morphology and Systematic 10: 137-163.

Abbreviations

AFN	Autoridade Florestal Nacional
bp	base pair
ca	circa
cytb	cytochrome b
COXI	Cytochrome c Oxidase Subunit I
DNA	Deoxyribonucleic Acid
D2D3	D2D3 region from 28S region
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediamine Tetraacetic Acid
EPN	Entomopathogenic Nematodes
EPPO	European and Mediterranean Plant Protection Organization
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
g	gravity acceleration
GHF	Glycosyl Hidrolase Family
GPS	Global Positioning System
ha	hectare
HGT	Horizontal Gene Transfer
Hsp70	Heat shock protein 70
IJ	Infective Juvenile
IGS	Intergenic Spacer
ISPM 15	Norma Internacional para as Medidas Fitossanitárias n.º 15 da FAO
ISSR	Inter Simple Sequence Repeats
ITS	Internal Transcribed Spacer
J2	Second stage juvenile (propagative)
J3	Third stage juvenile (propagative)
J_{III}	Third stage juvenile (dispersal)
J_{IV}	Four stage juvenile (dispersal)
Km	Kilometeres

min	minute
L	Liter
LM	Light Microscope
LSU	Large Subunit of Ribosomal RNA
ML	Maximum Likelihood
ml	mililiter
mtDNA	mitochondrial DNA
m^2	square meter
NUT	National Units of Territories
PCR	Polymerase Chain Reaction
PROLUNP	Programa Nacional de Luta Contra o Nemátode da Madeira do Pinheiro
PWD	Pine Wilt Disease
PWN	Pinewood Nematode
PWNSC	Pinewood Nematode Species Complex
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
Sec	seconds
SEM	Scanning Electronic Microscope
SSRs	Simple Sequence Repeats
SSU	Small Subunit of Ribosomal RNA
U	Unit

Abstract

Entomopathogenic nematodes are used as biocontrol agents. To understand their diversity, a survey was undertaken in Portugal. Five species, namely *Steinernema feltiae*, *S. intermedium*, *S. kraussei*, *Steinernema* sp. and *Heterorhabditis bacteriophora* were identified. The ITS, 28S rRNA D2D3 region, COXI and *cytb* sequences, used to study the genetic diversity of the two most abundant species, *S. feltiae* and *H. bacteriophora*, showed no significant differences among the isolates.

Bursaphelenchus xylophilus causes severe disease in pine trees and was detected for the first time in Europe and in Portugal in 1999. To evaluate the genetic diversity of Portuguese isolates and identify disease spread pathways, the sequence of 5.8S rRNA IGS region, *cytb* and cellulase genes, combined with ISSR fingerprints were used. ISSR fingerprints show a high genetic variability among recent Portuguese isolates, suggesting the possibility of a new introduction. Phylogenetic trees based on cellulase and *cytb* genes suggests an Asian origin for Portuguese isolates.

Resumo

"Diversidade genética dos nemátodes entomopatogénicos (Nematoda: Steinernematidae e Heterorhabditidae) e do nemátode *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididade) em Portugal continental"

Os nematodes entomopatogénicos são utilizados como agentes de controlo biológico. Para compreender a sua diversidade, foi realizada uma prospecção em Portugal. Cinco espécies, nomeadamente *Steinernema feltiae*, *S. intermedium*, *S. kraussei*, *Steinernema* sp. e *Heterorhabditis bacteriophora* foram identificadas. As sequências de ITS, região D2D3 do 28S rRNA, COXI e *cytb* foram utilizadas para estudar a diversidade genética das duas espécies mais abundantes, *S. feltiae* and *H. bacteriophora*, não tendo sido encontradas diferenças significativas entre isolados.

O nemátode da madeira do pinheiro, *Bursaphelenchus xylophilus,* provoca doença nos pinheiros tendo sido detectada pela primeira vez na Europa e em Portugal em 1999. Para avaliar a diversidade genética dos isolados Portugueses e identificar o padrão de propagação da doença, foram utilizadas a sequência da região IGS do 5.8S rRNA, e os genes *cytb* e cellulase, combinados com os padrões ISSR. Os padrões de ISSR mostraram elevada diversidade genética entre os recentes isolados Portugueses, sugerindo a possibilidade de uma nova introdução. As árvores filogenéticas dos genes da celulase e *cytb* sugeriram uma origem Asiática para os isolados Portugueses.

List of Figures

Chapter.III. State of art of entomopathogenic nematodes – Families Steinernematidae and Heterorhabditidae

IV.4. A survey of entomopathogenic nematodes species in continental Portugal reveals predominance of *Steinernema feltiae* and *Heterorhabditis bacteriophora*

VI.1. The nematode, *Bursaphelenchus xylophilus*, in Portugal: possible introductions and spread routes of a serious biological invasion revealed by molecular methods

VII.2. Molecular characterization of Portuguese populations of the pinewood nematode *Bursaphelenchus xylophilus* using cytochrome b and cellulase genes

replicates); scale bar indicates 0.1 substitutions per site. The Tamura & Nei model (Tamura & Nei, 1993) was used. Symbols represent isolates collected until 2008 ◘, 2009 ●, 2010 ʘ (pg. 155).

Figure.VII3. Maximum likelihood cellulase phylogenetic tree based on nucleotide sequences of 26 isolates of B. xylophilus and reference strains (alignment length 787 bp). Percentage bootstrap is indicated on internal branches (100 replicates); scale bar indicates 0.1 substitutions per site. The Kimura two-parameter model (Kimura, 1980) was used. Symbols represent isolates collected until 2008 ◘, 2009 ●, 2010 ʘ (pg. 156).

List of Tables

Chapter.IV. Identification of entomopathogenic nematodes species in continental Portugal

IV.2. First report of the genus *Heterorhabdithis* (Nematoda: Heterorhabditidae) from continental Portugal

IV.3. Three species of entomopathogenic nematodes of the family Steinernematidae (Nematoda: Rhabditida) new to continental Portugal

IV.4. A survey of entomopathogenic nematodes species in continental Portugal reveals predominance of *Steinernema feltiae* and *Heterorhabditis bacteriophora*

Chapter.VI. Study of the genetic diversity of geographic populations of *Bursaphelenchus xylophilus* from continental Portugal and Madeira Island

VI.1. The nematode, *Bursaphelenchus xylophilus*, in Portugal: possible introductions and spread routes of a serious biological invasion revealed by molecular methods

VII.2. Molecular characterization of Portuguese populations of the pinewood nematode *Bursaphelenchus xylophilus* using cytochrome b and cellulase genes

Table of Contents

Chapter I. General introduction

I.1. General Introduction

Nematodes are simple roundworms belonging to the Phylum Nematoda, the most diverse phylum of pseudocoelomates, and one of the most diverse of all animals. They are considered the most abundant group of metazoan in the biosphere, representing 80% of all metazoan animals (Lambshead, 1993). Nematodes are unsegmented organisms, with no appendages, including free-living, predaceous, or parasitic organisms. Several parasitic nematodes cause serious diseases in plants, animals and also in humans. Others are beneficial, due to their potential as biocontrol agents in the control of insect pests. Nematodes are well succeeded organisms, a fact demonstrated by the high species diversity. They are successfully adapted to nearly every ecosystem from marine to fresh water, from the polar regions to the tropics, as well as from the highest to the lowest of altitudes. They can be found in locations as diverse as mountains, deserts, oceanic trenches, and within the earth's lithosphere. The effects on Man, animals and agricultural management are significative.

Nematodes include organisms that have beneficial effects, such as the entomopathogenic nematode (EPN) *Steinernema feltiae* (Simões *et al*., 1994; Rosa and Simões, 2004), but also capable of causing serious economic and ecological impacts, like *Bursaphelenchus xylophilus,* the pinewood nematode (PWN). Parasitic nematodes of plants and insects, belong to the order Rhabditida. They can parasite insects – entomopathogenic nematodes, while others such as *B. xylophilus* – are mycophagous. In the present work, EPN and PWN were studied due to their economical importance in ecosystems, nationally and worldwide.

The order Rhabditida comprises many families, including Steinernematidae and Heterorhabditidae, commonly referred EPN. These multicelular metazoans possess an optimal balance of biological attributes and occupy a biocontrol middle ground between microbial pathogens and predators/parasitoids, and are frequently lumped with pathogens, presumably because of their symbiotic relationship with bacteria. EPN are extraordinarily lethal to many important

insect pests, yet are safe for plants and animals. Most of the currently used biological control agents require days or weeks to kill their hosts, while EPN, working with their symbiotic bacteria, can kill insects within 24-48 hours. Dozens of different insect pests are susceptible to EPN infection, but no adverse effects have been shown against beneficial insects or other non-targets in field studies (Georgis *et al.,* 1991; Ahkurst and Smith, 2002). EPN are able to be mass produced and do not require specialized application equipment as they are compatible with standart agrochemical equipment, including various sprayers and irrigation systems. As no information was available to continental Portugal about EPN, a national survey was undertaken from 2006-2009 to determine the indigenous species of the country, and to determine the genetic diversity of species.

B. xylophilus (Steiner and Bührer, 1934; Fuchs, 1937; Nickle, 1970) is indigenous from North America and is widespread in natural coniferous forests in Canada and in the USA (Sutherland and Peterson, 1999). Presently, *B. xylophilus* is considered one of the most important pests and pathogens in the world (Mota and Vieira, 2008). The general fear of establishment of this pest into countries, where conifer forests assume great importance, stems from the devastating damage caused by this nematode to pine forests (Mamiya, 2004; Shin *et al.,* 2006). The introduction of pinewood nematode (PWN) into non-native areas (outside North America) is primarily associated with trade and the global flow of the forest products (Bergdahl and Halik, 1999; Webster, 2004), resulting in huge annual losses due to the increased mortality and growth loss of the pine forest, and to the increased costs in management procedures and disease control (Mamiya, 2004; Shimazu, 2006). In 1999, the PWN was reported for the first time in Portugal and in Europe (Mota *et al.,* 1999), and more recently in Madeira Island (Fonseca *et al*., 2012). Recently, it was detected in Spain as well (Abelleira *et al.,* 2011; Robertson *et al.,* 2011). Therefore, it is of major importance to determine the origin of the new isolates and their spread routes, to prevent further dissemination of the disease. Due to the importance of this nematode to the national economy, the present study attempted to understand the genetic diversity of Portuguese populations and determine the introduction points and spread pathways of the disease.

In conclusion, among nematodes we can find species that represent serious problems to ecological ecosystems with huge economic impacts, like *B. xylophilus*, but also nematodes that have benificial effects under the ecosystem stability avoiding the use of insecticides to control natural insect pests, like EPN. In this study, a national survey of nematodes, *B. xylophilus* and EPN were undertaken and their genetic diversity studied with the goal of understanding the spread of *B. xylophilus* disease and the biodiversity of Steinernematidae and Heterorhabditidae species in continental Portugal.

The understanding and knowledgement of the native biodiversity of EPN in Portugal may represent a possibility to use these nematodes in the biological control of the insect vector of *B. xylophilus*, because so far it has been proved that is impossible to control *B. xylophilus* once introduced into a tree. Previous studies in Japan, as already try the use of *S. carpocapsae* to control the insect vector of PWN, with some promisor results (Phan, 2008).

The present work represents an innovation since it is the first time that EPN were surveyed in continental Portugal and the genetic diversity of species were analyzed and simultaneously it represents an intensive study using different geographic Portuguese isolates of *B. xylophilus*, using different molecular markers, with the goal of understanding the genetic diversity and spread pathways within Portugal. This was also the first study employing *cytb* and cellulase genes to characterize PWN populations. It also represents the most systematic and extensive survey made for the first time in continental Portugal to evaluate the indigenous species of EPN.

I.2. Literature cited

Abelleira, A., Picoaga, A., Mansilla, J.P. and Aguin, O. (2011). Detection of *Bursaphelenchus xylophilus*, causal agent of pine wilt disease on *Pinus pinaster* in North-western Spain. EPPO Bulletin 39:344.

Akhurst, R. and Smith, K. (2002) Regulation and safety. In: Gaugler, R.(Ed.). Entomopathogenic Nematology. CABI Publishing, Oxon, UK. pp. 311-332.

Bergdahl, D.R. and Halik, S. (1999). Inoculated *Pinus sylvestris* serve as long-term hosts for *Bursaphelenchus xylophilus*. In: Futai, K., Togashi, K. and Ikeda, T. (Eds). Sustainability of Pine Forest in Relation to Pine Wilt and Decline: proceedings of international symposium. Tokyo, Japan, Shokada, pp. 73-78.

Fonseca, L., Cardoso, J.M.S., Lopes, A., Pestana, M., Abreu, F., Nunes, N., Mota, M. and Abrantes, I. (2012). The pinewood nematode, Bursaphelenchus xylophilus, in Madeira Island. Helminthologia 49(2): 96-103.

Füchs, A.G. (1937). Neue parasitische und halbparasitische Nematoden bei Borkenkafern und einige andere Nematoden. I. Teil (in German). Zoologische Jahrbuecher Abteilung fuer Systematik Oekologie und Geographie der Tiere 70, 291-380.

Georgis, R., Kaya, H. and Gaugler, R. (1991). Effect of steinernematid and heterorhabditid nematodes on no target arthropods. Environmental Entomology 20: 815-22.

Lambshead, P.J.D. (1993). Recent developments in marine benthic biodiversity research. Oceanis 19 (6): 5–24.

Mamiya, Y. (2004). Pine wilt disease in Japan. In: Mota, M. and Vieira, P. (Eds). The nematode, *Bursaphelenchus xylophilus*. Nematology Monographs and Perspectives, vol. 1. Leiden-Boston, Brill Academic Publishers, pp 9-20.

Mota, M. and Vieira, P. (2008). Pine Wilt Disease: a worldwide threat to forest ecosystems. Springer, pp. 428.

Mota, M., Braasch, H., Bravo, M.A., Penas, A.C., Burgermeister, W., Metge, K. and Sousa, E. (1999). First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. Nematology 1: 727-734.

Nickle, W.R. (1970). A taxonomic review of the genera of the Aphelenchoidea (Fuchs, 1937) Thorne, 1949 (Nematoda: Tylenchida). Journal of Nematology 2: 375-392.

Phan, L.K. (2008). Potential of Entomopathogenic Nematodes for Controlling the Japanese Pine Sawyer, *Monochamus alternatus*. In: Zhao B, Futai K, Sutherland JR, Takeuchi Y (Eds). Pine wilt disease Japan Springer, Japan, pp. 371-381.

Robertson, L., Arcos, C.S., Escuer, M., Merino, S.R. Esparrago, G., Abelleira, A. and Navas, A. (2011). Incidence of the nematode *Bursaphelenchus xylophlius* Steiner and Buhrer, 1934 (Nickle, 1970) in Spain. Nematology 13 (6): 755-757.

Rosa, J.S. and Simões, N. (2004). Evaluation of twenty-eight strains of *Heterorhabditis bacteriophora* isolated in Azores for biocontrol of the armyworm, *Pseudaletia unipuncta* (Lepidoptera: Noctuidae). Biological Control 29: 409-417.

Shimazu, M. (2006). Current status on research and management of pine wilt disease in Japan. Han, H.R., Koh, S.H., Jung, C.S., Chung, Y.J. and Shin, S.C. (Eds). Current Status on Research and Management of Pine Wilt Disease. Seoul, Korea, Korea Forest Research Institute, pp. 1-18.

Shin, S., Shokado, S. and Han, H. (2006). Current status on research and management of pine wilt disease in Korea. Current status on research and management of pine wilt disease, International Symposium, October 20. Korea Forest Research Institute, Seoul, Korea, 31-44.

Simões, N., Laumond, C. and Bonifassi, E. (1994). Effectiveness of *Steinernema* spp. and *Heterorhabitis bacteriophora* against *Popillia japonica* in Azores. Journal of Nematology 25: 480-485.

Steiner, G. and Buhrer, E.M. (1934) *Aphelenchoides xylophilus* n. sp., a nematode associated with blue-stain and other fungi in timber. Journal of Agricultural Research 48: 49-951.

Sutherland, J.R. and Peterson, M. (1999). The nematode in Canada: history, distribution, hosts, potential vectors and research. In: Futai K, Togashi K, Ikeda T (Eds). Sustainability of pine forests in relation to pine wilt and decline. Proccedings of the Symposium Shokado Shoten Kyoto, Japan, Shokado Shoten, pp. 247-253.

Webster, J.M. (2004). The pinewood nematode: implications of factors past and present for pine wilt disease. In: Mota M and Vieira P (Eds). The nematode, *Bursaphelenchus xylophilus*. Nematology Monographs and Perspectives, vol 1.Leiden-Boston: Brill Academic Publishers, pp. 55-64.

Chapter II. Objectives and thesis organization

II.1. Aims of this thesis

Entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae are lethal parasites of some insects and a potential alternative to insecticides, since they present excellent qualities as biocontrol agents. This biological attribute makes the characterization and study of their genetic diversity extremely important since new species and/or isolates may be useful to be used as biological control agents against agriculturally important pests. The main goals in this subject were:

1. Perform a national survey in continental Portugal to evaluate the biodiversity of EPN;

2. Identify the occurring species using molecular techniques;

3. Determine the genetic diversity of species with different molecular markers.

The diversity and distribution of EPN was studied in continental Portugal from 2006 until 2009, throughout the five NUT (National Units of Territory) of the country, covering both natural and agricultural habitats. Soil samples were tested for the presence of nematodes by means of baiting with the larvae *Galleria mellonella.* Genetic studies used genetic markers, namely, D2D3 region from 28S, internal transcribed spacer (ITS) from ribosomal DNA and cytochrome c oxidase subunit I gene (COXI) and the gene cytochrome b (*cytb*) from mitochondrial DNA, to identify species and to study their genetic diversity.

B. xylophilus was detected in Portugal for the first time in 1999 (Mota *et al.,* 1999), causing serious damages in *Pinus pinaster* forests since that time. Presently, pine wilt disease (PWD), spreads to different parts of the country and recently, it has also been found in Madeira Island (Fonseca *et al.,* 2012). The importance of this nematode to the national economy, stressed the need to understand the genetic diversity of Portuguese populations and determine the

introduction and spread pathways of the disease. The main goal of the present work was:

1. Characterize the different geographic populations from continental Portugal and Madeira Island in order to understand the possible origin of the new outbreaks of the disease. For that propose several genetic markers were used, namely, IGS (intergenic spacers), *cytb* and cellulase genes and the ISSR fingerprints.

II.2. Thesis structure

This thesis includes seven chapters, with a listing of the bibliographic references presented at the end of each chapter. The results are presented in Chapter IV and Chapter VI in the form of six scientific papers (corresponding to sub-chapters), following the chronological order in which they were submitted and/or accepted for publication. These sub-chapters should be addressed as interdependent and integrated units, with the studies of the last papers already taking into consideration the results of the previous ones.

All published papers have been peer-reviewed international journals, and each following the journal´s specific guidelines. Although presenting different formatting, all papers include an introduction, description of material and methods, result, discussion of the results and the cited literature.

Chapter I is a general introduction presenting the subject of this thesis and the importance of study two different groups of nematodes, EPN and PWN.

Chapter II presents the aims and thesis structure.

Chapter III reviews the current literature on entomopathogenic nematodes, namely its current taxonomy, morphological characterization, geographic distribution, species diversity, general bio-ecology and life cycle and molecular methods used in the study of genetic diversity.

Chapter IV presents the studies on the EPN national survey in continental Portugal, and is divided in four sub-chapters:

Sub-chapter IV.1 represents the first report of an entomopathogenic nematode in continental Portugal, *Steinernema feltiae,* based on molecular data, sequences of internal transcribed spacer (ITS) region from ribosomal DNA.

Sub-chapter IV.2 is the first report of the genus *Heterorhabditis* (*H. bacteriophora)* in continental Portugal, based on morphological characters and ITS sequence.

Sub-chapter IV.3 reports the identification of three species of EPN for continental Portugal (*S. kraussei, S. intermedium* and an undescribed species *Steinernema* sp.) based on molecular sequence of ITS, and D2D3 region from 28S from ribosomal DNA and a gene from mitochondrial DNA, cytochrome c oxidase subunit I (COXI) as well as, morphological and morphobiometric analysis.

Sub-chapter IV.4 describes the study of genetic diversity of the two most abundant species in continental Portugal: *S. feltiae* and *H. bacteriophora* based on ITS, D2D3 region from 28S, COXI and cytochrome b (*cytb*). It also evaluates the possibility of abiotic/biotic factors influence the distribution of these species.

Chapter V reviews the current literature on *Bursaphelenchus xylophilus*, namely its current taxonomy, morphological characterization, geographic distribution, general bio-ecology and molecular methods used in the study of genetic diversity of PWN.

Chapter VI presents the studies on *B. xylophilus* geographic populations from continental Portugal and Madeira Island, and is divided in two sub-chapters:

Sub-chapter VI.1 studies the genetic diversity of *B. xylophilus* geographic populations from continental Portugal and Madeira Island trying to understand the possible origin of the new outbreaks of the disease and the widespread patterns of *B. xylophilus* isolates.

Sub-chapter VI.2 is based on the characterization of PWN Portuguese populations, from continental Portugal and Madeira Island, using *cytb* and cellulase gene sequences. *Cytb* and cellulase sequences were compared with sequences from *B. xylophilus* from other countries, such as USA, Japan, China and South Korea, in order to understand the possible origin of the new outbreaks of the disease in continental Portugal and Madeira Island.

Chapter VII comprehends the discussion of the results from the previous chapters and publications referring the most important data and conclusions. A general conclusion of the results from the present thesis together with future perspectives and further studies that can be undertaken, are also referred.

II.3. Literature cited

Fonseca, L., Cardoso, J.M.S., Lopes, A., Pestana, M., Abreu, F., Nunes, N., Mota, M. and Abrantes, I. (2012). The pinewood nematode, Bursaphelenchus xylophilus, in Madeira Island. Helminthologia 49(2): 96-103.

Mota, M., Braasch, H., Bravo, M.A., Penas, A.C., Bürgermeister, W., Metge, K. and Sousa, E. (1999). First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. Nematology 1: 727-734.

Chapter III: State of the art of entomopathogenic nematodes – Families Steinernematidae and Heterorhabditidae

III.1. Foreword

Entomopathogenic nematodes (EPN) inhabit a large diversity of soil habitats being worldwide distributed. EPN have an association with symbiotic bacteria, causing insect death, thus the designation of EPN. These nematodes belong to the families Steinernematidae and Heterorhabditidae, being currently used as biological agents on integrated management projects. Nematodes parasites of insects are known since the XVIIth century and include more than 30 families (Grewal *et al.,* 2005). With the advent of synthetic insecticides in the 1950s, easy control of insect pests appeared at hand. However, it soon became obvious that there were problems associated with their use. Some insect pests became resistant, and some non-target organisms were adversely affected, and pest resurgence occurred. Additionally, environmental and health concerns arose.

All insect pests have natural enemies. Conservation of natural enemies is probably the most important biological control practice readly available. Through the use of selective insecticides and the judicious use of broader spectrum materials, natural enemies can exist and exert their impact on pest populations. EPN have a great potential in biological control, because they are parasites and pathogens. They are good candidates to biological control since, according to Poinar (1979): they carry symbiotic bacteria that are released inside the insect host haemocoel; they can parasitize many different orders and families of insects; they are easily produced in a large scale; they can kill an insect in 48h; their third-stage infective juvenile (IJ), is a free-living stage and can remain in the soil during long periods of time, under extreme conditions, until they find a suitable host; EPN can be stored during long periods of time and they are not damaging for plants or animals. The interest in the use of these nematodes as biological pest control agents has increased exponentially over the past two decades and their success made them now commercially mass-produced in six of the seven continents to treat pest problems in agriculture, veterinary, horticulture, forestry and health (Grewal *et al.,* 2005).

Much more emphasis needs to be placed on investigating indigenous natural enemies and their impact on the pests they attack. With this information it may be possible to enhance the efficacy of natural enemies through manipulation of the crop habitat, changes in cultural practices, or changes in pesticide application practices (Hoffmann and Frodsham, 1993).

Along with the large diversity of cultivated crops, Portugal has a huge diversity of insect pests, which cause serious economic damage. In agriculture, insects are of great interest and farmers often have to use insecticides to protect their crops. Insects, on the other hand, are ever more resistant to insecticides (Ahmad *et al.,* 2003), and farmers need to apply greater amounts of these products and use more toxic insecticides. With this, the environment becomes more polluted and the concentration of toxic chemicals in nature's food chain increases. Insecticides can also kill natural enemies of insects (Delbeke *et al.,* 1997).

The different species of both genera in the Steinernematidae and Heterorhabditidae look so similar that the use of molecular biology to distinguish between genera and species within each genus level became common, popular and easy, because they do not require a systematic taxonomist. In spite of excellent morphological keys (Nguyen and Hunt, 2007; Hominick *et al.,* 1997), the requirements to make morphologically studies are so demanding due to the number of specimens required and the different generations that can only be achieved using an insect vector to reproduce EPN and obtain the different generations. The recent advances in molecular biology can provide nematologists with additional tools for identifying, delimiting and representing species of entomopathogenic nematodes (Liu *et al.,* 2000). Recent advances in molecular biology, allow the study of intraspecific genetic diversity, and the establishement of phylogenetic relationships between species. Currently, the most suitable approach, not only for assessing phylogenetic relationships, but also for species delimitation is the sequencing of different regions of the genome (Stock, 2009).

III.2. Taxonomy and Systematics of EPN

There are only two families of EPN: the family Steinernematidae that includes two genera, *Steinernema* Travassos, 1927 (Poinar, 1990) and *Neoaplectana* Nguyen and Smart, 1994 (Nguyen and Smart, 1994) and the family Heterorhabditidae that includes a single genus, *Heterorhabditis* Poinar, 1976 (Poinar, 1990). These nematodes have been surveyed in several countries and used as biocontrol agents against economically insect pests in various crops.

Gotthold Steiner (1886-1961) identified the first entomopathogenic nematode, *Aplectana* (= Steinernema) *kraussei* (Steiner, 1923) giving major contributions to the systematics of nematodes parasites of insects, thus being considered the "father of entomopathogenic nematodes" (Poinar, 1990). Rudolf Glaser, produced EPN for the first time in solid medium (Glaser, 1931) and axenically (Glaser *et al.,* 1940). In 1929, in New Jersey, he discovered a nematode parasitising the Japanese beetle, *Popillia japonica* and sent it to Steiner, who described it as *Neoaplectana glaseri*, today classified as *S. glaseri* (Steiner, 1929).

III.2.1. Family Steinernematidae Chitwood and Chitwood, 1937

The first entomopathogenic nematode species, *Aplectana kraussei*, was described for the first time by Steiner (1923) and changed by Travassos (1927) to *S. kraussei*. After a few years, Chitwood and Chitwood raised the subfamily Steinernematidae to the family level (Chitwood and Chitwood, 1937). Presently, Nguyen and Hunt (2007) consider the family with two genera: *Steinernema* with more than 60 described species and *Neosteinernema* with just one species, *N. longicurvicauda* (Nguyen and Hunt, 2007) (Fig.III1.).

Phylum: Nematoda

Class: Chromadorea

Order: Rhabditida

Sub-order: Tylenchina

Infra-order: Panagrolaimomorpha

Super-family: Strongyloidoidea

Family: Steinernematidae

Genera: *Steinernema*

Neosteinernema

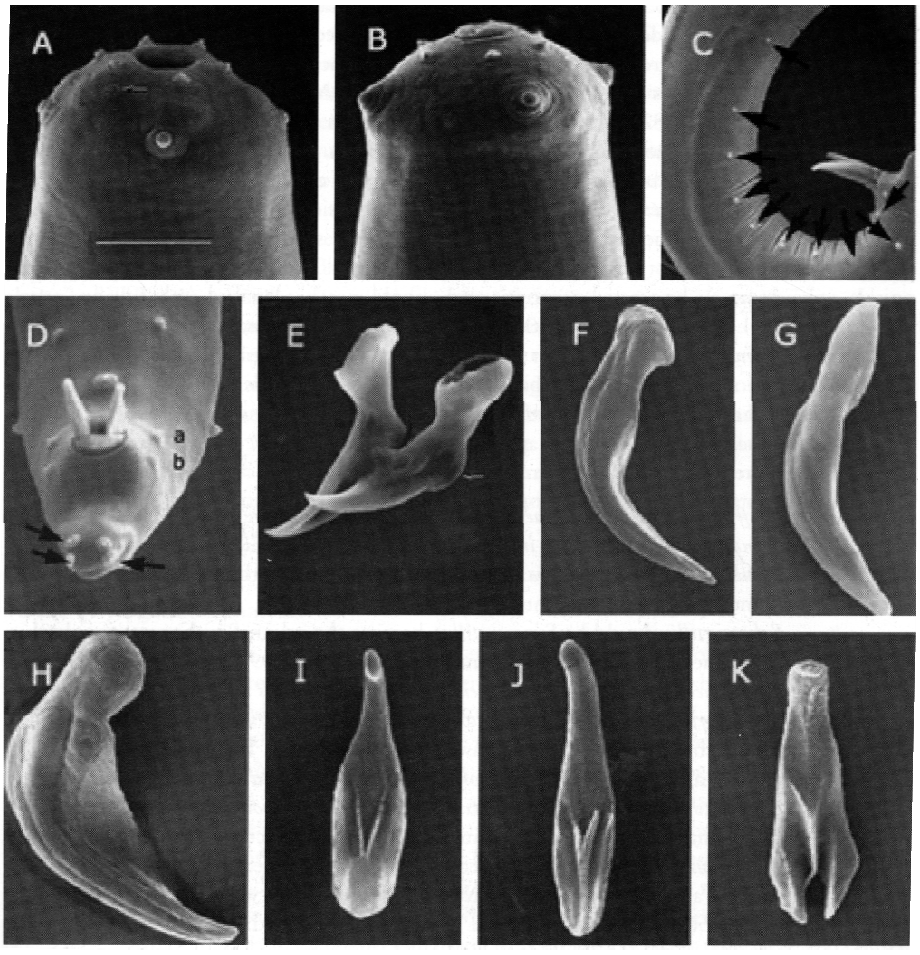


Figure.III1. SEM micrographs of male morphological structures of the family Steinernematidae. A, B, Head showing stomal opening, labial and cephalic papillae; C, Spicules and pre-anal genital papillae (arrows); D, Posterior region; E, Spicules of *Neosteinernema*; F-H, Spicules of *Steinernema*; I-K, Gubernacula of *Steinernema*. Scale bars: A, B =10 µm, C = 50 µm, D-K = 22 µm. (From Nguyen and Hunt, 2007).

III.2.1.1 Genus *Steinernema* Chitwood and Chitwood, 1937

Nematodes from genus *Steinernema* are obligate insect parasites, with the infective juveniles (IJ) carrying the symbiotic bacteria of the genus *Xenorhabdus,* in a special chamber in the ventricular part of the intestine. IJ or *dauer* larvae (third stage juvenile- J3) are the only free living stage in the soil. Both males and females are needed for reproduction (Nguyen and Hunt, 2007). Generally, they have two or three generations of amphimitic adults inside the host, and finally the J3 emerges from the insect cadaver searching for a new insect host. The anterior end is rounded or truncated and has normally six fused lips each one with a labial papillae (Fig.III.1) (Nguyen and Hunt, 2007). They usually have a typical rhabditoid esophagus and a perioral disc, with a slightly swollen metacorpus, an isthmus surrounding the nerve ring and a large basal bulb with reduced valve. Excretory pore opening distinct (Nguyen and Hunt, 2007).

Male morphological characters are the most important in the taxonomy of Steinernematidae. They are monorchic with single and reflexed testis. Spicules are paired and symmetrical, with a long gubernaculum and no bursa (Fig.III.1). The end of the tail is rounded, digitated or mucronated. The head is swollen or not, with four cephalic papillae and six labial papillae (Fig.III.1) (Nguyen and Hunt, 2007).

Females are large, with variable size and do not have lateral fields. On the other hand, we may see the presence or absence of the epiptygma, a structure located in the vulva (Fig.III2.C). The tail tip has a small mucron that can be used as a diagnostic character (Fig.III2B). They have a distinct excretory pore, anterior to the nerve ring. The head is rounded or truncated, with six lips fused, completely or not. The reproductive system is didelphic, amphidelphic and reflexed, with the vulva mid–body, sometimes with a protuberance. Females are oviparous or ovoviviparous with the developing infective juveniles inside the body before emerging (Nguyen and Hunt, 2007).

IJ have a head with four cephalic papillae and six or four labial papillae. The lateral field from head to tip and its characteristics (number of lines) is a very

important character. Phasmids may be small, large or can be absent. In the third phase of IJ (J3) it can be observed the excretory pore before the nerve ring (Nguyen and Hunt, 2007).

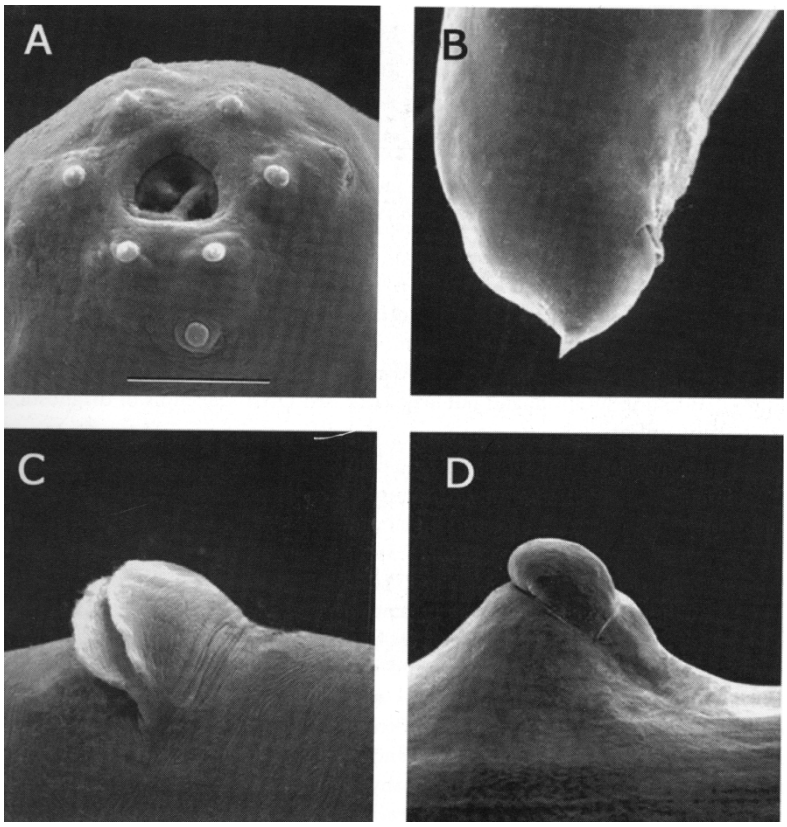


Figure.III2. SEM micrographs of morphological structures of female of *Steinernema*. A, Face view showing labial and cephalic papillae; B, Tail with a mucron; C, Double-flapped epiptygma; D, Vulva with a thick flap. Scale bars: A = 10 µm, B = 52 µm C, D = 20 µm. (From Nguyen and Hunt, 2007).

This genus is worldwide distributed and can be easily diagnosed by the presence in the male of a small mucron in the tail and spicules with a *capitulum.* The IJs can be identified by their length and the presence of the excretory pore in the anterior position (Poinar, 1990). Geographically, this genus can be found in North America, South America, Australia, New Zealand and Europe.

Type species: *Steinernema kraussei* (Steiner, 1923) Travassos, 1927

III.2.1.2. Genus *Neosteinernema* Nguyen and Smart, 1994

Adults of this genus have prominent phasmids located posterior to tail.

Females are ovoviviparous, with the eggs hatching inside the female body. The juveniles became IJ before hatching from female. The tail is longer than body width at anus (Fig.III3B). There is no second generation female.

Males are smaller than females. The posterior region has one ventral and 13-14 pairs of genital papillae. Phasmids prominent and tail tip digitated. Spicule foot-shaped with the gubernaculum as long as the spicule (Fig.III3C).

The IJ are characterized for the presence of a swollen head (Fig.III3A), a large phasmid and an elongated filiform tail, usually curved at the end (Fig.III3F).

This genus will not be mentioned further, because the only species was not diagnosis in the above mentioned studies and also because bibliographic references only referred to *Steinernema* and *Heterorhabditis* genus.

Type species: *Neosteinernema longicurvicauda* (Nguyen and Smart, 1994).

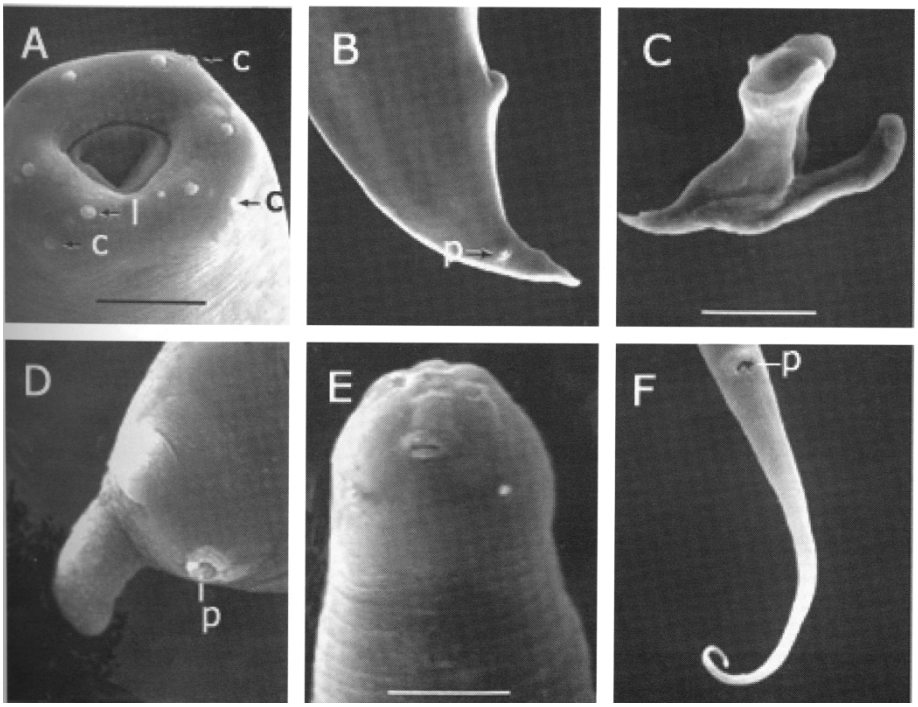


Figure.III3. SEM micrographs of *Neosteinernema longicurvicauda*. A, Cephalic papillae; B, Female tail; C, Foot-shaped spicules and gubernaculum; D, Tail end with digitate tail tip; E,

Head of infective juvenile; F, tail of infective juvenile. Scale bars: A =12 µm, B =30 µm C = 23 µm, D =5 µm, E =3 µm F = 17 µm. (From Nguyen and Hunt, 2007).

III.2.2. Family Heterorhabditidae Poinar, 1976

2.2.2.1. Genus *Heterothabditis* (Poinar, 1976)

Phylum: Nematoda

Class: Chromadorea

Order: Rhabditida

Sub-order: Rhabditina

Infra-order: Rhabditomorpha

Super-family: Strongyloidoidea

Family: Heterorhabditidae

Genus: *Heterorhabditis*

Adults from this genus present an anterior region with six well developed and separated lips, each one surrounding oral aperture and with terminal papillae. They have a symbiotic association with bacteria from the genus *Photorhabdus* that are located in the intestine. Once inside the body host these bacteria are bioluminescent. In this genus we may find both hermaphroditic and amphimitic females.

In hermaphroditic females (first generation) (Fig.III4A), the head is truncated or slightly rounded, with six conical lips, well developed each of them with a terminal papilla. The vulva is median, surrounded by elliptical rings (Fig.III4B). Ovotestis is amphidelphic and reflexed. Females are oviparous, later becoming ovoviviparous. The reproductive system is amphidelphic and reflexed. The vulva is not functional for deposition of eggs, with the hatching occurring inside the female body. In spite of that, vulva is functional for mating.

Males have one pair of spicules and a gubernaculum. Bursa is present with nine pairs of genital papillae and is similar in all species. Spicules and gubernaculum can be used as diagnostic characters. Males have just one reflexed testis. Spicules are paired, separated and slightly curved (Fig.III4E). The spicule head is shorter and gubernaculum usually as half long as spicule length (Fig.III4F). The bursa is present with nine pairs of genital papillae (Fig.III4D).

In amphimictic females (second generation) (Fig.III4C) the vulva pattern is also a good diagnostic character as well as the spicule. Amphimictic females are similar to hermaphroditic ones, but smaller, having prominent labial papillae.

IJ from third stage (J3) present a dorsal denticular formation in the anterior region used as a diagnostic character (Fig.III4I). J3 usually retains the cuticle of the second stage juvenile (J2) (Fig.III4G,H). The mouth and anus are closed. The excretory pore is anterior to the nerve ring. Symbiotic bacteria are found inside the intestine. Tail terminus pointed.

Poinar (1976) described the family Heterorhabditidae when he described the type species *Heterorhabditis bacteriophora*. The family has only one genus, and all species are obligate insect parasites; the infective juveniles carry symbiotic bacteria of the genus *Photorhabdus.*

Type species: *Heterorhabditis bacteriophora* Poinar, 1976.

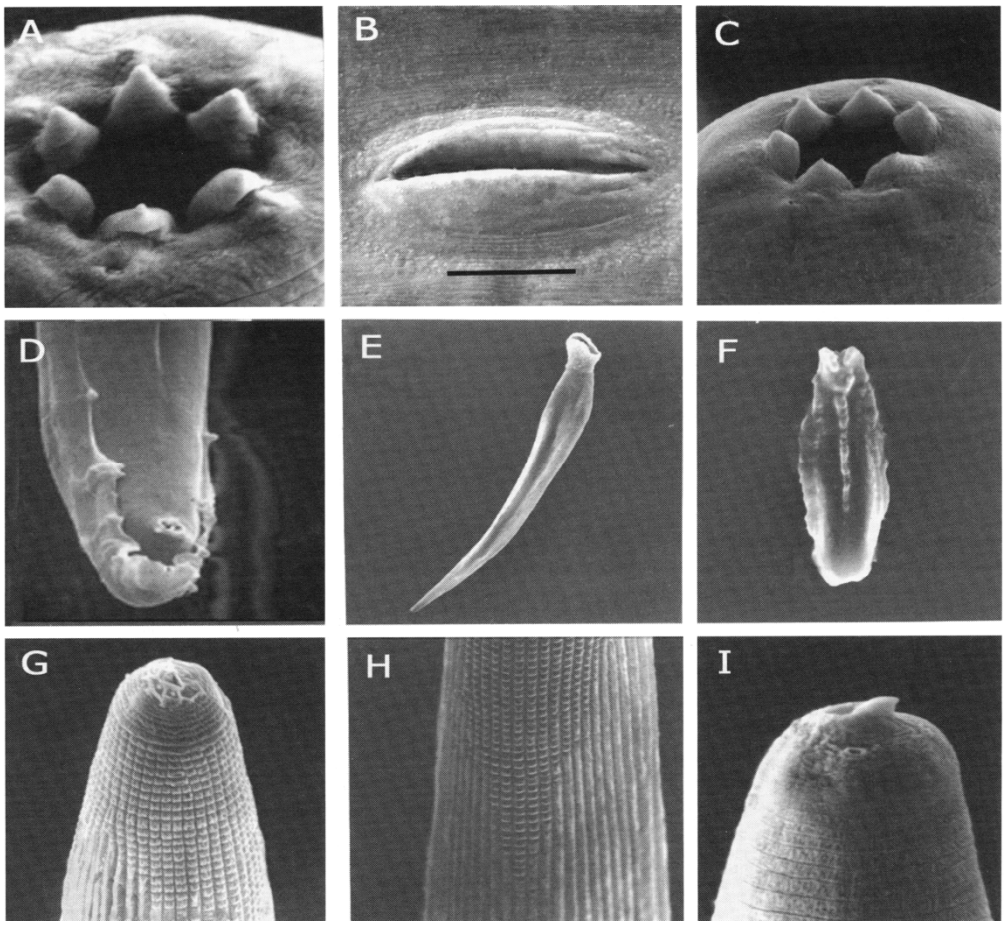


Figure.III4. SEM micrographs of *Heterorhabditis* spp. A, Hermaphroditic female face view; B, Vulval appearance; C, Face of amphimictic female; D, Bursa; E, Spicule; F, Gubernaculum; G, H, Anterior region of a third stage infective juvenile with the cuticule of the second stage juvenile. Note tessellate pattern and longitudinal ridges; I, Dorsal tooth of infective juvenile third stage. Scale bars: A = 5 µm, B =8.6 µm C = 6 µm, D = 20 µm, E = 15 µm, F = 8.6 µm, G = 8.6 µm, H = 10 µm, I = 3.8 µm. (From Nguyen and Hunt, 2007).

III.3. Biogeography

Steinernema and *Heterorhabditis* are distributed worldwide, with the only exception of Antarctica (Poinar, 1990). The reason of such a distribution is not known, but it can be due to natural dispersion and human introduction. Natural dispersion can be made by infected insects and human dispersion can be due to the introduction of exotic plants into non-native ecosystems (Poinar, 1990).

Surveys of EPN have been conducted worldwide, and it has been demonstrated that they can be present in the most variable habitats, from tropical to arctic,

due to their ability to survive under extreme conditions. In previous studies, the recovery percentage of EPN have varied between 3.8% in Northern Ireland (Blackshaw, 1998), 3.9% in the Azores, Portugal (Rosa *et al.,* 2000), 4.6% in Korea (Choo *et al.,* 1995), 5% in Italy (Ehlers *et al.,* 1991), 6.8% in the Hawaiian Islands (Hara *et al.,* 1991), 10% in Japan (Yoshida *et al.,* 1998), 12.8% in Great Britain (Gwynn and Richardson, 1996), 13.2% in the Pampas region of Argentina (Stock, 1995), 20% in western Canada (Mráček and Webster, 1993), 20.2% in North Carolina (Akhurst and Brooks, 1984), 25% in Sweden (Burman *et al.,* 1986), 30% in the south-western coastal zone of Sri Lanka (Armarasinghe *et al.,* 1994), 35% in the Slovak Republic (Sturhan and Liscova, 1999), 36.8% in Czechoslovakia (Mráček, 1980), 48.6% in England (Hominick and Briscoe, 1990), 7% in Chile (Edgington *et al.,* 2010), 23.3%- 5.4% in Spain (Garcia Del Pino and Palomo, 1996; Campos-Herrera *et al.,* 2007), 7.4% in China (Ma *et al.,* 2010), 6% in Guadeloupe islands (Constant *et al.,* 1998), 10.5% in Nepal (Khatri-Chhetri *et al.,* 2010), 53.8% - 50.6% in Check Republic (Mráček *et al.,* 1999, 2005), 4.84% in Vietnam (Long *et al.,* 2001, 2003, 2005, 2006a,b), 26.3% in California, USA (Stock *et al.,* 1999), 16.2% in Belgium (Midutri *et al.,* 1996), as well as others.

Both EPN families could occupy the same continent, the same region and even the same square metre. They are able to coexist because they occupy different soil depths and/or depend on different insects to maintain their populations (Hominick, 2002). EPN distribution is affected by many biotic and abiotic factors. Habitat type is also important in the distribution of EPN, for example, *S. kraussei* and *S. intermedium* are more common in forests ecosystems (Spiridinov and Moens, 1999; Stock *et al.,* 1999; Sturhan, 1999), while *S. feltiae* and *S. affine* are mostly found in grasslands (Boag *et al.,* 1992; Hominick *et al.,* 1995; Stock *et al.,* 1999). Climatic conditions are also an important factor affecting EPN distribution. For example, *H. indica* is mostly found in tropical and sub-tropical regions, whereas *H. bacteriophora* occurs in the Mediterranean region and central Europe, Japan and Africa appearing to have a global distribution. *S. feltiae* and *S. kraussei* have a wide distribution in the temperate region (Hominick, 2002). Generally the prevalence of steinernematids seems to be highest in woodlands (Stock *et al.,* 1999; Sturhan, 1999, Sturhan and

Liskova, 1999). Soil type is essential to guarantee the existence of *Heterorabditis* spp. but less important in Steinernematids (Hominick, 2002).

III.4. General Biology

Steinernema and *Heterorhabditis* infection begin with IJ, that are morphological and physiologically adapted to live in soil for long periods of time until they find a suitable host. The survival of IJ depends on each species, in spite of abiotic factors affect their survival. Symbiotic bacteria have an important nutritional function and are found in a ventricular portion of the intestine in steinernematids and in the entire intestine in heterorhabditis (Nguyen and Hunt, 2007). Once they find a suitable host, the IJs move over short distances using CO_2 and other chemicals produced in waste products of insects as cues to find their host (Robinson, 1995; Hui and Webster, 2000). The IJs from *Steinernema* penetrate the host through the natural openings, such as mouth, anus and spiracles, and in *Heterorhabditis* the IJs posses a dorsal tooth, which can be used to perforate the exterior of the cuticle of the insect allowing penetration. Once IJs penetrate the host, they start to develop. Symbiotic bacteria are released from the anus and initiate their life cycle in insect haemocoel. The success, maturation and multiplication of nematodes end with IJ formation, which depends on the establishment of the bacterial population in the insect haemolymph. The bacteria produce nutrients to nematodes and antibiotics that avoid the establishment of other microorganisms inside the insect body. Usually, the insect dies within 24-48h (Kaya and Gaulgler, 1993). Bacteria are primarily responsible for insect death, but the nematode also produces a toxin that is lethal to the insect (Burman, 1982). The symbiotic relationship between nematodes and bacteria is complex: the nematode serves as a vector for bacteria to enter the insect host, and the bacteria contribute to this mutualistic relationship by providing nutrients available to nematode reproduction and development inside the insect cadaver, using secretions or enzymes that they transport and that promote degradation of host tissues. In spite of that, bacteria release substances with antibiotic activity that protects the insect´s cadaver

from the invasion of opportunistic organisms, such as other bacteria, fungi, or saprophytic nematodes (Gaugler, 2001).

EPN can have many generations during their life cycle but only the IJ can survive outside the insect host and move from one insect to another. Depending on the host insect, nematode life cycle is completed in 6-18 days, when temperatures range is 18-28ºC (Poinar, 1990). The IJs that enter the host became hermaphrodites in the first generation in *Heterorhabditis* and males/females in *Steinernema*. Several generations can develop inside the insect host until nutrient supply is depleted. At this time the nematodes enter third stage (IJs) and leave the insect cadaver looking for a new host.

III.4.1. Life Cycle

Steinernematids and heterorhabditids have similar life cycles. The non-feeding, developmentally arrested IJ seeks out insect hosts and initiates infection. After locating the host, the nematode penetrates the insect body cavity, via natural openings (mouth, anus, spiracles) or areas of the thin cuticle. Once inside the insect body, the haemocoel, a symbiotic bacterium (*Xenorhabdus* for steinernematids, *Photorhabdus* for heterorhabditis) is released from the nematode gut which rapidly multiplies causing insect death, by septicemia in the next 24-48H (Grewal *et al.,* 2005). In both genera, the invasive form of the nematode, the infective juvenile carries the symbiotic bacteria inside special vesicles in the intestine of *Steinernema* (*Xenorhabdus*) or free in the anterior part of the intestine *Heterorhabditis* (*Photorhabdus*). The nematodes feed upon the bacteria and liquefying host tissues, mature into adult's stage. When the IJs of *Steinernema* enter the haemocoel of the insect host, they reach the feeding stage (J3), feeding on the bacteria and moulting to J4 and then into adults males and females of first generation (Grewal *et al.,* 2005) (Fig.III5). Adults mate and females lay the eggs that hatch and complete the life stages until reaching adults forms again. Then, the second generation begins. After hatching, when food source starts to decrease, the eggs moult to J1 and them J2, ceasing to feed, and incorporating a pellet of bacteria in the bacterial

chamber, moulting to J3, but retaining the cuticle of J2 as a sheath, leaving the cadaver to search for a new host (Fig.III5). When it finds a new suitable host, the J3 move towards the insect, penetrates its body and releases the cuticle that surrounds its body and become infective juveniles, releasing the bacteria to the insect haemocoel and a new cycle starts again (Grewal *et al.,* 2005). The life cycles of *Steinernema* and *Neosteinernema* are equal except for the fact that, in the last case, there is only one generation and the infective juveniles emerge inside the female body (Gaugler, 2001). In *Heterorhabditis,* the life cycle is similar, but in the first generation we have hermaphroditic self fertilizing females (Gaugler, 2001). Only in the next generations, second or more, amphimictic females are present. In both genera, females lay some eggs externally but most of them emerge inside the female body and later *endotokia matricida* occurs (intra-uterine birth causing maternal death) (Grewal *et al.,* 2005).

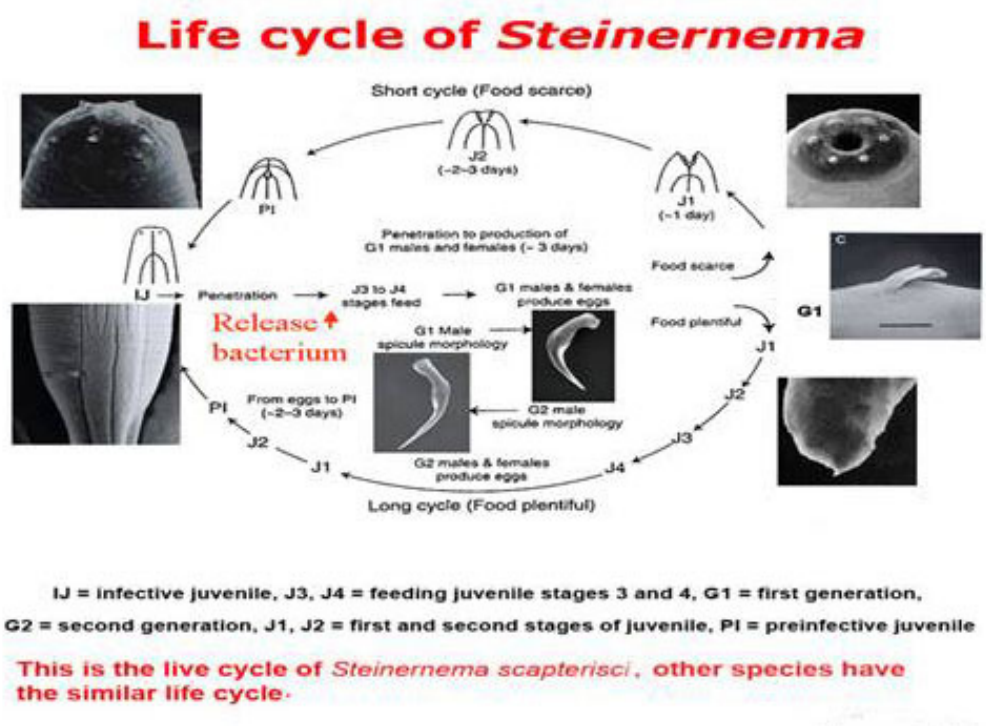


Figure.III5. Lyfe cycle of *Steinernema* sp. (From Nguyen and Hunt, 2007).

Entomopathogenic nematodes are a nematode-bacterium complex, where nematode growth and reproduction depend upon conditions established in the host cadaver by the bacterium. The bacterium also produces anti-immune

proteins to assist the nematode in overcoming host defenses, and anti-microbial that suppress colonization of the cadaver by competing with secondary invaders. Conversely, the bacterium lacks invasive power and is dependent upon the nematode to locate and penetrate the insect host (Akhurst and Smith, 2002; Grewal *et al.,* 2005).

The IJ, is free-living and a non-feeding stage, but once inside the insect host, it starts to feed on bacteria and moults into a third-stage juvenile. It starts to reproduce and changes to the various stages of the life cycle and for the diverse generations until food resources decrease. When this happens, *dauer* larvae – infective juvenile is formed, as a resistant stage and then reabsorb the bacteria into their body and leave the cadaver to the soil, searching for a new host. The infective juvenile can maintain itself in the soil under adverse conditions, during long periods of time, until it finds a suitable host.

In both cases, *Steinernema* and *Heterorhabditis,* during the life cycle of the nematode there are two different but "symbiotic" phases: the first during the infection period, when the nematode enters the insect haemocoel – pathogenic phase; the second where a relationship of mutualism between the nematode and the bacteria is established, and each organism take advantage from the presence of the other – mutualistic phase (Gaugler, 2001) (Fig.III6).

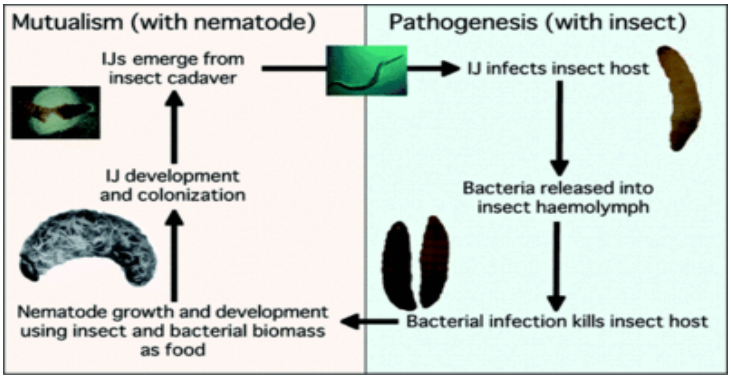


Figure.III6. Symbiotic phases of the bacteria that leave in mutualistic association with entomopathogenic nematodes. (From Nguyen and Hunt, 2007).

III.5. Bioecology - Survival Biology

Soil is a difficult environment for the persistence of any organism due to its complexity of physical, chemical and biological components. In spite of that, EPN have been isolated throughout the world. EPN, like other organisms, spend part of their life cycle in the soil, and so adopted unique survival mechanisms to resist environmental extreme and survive (Gaugler, 2001). These nematodes can survive under a dormant stage (IJ) which allows them to maintain their metabolism at low levels and with no need to feed. There are many factors that can promote the formation of this stage, and they all interact: absence of water, extreme temperatures, lack of oxygen, moisture, precipitation, texture among others (abiotic factors) and also the biotic factors, such as predators or other competitive organisms. All these factors interact and affect nematode survival. Wallace (1963) points out several soil factors that affect nematode survival. Texture and soil composition affect nematode survival because the available space between soil particles, and the low available oxygen reduces survival rate (Kaya, 1990). Temperature and moisture has a direct effect in nematode survival. With temperature increase, the survival of nematodes is reduced (Kaya, 1990; Molyneux, 1985). Water is also very important because it affects the mobility of IJs. Oxygen availability is very important and limitant to IJ survival (Kaya, 1990). The existence of antagonists is also fundamental in the nematode/ environment balance, bacteria, fungi and predators being the most important to be considered.

Abiotic factors

1. **Soil texture**: soil pores are occupied by water and oxygen, and inside them there are living organisms. Soil porosity depends on texture and depth (Wallace, 1963). Soil texture influences the survival, pathogenicity and IJ dispersion. Soils with high levels of clay are not well aerated and thus determine the survival of nematodes (Kaya, 1990). Soil deficiently aerated makes nematode dispersion and host finding difficult.
2. **Moisture** is another abiotic factor that affects nematode survival in soil. It represents the available water in soil (Kaya, 1990). Moisture is very important in the survival, dispersion, search for hosts and pathogenicity

of IJs. Moisture values present in nature don't seem to limit nematode life cycle, with steinernematids that are found in soils with the lowest moisture values (Kung *et al.,* 1990 a,b). This parameter allows nematodes to survive, affecting pathogenicity and host finding (Kaya, 1990). Extreme moisture values (low, in sandy soils; high in clay soils) inhibit infection capability of EPN.

Nematodes live inside the water available in soil pores, and thus exchanges in oxygen must occur (Kaya, 1990). Aeration influence nematode survival, pathogenicity and the occurrence of IJ in the soil. Oxygen is a limiting factor in soils with high levels of organic matter, with much water available or in clay soils (Kaya, 1990).

3. **Temperature** is other factor that influence the presence of nematodes is soil. Nematodes live in the upper horizons of soil, where temperature variations are faster and frequent. Nematode survival, life cycle, dispersion and host search depend on temperature. Lower temperatures are more suitable to nematode survival, than higher ones, because in higher temperatures, the available water decreases, oxygen concentration and moisture too, and nematodes have difficulty in moving towards soil pores and searching for insect hosts (Kaya, 1990).
4. **pH** values also affect nematode survival and dispersion. pH values, in the majority of soils varies between 4-8, and seems to have little influence in nematode activity.
5. **Organic matter**: the availability of organic matter in soils causes an increase of organisms in soil. Native populations of *S. feltiae* increase when organic supplements are available. Nevertheless, the best results are obtained at low levels of available organic matter (Akhurst and Bedding, 1986).

For example, sandy soils normally are more suitable for nematode presence than clay soils, because in these soils, usually, the aeration is better and consequently oxygen availability is also more favorable. It is also important to know the natural conditions where the native species are found, to use them in new environments.

Biotic factors

The effectiveness of EPN also depends on biotic soil factors, which include hosts (Kaya, 1990) and antagonists (Timper and Kaya, 1989; Van Sloun *et al.,* 1990). The result of these factors causes a reduction on EPN survival and establishment (Kaya, 1990).

Entomopathogenic nematodes are insect parasites, being the presence of the host needed to assure EPN persistence in soil (Kaya, 1990). EPN are able to infect more than 300 insect species (Poinar, 1979; Simões *et al.,* 1992). Insect host determines the mode of dispersion of the IJ, because IJs respond positively to the increase of CO_2 concentrations (Gaugler *et al.,* 1980), to temperature variation (Byers and Poinar, 1982) and to the excretion products produced by the insect hosts (Schmit and All, 1978).

EPN are biological control agents that affect the survival mechanisms of other organisms, called antagonists, which are present in the soil environment. Antagonists include antibiosis, competition, parasites, pathogens and predators (Gray, 1988; Stirling, 1988). Predators and parasites decrease the effectiveness of EPN, because they reduce their survival ability and the capacity to locate host insects (Timper *et al*, 1991).

III.6. Molecular systematics

The recent advances in molecular biology and evolutionary taxonomy, do not substitute the expertise of a taxonomist, but provide nematologists with additional tools for identification and delimiting entomopathogenic species (Stock*,* 2009). Molecular techniques have been developed to distinguish species, as well as to study genetic variability and phylogenetic relationships of organisms (Stock, 2009). Several studies have already integrated both, morphological and molecular data (Stock and Kaya, 1998; Van Luc *et al.,* 2000).

Selection of the appropriate genes is quite important. Among nuclear genes, ribosomal genes have been extensively used at different taxonomic levels.

These genes are found in high copy numbers and each gene evolves at different rates (Stock, 2009). Ribosomal genes include the 18S rRNA gene, the internal transcribed spacers (ITS1 and ITS2), the 28S rRNA gene and 5.8S rRNA gene, which include variable and conserved regions (Stock, 2009). The 5.8S rRNA gene is a highly conserved region, contrary to ITS1 and ITS2 regions that evolve at a higher rate than 18S and 28S rRNA genes, making them ideal for phylogenetic studies at the species and population levels (Nguyen *et al,* 2001; Stock, 2009). The 28S rRNA gene is more variable than the 18S rRNA gene and has fewer ambiguously aligned positions than ITS (Stock, 2009), having been used to study phylogenetic relationships among *Steinernema* species (Stock, 2009). 18S rRNA gene is characterized by having a slow evolutionary rate of change, due to its conservative nature. It is normally used for studying EPN at the species level (Stock, 2009; Stock *et al.,* 2001; Nadler *et al.,* 2006).

The genus *Steinernema* has been divided into five phylogenetic groups based on the 28S rRNA D2D3 region and infective juveniles' length (Nguyen and Hunt, 2007): "*bicornutum-group*", "*carpocapsae-group*", "*feltiae-group*", "*glaseri-group*", and "*intermedium-group*".

Mitochondrial genes have different evolutionary rates than chromosomal genes. Mitochondrial cytochrome c oxidase subunit I (COXI) and cytochrome b (*cytb*) genes evolve slowly, being better suited for deeper lineage phylogenies (Stock, 2009).

Cytochrome c oxidase subunit II (COXII) (Szalanski *et al.,* 2000) and ND4 (Liu *et al.,* 1999) have also revealed to be good markers to assess species identification.

Other markers, such as satellite DNA probes also proved to be a good diagnostic tool for the identification of species (Abad *et al.,* 1998). Determining intraspecific variability of EPN should be assessed carefully, especially if the marker is used in phylogenetic studies (Hominick, 2002). Sequences of microsatellite DNA seem to be a good marker used in the study of genetic variation between populations (Jarne and Lagoda, 1996). Random amplified

polymorphic DNA (RAPD) is another technique used in the study of genetic variation between populations of the same species (Hashmi and Gaugler, 1998). In spite of that, the sensitivity of this technique and the difficulty of produce reproducible results make it not very reliable.

Few studies have sampled multiple populations of a single EPN species. In *Heterorhabditis* populations, rRNA ITS (Adams *et al.,* 1998) and mtDNA ND4 locus (Liu *et al.,* 1999), revealed little intraspecific variation. For phylogenetic analysis at the species level several studies have already been made: RFLP, RAPD (Liu and Berry, 1996), sequences of 18S, ITS, COII and ND4. The 18S rRNA gene revealed to be too conserved to resolve relationships between *Steinernema* and *Heterorhabditis* species (Liu *et al.,* 1997). On the other hand, the mutations on ITS, COXII and ND4 are too high (Adams *et al.,* 1998, Nguyen *et al.,* 2001), so most of the aforementioned markers have been extensively studied, yet an ideal marker has yet to be found (Adams *et al.,* 1998; Liu *et al.,* 1999; Nguyen *et al.,* 2001).

III.7. Literature cited

Abad, M., Grenier, E., Laumond, C. and Abad, P. (1998). A species-specific satellite DNA from the entomopathogenic nematode *Heterorhabditis indicus*. Genome 41: 148-153.

Adams, B.J., Burnell, A.M. and Powers, T.O. (1998). A phylogenetic analysis of *Heterorhabditis* (Nemata: Rhabditidae) based on internal transcribed spacer 1 DNA sequence data. Journal of Nematology 30: 22–39.

Ahmad, M., Arif, M.I. and Denholm, I. (2003). High Resistance of Field Populations of the Cotton Aphid Aphis gossypii Glover (Homoptera: Aphididae) to Pyrethroid Insecticides in Pakistan. Journal of Economic Entomology 96: 875-878.

Akhurst, R. and K. Smith. (2002). Regulation and safety. In: R. Gaugler (Ed.), Entomopathogenic Nematology. CABI Publishing, Oxon, UK. pp. 311-332.

Akhurst, R.J. and Bedding, R.A. (1986). Natural occurrence of insect pathogenic nematodes (Steinernematidae and Heterorhabditidae) in soil in Australia. Australian Journal of Entomology 25: 241–244.

Akhurst, R.J. and Brooks, W.M. (1984). The distribution of entomophilic nematodes (Heterorhabditidae and Steinernematidae) in North Carolina. Journal of Invertebrate Pathology 44: 140-145.

Blackshaw, R.P. (1988). A survey of insect parasitic nematodes in Northern Ireland. Annals of Applied Biology 113: 561–565.

Boag, B., Neilson, R. and Gordon, S.C. (1992). Distribution and prevalence of the entomopathogenic nematode *Steinernema feltiae* in Scotland. Annals of Applied Biology 121: 355–360.

Burman, M., Abrahamsson, K., Ascard, J., Sjoberg, A. and Eriksson, B. (1986). Distribution of insect parasitic nematodes in Sweden. In: Samson, R.A., Vlak, J.M. and Peters, D. (Eds) Proceedings 4th International Colloquium of Invertebrate Pathology, Veldhoven, The Netherlands, pp. 312.

Burman, M. (1982). *Neoaplectana carpocapsae*: toxin production by axenic insect parasitic nematodes. Nematologica 28: 62 –70.

Byers, J.A. and Poinar, G.O. Jr. (1982). Location of insect hosts by the nematode, *Neoaplectana carpocapsae*, in response to temperature. Behavior 79: 1–10.

Campos-Herrera, R., Escuer, M., Labrador, S., Robertson, L., Barrios, L. and Gutiérrez, C. (2007). Distribution of the entomopathogenic nematodes from La Rioja (Northern Spain). Journal of Invertebrathe Pathology 95: 125–139.

Chitwood, B. G. and Chitwood, M. B. (1937). An introduction to nematology. Monumental Printing Company, Baltimore, Maryland, pp. 372.

Choo, H.Y., Kaya, H.K. and Stock, S.P. (1995). Isolation of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from Korea. Japanese Journal of Nematology 25: 44–51.

Constant, P., Marchay, L., Fischer-Le-Saux, M., Briand-Panoma, S. and Mauléon, H. (1998). Natural occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Guadeloupe islands. Fundamental and Applied Nematology 21 (6): 667-672.

Delbeke, F., Vercruysse, P., Tirry, L., De Clercq, P. and Degheele, D. (1997). Toxicity of diflubenzuron, pyriproxyfen, imidacloprid and diafenthurion to the predatory bug, Orius laevigatus (Heteroptera: Anthocoridae). Entomophaga 42: 349-358.

Edgington, S., Buddie, A.G., Moore, D., France, A., Merino, L., Tymo, L.M. and Hunt, D.J. (2010). Diversity and distribution of entomopathogenic nematodes in Chile. Nematology 12 (6): 915-928.

Ehlers, R.U., Deseo, K.V. and Stackebrandt, E. (1991). Identification of Steinernema spp. (Nematoda) and their symbiotic bacteria Xenorhabdus spp. from Italian and German soils. Nematologica 37: 360–366.

García del Pino, F. and Palomo, A. (1996). Natural occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Spanish soils. Journal of Invertebrate Patholoy 68: 84–90.

Gaugler, R. (2001). Entomopathogenic nematology. CABI Publishing, Bnew York, USA, pp. 388.

Gaugler, R., LeBeck, L., Nakagaki, B. and Boush, G.M. (1980). Orientation of the entomopathogenic nematodes, *Neoaplectana carpocapsae*, to carbon dioxide. Environmental Entomology 8: 649-652.

Glaser, R.W., McCoy, E.E. and Girth, H.B. (1940). The biology and economic importance of a nematode parasitic in insects. Journal of Parasitology 26: 479--495.

Glaser, R.W. (1931). The cultivation of a nematode parasiteof an insect. Science 73: 614-15.

Gray, N.F. (1988). Fungi attacking vermiform nematodes. In: Poinar, G.O.Jr. and Jasson, H.B. (Eds) Disease of nematodes, Vol.2. CRC Press, Boca Raton, Florida, pp. 3-33.

Grewal, P.S., Ehlers, R.-U. and Shapiro-Ilan, D.I. (2005). Nematodes As Biocontrol Agents. CABI Publishing, CAB International, Oxon, UK.

Gwynn, R.L. and Richardson, P.N. (1996). Incidence of entomopathogenic nematodes in soil samples collected from Scotland, England and Wales. Fundamental Applied Nematology. 19: 427–431.

Hara, A.H., Gaugler, R., Kaya, H.K. and Lebeck, L.M. (1991). Natural populations of entomopathogenic nematodes (Rhabditida: Heterorhabditidae, Steinernematidae) from the Hawaiian islands. Environmental Entomology 20: 211–216.

Hashmi, G. and Gaugler, R. (1998). Genetic diversity in insect-parasitic nematodes (Rhabditida: Heterorhabditidae). Journal of Invertebrate Pathology 72: 185-189.

Hoffmann, M.P. and Frodsham, A.C. (1993). Natural Enemies of Vegetable Insect Pests. Cooperative Extension, Cornell University, Ithaca, NY. pp. 63.

Hominick, W.M. (2002). Biogeography. In: Gaugler, R. (Ed.), Entomopathogenic Nematology. CABI Publishing, Wallinford, UK, pp. 115–143.

Hominick, W.M., Briscoe, B.R., Garcia del Pino, F., Heng, J., Hunt, D.J., Kozodoy, E., Mraček, Z., Nguyen, K.B., Reid, A.P., Spiridonov, S., Stock, P., Sturhan, D., Waturu, C. and Yoshida, M. (1997). Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. Journal of Helminthology 71: 271–298.

Hominick, W.M., Reid, A.P. and Briscoe, B.R. (1995). Prevalence and habitat specificity of steinernematid and heterorhabditid nematodes isolated during soil surveys of the UK and the Netherlands. Journal of Helminthology 69: 27–32.

Hominick, W.M. and Briscoe, B.R. (1990). Occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in British soils. Parasitology 100: 295–302.

Hui, E. and Webster, J.M. (2000). Influence of insect larvae and seedling roots on the host-finding ability of *Steinernema feltiae* (Nematoda: Steinernematidae). Journal of Invertebrate Pathology 75: 152-162.

Jarne, P. and Lagoda, P.J.L. (1996). Microsatellites, from molecules to populations and back. Trends in Ecology and Evolution 11: 424-429.

Kaya H.K. and Gaugler R. (1993). Entomopathogenic nematodes. Annual Review of Entomology 38: 181-206.

Kaya, H.K. (1990). Soil Ecology. In: Gaugler, R. And Kaya, H.K. (Eds) Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, Florida, pp. 93-115.

Khatri-Chhetri H.B., Waeyenberge L., Spiridonov S., Manandhar H.K. and Moens M. (2010). Two new species of *Steinernema* Travassos, 1927 with short infective juveniles from Nepal. Russian Journal of Nematology 19: 53-74.

Kung, S., R. Gaugler, and Kaya, H.K. (1990a). Soil type and entomopathogenic nematode persistence. Journal of Invertebrate Pathology 55: 401-406.

Kung, S., R. Gaugler, and Kaya, H.K. (1990b) Influence of soil pH and oxygen on persistence of *Steinernema* spp. Journal of Nematology 22: 440-445.

Liu, J., Poinar Jr., G.O., and Berry, R.E. (2000). Control of insect pests with entomopathogenic nematodes: the impact of molecular biology and phylogenetic reconstruction. Annual Review of Entomology 45: 287-306.

Liu, J., Berry, R.E. and Blouin, M.S. (1999). Molecular differentiation and phylogeny of entomopathogenic nematodes (Rhabditida: Heterorhabditidae) based on ND4 gene sequences of Mitochondrial DNA. The Journal of Parasitology 85: 709-715.

Liu, J., Berry, R.E. and Moldenke, A.F. (1997). Phylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) inferred from partial 18S rDNA gene sequences. Journal of Invertebrate Pathology 69: 246-252.

Liu, J.and Berry, R.E. (1996). Steinernema oregonensis n. sp. (Rhabditida: Steinernematidae) from Oregon, USA. Fundamental and Applied Nematology. 19: 375-380.

Ma, J., Shulong, C., Zou, Y., Xiuhua, L., Richou, H. and De Clercq, P. Moean, M., (2010). Natural occurrence of entomopathogenic nematodes in North China. Russian Journal of Nematology 18: 117-126.

Molyneux, A. S. (1985). Survival of infective juveniles of *Heterorhabditis* spp. and *Steinernema* spp. (Nematoda: Rhabditida) at various temperatures and their subsequent infectivity for insects. Revue de Nématologie 8:165–170.

Mráček Z., Bečvář S., Kindlmann P. and Jersáková J. (2005). Habitat preference for entomopathogenic nematodes, their insect hosts and new faunistic records for the Czech Republic. Biological Control 34: 27-37.

Mráček Z., Bečvář S. and Kindlmann P. (1999). Survey of entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) in the Czech Republic. Folia Parasit. 46, 145-148.

Mráček, Z. and Webster, J.M. (1993). Survey of heterorhabditidae and steinernematidae (Rhaditida, Nematoda) in Western Canada. Journal of Nematology 25 (4): 710–717.

Mráček, Z. (1980). The use of “Galleria traps” for obtaining nematode parasites of insects in Czechoslovaquia (Lepidoptera: Nematoda, Steinernematidae). Acta Entomologica 77: 378–382.

Nadler, S.A., Bolotin, E. and Stock, S.P. (2006). Phylogenetic relationships of Steinernema Travassos, 1927 (Nematoda: Cephalobine: Steinernematidae) based on nuclear, mitochondrial and morphological data. Systematic Parasitology 63: 161-181.

Nguyen, K.B. and Hunt, D. (2007). Entomopathogenic nematodes: Systematics, phylogeny and bacterial symbionts. Leiden, E. J. Brill, pp. 816.

Nguyen, K.B., Maruniak, J. and Adams, B.J. (2001). The diagnostic and phylogenetic utility of the rDNA internal transcribed spacer sequences of *Steinernema*. Journal of Nematology 33: 73–82.

Nguyen, K.B. and. Smart, G.C. (1994). Neosteinernema longicurvicauda n. gen., n. sp. (Rhabditida: Steinernematidae), a parasite of the termite Reticulitermes flavipes (Koller). Journal of Nematology 26: 162-174.

Poinar, G.O. (1990). Taxonomy and biology of Steinernematidae and Heterorhabditidae. In: Gaugler, R. and Kaya, H.K. (Eds) Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, Florida, pp. 23-61.

Poinar, G.O. (1979). Nematodes for biological control of insects. CRC Press, Boca Raton, Florida. pp. 270.

Poinar, G.O., Jr. (1976). Description and biology of a new insect parasitic rhabditoid *Heterorhabditis bacteriophora* n. gen., n. spp., (Rhabditida, n. ram.). Nematologica. 21: 463-470.

Robinson, A.F. (1995). Optimal release rates for attracting *Meloidogyne incognita*, *Rotylenchulus reniformis*, and other nematodes to carbon dioxide in sand. Journal of Nematology 27: 42–50.

Rosa J.S., Bonifassi E., Amaral J., Lacey L.A., Simões N. and Laumond C. (2000). Natural occurrence of entomopathogenic nematodes (Rhabditida: Steinernema, Heterorhabditis) in Azores. Journal of Nematology 32: 215–222.

Simões, N., Bettencourt, R., Laumond, C. and Boemare, N. (1992). Studies on immunoinhibitor released by *Steinernema carpocapsae*. In: In Proceedings of the European Society of Nematology 21st Meeting, Albufeira, Portugal, pp. 123-127.

Steiner, G. (1929). *Neoaplectana glaseri* n. g., n. sp. (Oxyuridae) a new nemic parasite of the Japanese beetle (*Popillia japonica* Newm.). Journal of the Washington Academy of Science 19: 436-440.

Steiner, G. (1923). *Aplectana kraussei* n. sp., eine in der Blattwespe Lyda sp. parasitierende Nematoden form, nebst Bomerkungen uber das Seitenorgan der parasitischen Nematoden. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Aby. 59: 14-18.

Stirling, G.R. (1988). Biological control of plant parasitic nematodes In: Poinar, G.O.Jr. nad Jansson, H.B. (Eds) Disease of nematodes, Vol.2. CRC Press, Boca Raton, Florida, pp. 93-139.

Stock, S.P. (2009). Molecular approaches and the taxonomy of insect-parasitic and pathogenic nematodes. In: Stock, S.P., Vandenburg, J., Glazer, I., Boemare, N. (Eds), Insect pathogens: molecular approaches and techniques. CAB International Press, pp. 71-100.

Stock S.P., Campbell J.F. and Nadler S.A. (2001). Phylogeny of Steinernema Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. Journal of Parasitology 87: 877-889.

Stock, S.P., Pryor, B.M. and Kaya, H.K. (1999). Distribution of entomopathogenic nematodes (Steinernematidae, Heterorhabditidae) in natural habitats in California. Biodiversity and Conservation 8: 339–345.

Stock, S.P. and Kaya, H.K. (1998). *Steinernema siamkayai* sp. n. (Rhabditida: Steinernematidae), an entomopathogenic nematode from Thailand. Systematic Parasitology 41: 105-113.

Stock, S.P. (1995). Natural populations of entomopathogenic nematodes in the Pampean region of Argentina. Nematropica 25: 143-148.

Sturhan, D. (1999). Prevalence and habitat specificity of entomopathogenic nematodes in Germany. In: Gwynn, R.L., Smits, P.H., Griffin, C., Ehlers, R.U., Boemare, N. and Masson, J.P. (Eds) COST 819 – Entomopathogenic

Nematodes – Application and Persistence of Entomopathogenic Nematodes. European Commission, Luxembourg, DG XII, pp. 123–132.

Sturhan, D. and Liskova, M. (1999). Occurrence and distribution of entomopathogenic nematodes in the Slovak Republic. Nematology 1: 273–277.

Szalanski, A.L., Taylor, D.B. and Mullin, P.G. (2000). Assessing nuclear and mitochondrial DNA sequence variation within *Steinernema* (Rhabditida: Steinernematidae). Journal of Nematology 32: 229-233.

Timper, P., Kaya, H.K. and Jaffee, B.A. (1991). Survival of entomogenous nematodes in soilinfested with the nematode parasitic fungus *Hirsutella rhossiliensis* (Deuteromycotina: Hyphomycetes). BiologicalControl 1: 42-50.

Timper, P. and Kaya, H.K. (1989). Role of the 2nd-stage cuticule of entomogenous nematode in preventing infection by nematophagous fungi. Journal of Invertebrate Pathology 54: 314-321.

Travassos, L. (1927). Sobre o genera Oxysomatium. Boletim Biológico. 5: 20-21.

Van Luc, P., Nguyen, K.B., Reid, A.P.and Spiridonov, S.E. (2000). *Steinernema tami* sp. n. (Rhabditida: Steinernematidae) from Cat Tien Forest, Vietnam. Russian Journal of Nematology 8: 33-43.

Van Sloun, P. Nicolay, R., Lohmann, U. and Sikora, R.A. (1990). Anfälligkeit von entomopathogenen nematode gegnüber nematodenfangenden und endoparasitären Pilzen. Journal of Phytopathology 129: 217-227.

Wallace, H.R. (1963). The biology of plant parasitic nematodes. London: Edward Arnold.

Yoshida, M., Reid, A.P., Briscoe, B.R. and Hominick, W.M. (1998). Survey of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Japan. Fundamental and Applied Nematology 21:185-198.

Chapter IV. Identification of entomopathogenic nematodes species in continental Portugal

Sub-chapter IV.1.

Valadas, V., Boyle, S., Vieira, P., Kakouli-Duarte, T. and Mota, M. (2007). First report of an entomopathogenic nematode from continental Portugal. Helminthologia 44 (4): 226-229.

Sub-chapter IV.2.

Valadas, V., Vieira, P., Oliveira, S. and Mota, M. (2009). First report of the genus *Heterorhabditis* (Nematoda: Heterorhabditidae) from continental Portugal. Helminthologia 46 (1): 45–48.

Sub-chapter IV.3.

Valadas, V., Mráček, Z., Oliveira, S. and Mota, M. (2011). Three species of entomopathogenic nematodes of the family Steinernematidae (Nematoda: Rhabditida) new to continental Portugal. Nematologia Mediterranea 39 (2): 169-178.

Sub-chapter IV.4.

Valadas, V., Laranjo, M., Mota, M. and Oliveira, S.. A survey of entomopathogenic nematodes species in continental Portugal reveals predominance of *Steinernema feltiae* and *Heterorhabditis bacteriophora (submitted).*

A survey of entomopathogenic nematodes species in continental Portugal reveals predominance of *Steinernema feltiae* and *Heterorhabditis bacteriophora*

Vera Valadas [a], Marta Laranjo [b,c], Manuel Mota [a], Solange Oliveira [b]

[a] NemaLab-ICAAM & Departamento de Biologia, Universidade de Évora, Évora, Portugal

[b] Lab. Microbiologia do Solo-ICAAM & Departamento de Biologia, Universidade de Évora, Évora, Portugal

[c] IIFA (Instituto de Investigação e Formação Avançada), Universidade de Évora, Évora, Portugal

(Corresponding author: S. Oliveira, Fax-+351266760914, E-mail: ismo@uevora.pt)

Abstract

Entomopathogenic nematodes (EPN) are lethal parasites of insects used as biocontrol agents. The objectives of this work were to survey the presence of EPN in continental Portugal and to evaluate the genetic diversity of *Steinernema feltiae* and *Heterorhabditis bacteriophora*, the two most abundant species. Of the 791 soil samples collected throughout continental Portugal, 53 were positive for EPN. Analysis of EPN geographical distribution revealed an association between nematode species and vegetation type. *H. bacteriophora* was mostly found in the Alentejo region while *S. feltiae* was present in land occupied by agriculture with natural vegetation, broad-leaved forest, mixed forest, transitional woodland-shrub, agro-forestry areas, complex cultivated patterns and non-irrigated arable land. Although no clear association was found between species and soil type, *S. feltiae* was typically recovered from cambisols and *H. bacteriophora* was more abundant in lithosols. Sequencing of the ITS region indicated that *S. feltiae* was the most abundant species, followed by *H. bacteriophora*. *S. intermedium* and *S. kraussei* were each isolated from one site and *Steinernema* sp. from two sites. *H. bacteriophora* was found mainly in forest and agricultural fields, while *S. feltiae* was present in several habitats. Phylogenetic analyses of ITS, D2D3 expansion region of the 28S rRNA gene, as well as COXI and cytb genes, was performed to evaluate the genetic diversity of *S. feltiae* and *H. bacteriophora*. No significant genetic diversity was found among *H. bacteriophora* isolates. However, COXI seems to be the best marker to study genetic diversity of *S. feltiae*. This survey contributes to the understanding of EPN distribution in Europe.

Keywords: *Steinernema feltiae, Heterorhabditis bacteriophora*, genetic diversity, ITS rDNA, D2D3, COXI, *cytb*.

Introduction

Entomopathogenic nematodes (EPN) are a group of nematode families which are insect parasites and possess many attributes that enable their commercial use as biocontrol agents (Kaya & Stock, 1997). They are promising candidates for biocontrol of insects due to their ability to search for hosts, safety to non-target organisms and the environment, high reproductive potential, capacity to be mass produced and ability to be used with other agricultural chemicals (Koppenhöfer, 2000). Only the infective third-stage juveniles (IJ) of these nematodes are found free living in soil and non-feeding stages, under diverse ecological conditions and in all kinds of habitats (Hominick *et al*., 1996), being able to survive in soil during extended periods, until they find a suitable host. EPN are distributed worldwide, and include more than 16 species of *Heterorhabditis* and at least 60 species of *Steinernema* (Nguyen & Hunt, 2007). They are obligate parasites of insects that form complex, highly virulent symbiotic relationships with enterobacteria, *Xenorhabdus*-Steinernematidae and *Photorhabdus*-Heterorhabditidae (Boemare, 2002). As parasites of insects, they have a wide range of hosts (Kaya & Gaugler, 1993) being used in crop protection in Australia, Europe, USA and Asia (Georgis *et al*., 2006).

The study of their genetic diversity is extremely important because new species and/or isolates may be useful as biological control agents against agriculturally important pests (Stock, 2009). The detection and identification of indigenous EPN isolates is of major importance, due to differences in strain virulence against natural and local insect pests, environmental conditions that may affect their survival and reproductive potential (Stock, 2009). Several surveys on EPN have already been conducted in Mediterranean countries, namely Egypt (Shamseldean & Abd- Elgawad, 1994), Greece (Menti *et al*., 1997), Italy (Triggiani & Tarasco, 2000), Palestine (Iraki *et al*., 2002), Spain (García del Pino, 2005) and Turkey (Hazir *et al*., 2003). Recently, surveys have been conducted in several European countries, such as Austria (Hozzank *et al*., 2003), Belgium (Midituri *et al*., 1997), Bulgaria (Shishiniova *et al.,* 2000), Czechoslovakia (Mráček *et al*.,1999), Denmark (Nielsen & Philipsen, 2003), Germany (Sturhan & Ruess, 1999), Poland (Bednarek, 1998), Russia (Ivanova *et al*., 2000), Slovakia (Sturhan & Liskova, 1999), Switzerland (Steiner, 1996) and United Kingdom (Gwynn & Richardson, 1996). Entomopathogenic nematodes show significant variation in behaviour, host range,

infectivity, reproduction and tolerance to adverse environmental conditions and therefore it is of major interest to fully characterize natural populations (Stock, 2009).

Entomopathogenic species identification should combine both molecular and morphological data (Adams *et al*., 2007). Cross-breeding tests have also been used, but since the discovery of hermaphroditism in steinernematids by Griffin *et al*. (2001), the consideration of hybridization assays to test validity of biological species has been reduced in importance (Stock *et al*., 2002). Morphological analysis is the basis of nematode classification, but molecular markers provide faster results which should be carefully analyzed, according to the DNA region used (Stock *et al.,* 2002). Molecular techniques used in EPN studies have been developed to distinguish species, races and biotypes, as well as to study genetic diversity (Stock, 2009).

Studies on the genera *Steinernema* and *Heterorhabditis* have been conducted using molecular methods, such as RAPD (Liu & Berry, 1996) and RFLP (Reid *et al*., 1997). After several tested methods, sequencing of different regions of the genome has became the most suitable approach, not only for assessing phylogenetic relationships, but also for species delimitation (Stock, 2009). Among nuclear genes, ribosomal genes have been extensively used at different taxonomic levels. Ribosomal genes include the 18S rRNA gene, the internal transcribed spacers (ITS1 and ITS2), the 5.8S and the 28S rRNA gene, which contain variable and conserved regions (Stock, 2009). The 5.8S rRNA gene is a highly conserved region, contrary to the ITS1 and ITS2 regions, which evolve at a higher rate than the 18S and 28S rRNA genes, making them ideal for phylogenetic studies at species and population levels (Nguyen *et al*, 2001; Spiridonov *et al.*, 2004; Stock, 2009). On the other hand, mitochondrial cytochrome c oxidase subunit I (COXI) and cytochrome b (*cytb*) genes evolve more slowly, being better suited for deeper lineage phylogenies (Stock, 2009). The genus *Steinernema* is divided into five phylogenetic groups based on the D2D3 expansion region of the 28S rRNA gene and infective juveniles' length (Nguyen & Hunt, 2007): "*bicornutum-group*", "*carpocapsae-group*", "*feltiae-group*", "*glaseri-group*", and "*intermedium-group*".

Earlier reports of EPN in Portugal were conducted in the Azores archipelago (Rosa & Simões, 2004; Simões *et al*., 1994), where several surveys have been undertaken as part of a wide program to find endemic biological agents to control insect pests of pastures. These previous studies describe the presence of *Steinernema carpocapsae, S. glaseri*

and *Heterorhabditis bacteriophora* in the Azores (Rosa & Simões, 2004; Simões *et al*., 1994). Until 2006, there were no studies on entomopathogenic nematodes from continental Portugal. The first published report was on the identification of three isolates (I1, I8, H9) of *S. feltiae* based on ITS sequence (Valadas *et al*., 2007). Later, three other isolates (I3, R7, X7), were identified as *H. bacteriophora* using both ITS sequence and morphological characterization (Valadas *et al*, 2009). More recently, three other species, *S. intermedium* (isolate 2B), *S. kraussei* (isolate 20F) and *Steinernema* sp. (isolates 59F and 15G) have been described (Valadas *et al*., 2011), using morphological and molecular data.

It is known that EPN distribution depends on temperature and precipitation and is closely related to vegetation type and presence of insect hosts. Soil type and texture are also very important parameters which influence EPN distribution (Campos Herrera *et al*., 2011; El Borai *et al*., 2012). Continental Portugal has a wide diversity of crops, such as fruit trees, cereals and vegetables, and natural habitats, such as conifer forests and grasslands. These habitats are subject to insect pests which every year cause significant losses in agricultural production.

The major objectives of this research were to determine the distribution of EPN in continental Portugal and to evaluate the genetic diversity of *S. feltiae* and *H. bacteriophora*, the two most abundant species.

Materials and methods

Survey zones and sampling procedure

Between 2006 and 2009, 791 soil samples were haphazardly collected across continental Portugal, divided into five NUTS (Nomenclature of Territorial Units for Statistics): Norte, Centro, Lisboa e Vale do Tejo (herein designated as Lisboa), Alentejo and Algarve (http://www.igeo.pt/atlas/Cap3/Cap3f_1.html).

According to Köppen's climate classification (Köppen & Geiger, 1928), continental Portugal is divided in two regions: one temperate with rainy winters and dry, hot summers (Csa) and another temperate with rainy winters and dry, cool summers (Csb). Vegetation is affected by climate and thus, continental Portugal has three kinds of

influence: Atlantic, Continental and Mediterranean, Atlantic being predominant. Soil samples were collected from both cultivated and non-cultivated areas, covering the two climatic regions of continental Portugal, including different vegetation types, such as irrigated land, forests, grasslands, cultivated fields, among others. Three to four subsamples were collected at 0-20 cm and used to create a single sample representative of 200m^2. Soil samples were properly dated and identified with GPS (Global Positioning System) (Garmin, USA) location.

Soil and vegetation types were mapped with ArcGIS software version 10.0 (Esri) using FAO soil classification (FAO, 2006) and CORINE land cover classification (Caetano *et al*., 2009). Temperature and precipitation data were obtained from "PORDATA, Base de Dados Portugal Contemporâneo" (http://www.pordata.pt).

Nematode recovery, propagation and identification

EPN were recovered from soil samples using the baiting technique, described by Bedding & Akhurst (1975). Before processing, samples were homogenized and then baited with ten last instar larvae of *Galleria mellonella* placed inside a perforated metal tea bag, partly filled with soil which was embedded in the soil sample. Soil samples were stored in the dark at 25ºC and dead *G. mellonella* were removed and replaced every four days for twelve total days of baiting. Collected *G. mellonella* were transferred to White traps (White, 1927) and IJ recovered for the five to twelve following days. IJ were stored in distilled water at 10ºC. To establish new cultures, emerging nematodes were pooled for each sample and used to infect new *G. mellonella* larvae. Only IJ collected during the week after the first emergence from the insect cadavers were used to establish new cultures. EPN were preliminarily identified to genus according to the color of *G. mellonella* cadavers, which ranges from cream to brown (*Steinernema* spp.) or red (*Heterorhabditis* spp.) within 24-48 h after nematode penetration. Isolates with atypical discoloration were identified to genus following the method of Nguyen & Hunt (2007). Further identification of isolates was carried out by sequencing the ITS region of rRNA (Nguyen *et al*., 2001).

DNA extraction

For each isolate, genomic DNA was extracted from a suspension of 50 µl with more than 10 000 nematodes. Total DNA was extracted with the JETQUICK Tissue DNA

Spin Kit extraction kit (Genomed), according to the manufacturer's protocol. DNA was used for sequence analysis of ribosomal regions ITS and D2D3 region of the 28S rRNA gene, and mitochondrial genes *cytb* and COXI. Nematode DNA was kept at -20ºC for further use.

Sequencing of ribosomal regions and mitochondrial genes

Fifty µl PCR reactions containing 1X PCR buffer (Fermentas), 1.5 mM $MgCl_2$ (Fermentas), 200 µM each dNTP (Fermentas), 0.4 µM of each primer, with the exception of *cytb* primers, were used at a concentration of 1 µM each (STABvida), 2.5 units of *Taq* DNA Polymerase (recombinant) (Fermentas) and 5 µl template DNA (10-20 ng) were used. Primers and PCR amplification conditions for each region and gene are those presented in table 1.

Table 1. Primers used for sequencing reactions of ribosomal regions and mitochondrial genes. PCR conditions for amplification of the different regions and genes.

Target	**Primer**	**Sequence (5´-3´)**	**Product (bp)**	**Reference**	**Innitial denaturation**	**Cycles**	**Denaturation**	**Annealing**	**Elongation**	**Final extension**
ITS rRNA	TW81-F AB28-R	5´-GTTTCCGTAGGTGAACCTGC-3' 5´-ATATGCTTAAGTTCAGCGGGT-3´	1000	Curran et al., 1994	5min, 94ºC	30	1min, 94ºC	1min 30s, 55ºC	2min, 72ºC	5min, 72ºC
D2D3 expansion	#391-F #501-R	5´-AGCGGAGGAAAAGAAACTAA – 3´ 5´-TCGGAAGGAACCAGCTACTA- 3´	1100	Nadler et al., 2006	3min, 94ºC	33	30s, 94ºC	30s, 52ºC	1min, 72ºC	7min, 72ºC
COXI	#507-F #588-R	5´-AGTTCTAATCATAARGATATYGG- 3´ 5´-TAAACTTCAGGGTGACCAAAAAATCA- 3´	1000	Nadler et al., 2006	3min, 94ºC	37	30s, 94ºC	30s, 50ºC	45s, 72ºC	7min, 72ºC
cytb	CytBHetF-F CytBHetR-R	5´-TTTTGTAAATTCTCTTGTT–3´ 5´-AAATAGAAAACAAATAACTCAAA- 3´	1200	this study	3min, 95ºC	35	1min, 95ºC	1min 30s, 45ºC	1min, 72ºC	5min, 72ºC

All PCR products were analyzed by electrophoresis in 1% agarose gels with Tris-borate buffer, stained in ethidium bromide, purified with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced at STABvida, Portugal.

Phylogenetic analysis

Multiple sequence alignments of ITS, D2D3 region of the 28S rRNA gene, COXI and *cytb* sequences were assembled using the ClustalW algorithm as implemented in BioEdit version 7.1.3.0 (Hall, 1999), under default alignment parameters. The best-fitting evolutionary model of nucleotide substitutions was determined, using MEGA5 version 5.05 (Tamura *et al*., 2011) and phylogenetic relationships among isolates were reconstructed by Neighbor-Joining (Saitou & Nei, 1987) and Maximum Likelihood (ML) methods. Gaps or indels were treated using the complete deletion option. Bootstrap analysis was performed with 100 replicates (Felsenstein, 1985). Sequences were compared with those from reference organisms/strains available in the GenBank database.

Statistical analysis

Statistical analysis was performed using PASW Statistics 18 release 18.0.0 software (SPSS Inc., Chicago, IL, USA). Relationships between species and NUTS, vegetation or soil types were determined using the chi-square test of association. Results are presented as the test statistic ($\chi 2$), degrees of freedom (df), and probability of equal or greater deviation (P). Correspondence analysis (CA) was used as an explorative method to study associations and to reveal interdependencies between each two of the abovementioned variables. Visualization using CA is based on representing chi-square distances among species and NUTS, vegetation or soil types.

Results

EPN distribution

EPN were recovered from 53 of the 791 sampled sites (6.7%) (fig. 1). Nine soil samples (17%) were positive for the occurrence of heterorhabditids and 44 (83%) for

steinernematids, identified as described. Nematodes isolated from the positive samples were further identified to the species level based on ITS sequences.

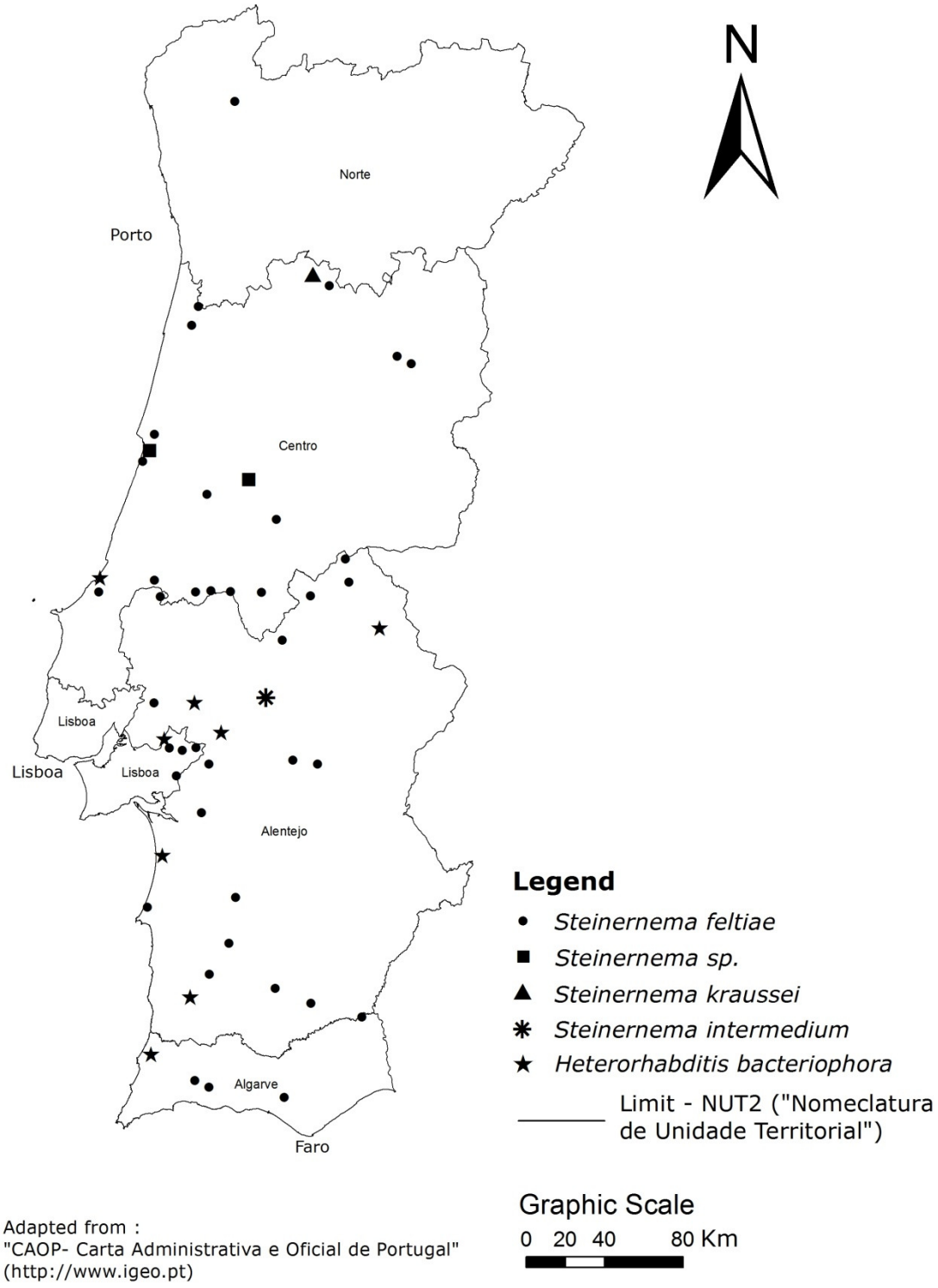


Fig. 1. Entomopathogenic nematode distribution across the five NUTS (Nomenclature of Territorial Units for Statistics) of continental Portugal. Each point is marked with the name of the isolate and the corresponding species. Map based on the CAOP (http://www.igeo.pt).

Although there is no association between species and NUTS ($P > 0.1$) (table 2), the CA biplot revealed that *H. bacteriophora* isolates are more abundant in the Alentejo region (data not shown).

Table 2. Occurrence of entomopathogenic nematodes by NUTS (Nomenclature of Territorial Units for Statistics) (CAOP; http:///www.igeo.pt). The table presents information about the area of each NUTS compared to the total area of continental Portugal and the number of samples collected, as well as the percentage of EPN species recovered ([1] *Steinernema feltiae*; [2] *S. intermedium*; [3] *S. kraussei*; [4] *Steinernema* sp.; [5] *Heterorhabditis bacteriophora*).

NUTS	**Area (Km^2)**	**Area (%)**	**Number of collected samples**	**Number of EPN**	**Positive samples (%)**
Norte	21285.876	23.89	268	1	1.9[1]
Centro	28199.404	31.65	207	19	28[1]; 1.9[3]; 3.8[4]; 1.9[5]
Lisboa e Vale do Tejo	3001.938	3.37	84	5	7.5[1];1.9[5]
Alentejo	31604.906	35.48	181	23	30.2[1]; 1.9[2]; 11.3[5]
Algarve	4996.795	5.61	51	5	7.5[1]; 1.9[5]

The nine positive samples for *H. bacteriophora* were recovered from sites below the altitude of 400 m. *S. feltiae* was detected from 40 sites (75.5%) in locations where altitude varies between 13-878 m. No association was found between species and climate ($P > 0.05$).

Habitat and soil type

Positive samples were most commonly found in mixed forest, land occupied by agriculture with natural vegetation, broad-leaved forest and transitional woodland-shrub (table 3). *H. bacteriophora* was found in a wide range of habitats including: mixed forest, transitional woodland-shrub, vineyards, broad-leaved forest, land occupied by agriculture with natural vegetation and permanently irrigated land (table 3).

S. feltiae was present in all kinds of habitats containing EPN, being more abundant in land occupied by agriculture with natural vegetation, broad-leaved forest, mixed forest, transitional woodland-shrub, agro-forestry areas, complex cultivated patterns and non-

irrigated arable land (table 3). S. intermedium was found in a mixed forest and S. kraussei in moors and heathland habitat (table 3). The Steinernema sp. isolates were found in a broad-leaved forest and in a mixed forest (table 3).

Table 3. Occurrence of entomopathogenic nematodes in the studied vegetation type according the "Land Cover Nomenclature" (Caetano et al., 2009). The table presents information about the number of samples collected from each vegetation type (vegetation code), as well as the species percentage recovered ([1] *Steinernema feltiae*; [2] *S. intermedium*; [3] *S. kraussei*; [4] *Steinernema* sp.; [5] *Heterorhabditis bacteriophora*).

Vegetation type			Number of collected samples	Number of EPN	Positive samples (%)
(type I)	**(type II)**	**(type III)**			
artificial surfaces	urban fabric	Discontinuous urban fabric (112)	47	2	3.8[1]
artificial surfaces	industrial, commercial and transport units	Industrial or commercial units (121)	6	0	0
artificial surfaces	industrial, commercial and transport units	Road and rail networks and associated land (122)	1	0	0
artificial surfaces	Mine, dump and construction sites	Construction sites (133)	1	0	0
agricultural areas	Arable lands	Non-irrigated arable land (211)	47	3	5.7[1]
agricultural areas	Arable lands	Permanently irrigated land (212)	39	3	3.81: 1.95
agricultural areas	Arable lands	Rice fields (213)	10	0	0
agricultural areas	Permanent crops	Vineyards (221)	15	2	3.8[5]
agricultural areas	Permanent crops	Fruit trees and berry plantation (222)	13	2	3.8[1]
agricultural areas	Permanent crops	Olive groves (223)	20	2	3.8[1]
agricultural areas	pastures	Pastures (231)	2	0	0
agricultural areas	pastures	Artificial grasslands (232)	1	0	0
agricultural areas	Heterogenous agricultural areas	Annual crops associated with permanent crops (241)	71	0	0
agricultural areas	Heterogenous agricultural areas	Complex cultivation patterns (242)	89	3	5.7[1]
agricultural areas	Heterogenous agricultural areas	Land occupied by agriculture with natural vegetation (243)	43	7	11.3[1]; 1.8[5]
agricultural areas	Heterogenous agricultural areas	Agro-forestry areas (244)	21	3	5.7[1]
forest and semi-natural areas	Forests	Broad-leaved forest (311)	77	6	7.5[1]; 1.9[4]; 1.9[5]
forest and semi-natural areas	Forests	Coniferous forest (312)	57	1	1.9[1]
forest and semi-natural areas	forests	Mixed forest (313)	58	8	7.5[1]; 1.9[2]; 1.9[4]; 3.8[5]
forest and semi-natural areas	Scrub and/or herbaceous vegetation associations	Natural grasslands (321)	30	2	3.8[1]
forest and semi-natural areas	Scrub and/or herbaceous vegetation associations	Moors and heathland (322)	16	1	1.9[3]
forest and semi-natural areas	Scrub and/or herbaceous vegetation associations	Sclerophyllous vegetation (323)	14	0	0
forest and semi-natural areas	Scrub and/or herbaceous vegetation associations	Transitional woodland-shrub (324)	94	6	7.5[1]; 3.8[5]
forest and semi-natural areas	Open spaces with little or no vegetation	Beaches, dunes, sands (331)	3	0	0
forest and semi-natural areas	Open spaces with little or no vegetation	Burnt areas (334)	2	0	0
wetlands	Maritime wetlands	Salt marshes (421)	1	0	0
water bodies	Inland waters	Water bodies (511)	3	1	1.9[1]
water bodies	Marine waters	Estuaries (522)	10	1	1.9[1]

There is an association between species and vegetation type ($\chi 2$=83.438, df=60, P>0.05) (fig. 2). Furthermore, the CA biplot showed that the most abundant species, *S. feltiae*, is present mainly in land occupied by agriculture with natural vegetation, broad-leaved forest, fruit trees and berry plantations, olive groves and non-irrigated arable land (fig. 2).

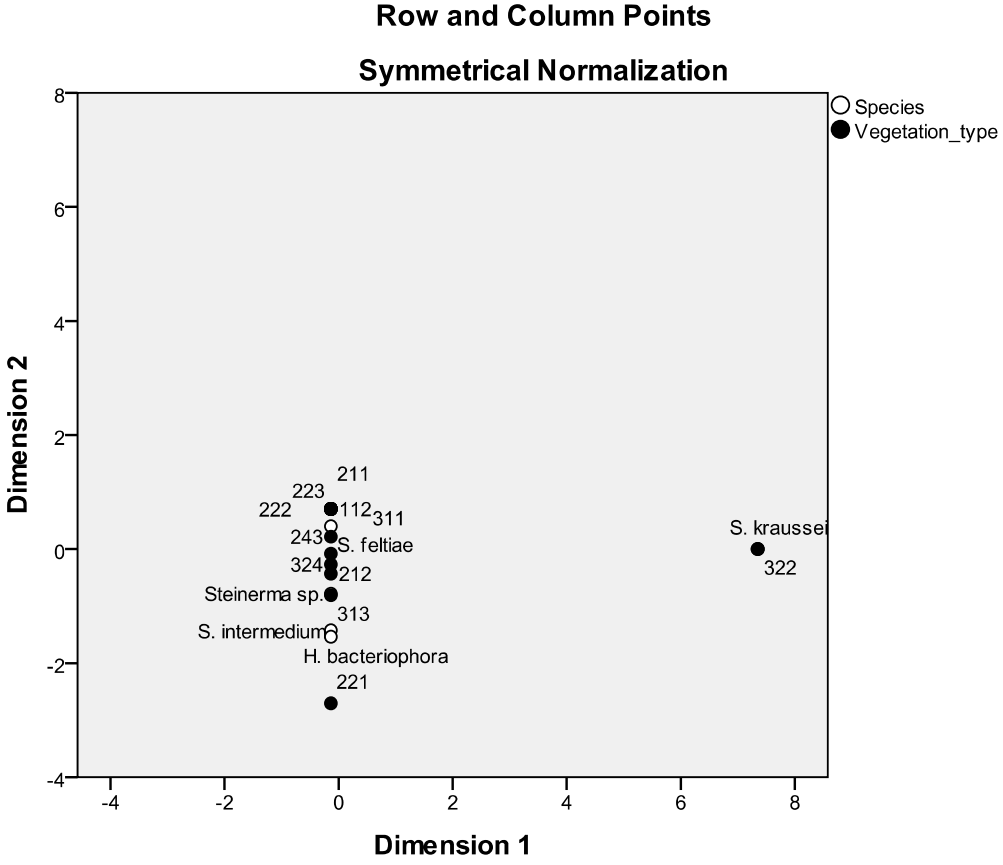


Fig. 2. CA biplot of the relationship between species and (a) NUTS (Nomenclature of Territorial Units for Statistics), (b) vegetation type, (c) soil type. Note: some dots are overlaid.

S. feltiae was found in almost all soil types, namely cambisols, podzols, luvisols, lithosols and regosols, whereas *H. bacteriophora* was only found in lithosols, podzols, cambisols and luvisols (table 4). The *Steinernema* sp. isolates were found in a lithosol and a cambisol. *S. kraussei* and *S. intermedium* isolates were found in a cambisol and a podzol, respectively (table 4).

Table 4. Occurrence of entomopathogenic nematodes in the different soil types (FAO, 2006). The table presents information about the number of samples collected from each

soil type, as well as the percentage of EPN species recovered ([1] *Steinernema feltiae*; [2] *S. intermedium*; [3] *S. kraussei*; [4] *Steinernema* sp.; [5] *Heterorhabditis bacteriophora*).

Soils Type	**Number of collected samples**	**Number of EPN**	**Positive samples (%)**
podzols	99	12	17[1]; 1.9[2]; 3.8[5]
cambisols	359	24	36[1]; 1.9[3]; 1.9[4]; 3.8[5]
luvisols	111	11	17[1];3.8[5]
lithosols	155	6	3.8[1]; 1.9[4]; 5.7[5]
regosols	12	1	1.9[1]
solonchaks	2	0	0
fluvisols	15	0	0
no classification	38	0	0

No association was found between species and soil type ($P > 0.1$). However, the CA biplot indicates that the two most abundant species are not randomly distributed by the different soil types: *S. feltiae* was mostly recovered from luvisols, but also from cambisols and regosols; *H. bacteriophora* is more abundant in lithosols. *H. bacteriophora* was found in soil samples with pH values between 4.37 and 7.92, whereas *S. feltiae* was present in soils with pH values between 4.02 and 8.11. *S. intermedium* and *S. kraussei* were found in soils with pH 4.17-5.34. *Steinernema* sp. isolates were recovered in soils with pH values of 4.90 and 6.23. No association was found between species and the soil's physical and chemical characteristics ($P > 0.05$).

Phylogenetic analysis of ITS, D2D3, COXI and cytb

Nematodes isolated from the positive samples were identified based on ITS sequences as *S. feltiae* (40 isolates), *S. intermedium* (one isolate), *S. kraussei* (one isolate), *Steinernema* sp. (two isolates) and *H. bacteriophora* (nine isolates). Because *S. feltiae* and *H. bacteriophora* were the two most abundant EPN species, D2D3 region of the 28S rRNA gene, COXI and *cytb* genes were used to further evaluate the genetic diversity of the populations. Repeated attempts to amplify and sequence some genes for some isolates were unsuccessful, thus justifying the discrepant number of isolates in the different phylogenies.

ITS phylogenies of S. feltiae and H. bacteriophora

For 37 *S. feltiae* and eight *H. bacteriophora* Portuguese isolates, a PCR product of approximately 1000 bp, containing the partial sequence of ITS1 and ITS2, and the whole 5.8S rRNA gene, was obtained and sequenced. *H. bacteriophora* was included as outgroup (fig. 3).

Only 34 *S. feltiae* isolates were used for phylogenetic analysis. Isolates that share 100% sequence identity were not considered (isolate 48A stands for isolates 59A and 87A; isolate 81A represents isolates W1 and 97A).

Comparing the 34 Portuguese with foreign *S. feltiae* isolates, few polymorphisms were found. All Portuguese and foreign *S. feltiae* isolates group in the same cluster, together with the other species from the *"feltiae-group" (S. oregonense, S. kraussei* and *S. monticolum)* sharing sequence identities between 76-99% (fig. 3). There is no distinction between *S. feltiae* Portuguese isolates, which are all almost 100% identical. Contrary to *S. monticolum*, which is the most distant species from the *"feltiae-group"*, *S. kraussei*, *S. oregonense*, as well as *S. kraussei* isolate 20F, are placed within the *S. feltiae* isolates. This suggests that ITS phylogeny has low resolution inside this group. Isolates 15G and 59F share the highest identity with species of the *"glaseri-group"* (69-80%), suggesting that *Steinernema* sp. belong to this group (fig. 3). Grouping with the major cluster are two species from the *"intermedium-group"*, *S. intermedium* and *S. affine*, also with Portuguese isolate 2B of the species *S. intermedium*. Species from *"intermedium-group"* share identities between 93-99% and with species from *"feltiae-group"* 61-68% identity (fig. 3).

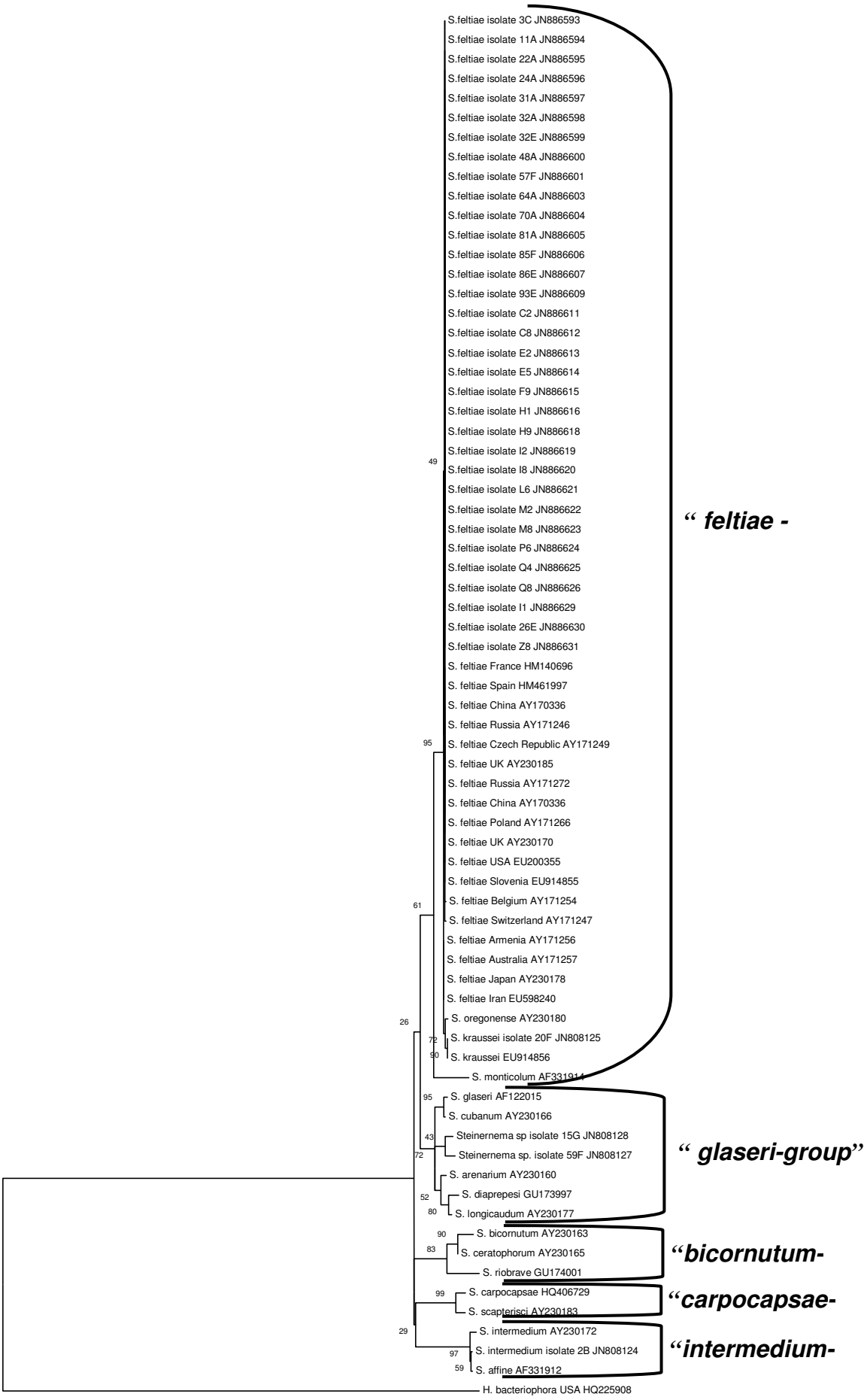

S.feltiae isolate 3C JN886593
S.feltiae isolate 11A JN886594
S.feltiae isolate 22A JN886595
S.feltiae isolate 24A JN886596
S.feltiae isolate 31A JN886597
S.feltiae isolate 32A JN886598
S.feltiae isolate 32E JN886599
S.feltiae isolate 48A JN886600
S.feltiae isolate 57F JN886601
S.feltiae isolate 64A JN886603
S.feltiae isolate 70A JN886604
S.feltiae isolate 81A JN886605
S.feltiae isolate 85F JN886606
S.feltiae isolate 86E JN886607
S.feltiae isolate 93E JN886609
S.feltiae isolate C2 JN886611
S.feltiae isolate C8 JN886612
S.feltiae isolate E2 JN886613
S.feltiae isolate E5 JN886614
S.feltiae isolate F9 JN886615
S.feltiae isolate H1 JN886616
S.feltiae isolate H9 JN886618
S.feltiae isolate I2 JN886619
49
S.feltiae isolate I8 JN886620
S.feltiae isolate L6 JN886621
S.feltiae isolate M2 JN886622
S.feltiae isolate M8 JN886623
S.feltiae isolate P6 JN886624
S.feltiae isolate Q4 JN886625
S.feltiae isolate Q8 JN886626
S.feltiae isolate I1 JN886629
S.feltiae isolate 26E JN886630
S.feltiae isolate Z8 JN886631
S. feltiae France HM140696
S. feltiae Spain HM461997
S. feltiae China AY170336
S. feltiae Russia AY171246
95
S. feltiae Czech Republic AY171249
S. feltiae UK AY230185
S. feltiae Russia AY171272
S. feltiae China AY170336
S. feltiae Poland AY171266
S. feltiae UK AY230170
S. feltiae USA EU200355
S. feltiae Slovenia EU914855
61
S. feltiae Belgium AY171254
S. feltiae Switzerland AY171247
S. feltiae Armenia AY171256
S. feltiae Australia AY171257
S. feltiae Japan AY230178
S. feltiae Iran EU598240
26
S. oregonense AY230180
72
S. kraussei isolate 20F JN808125
90
S. kraussei EU914856
S. monticolum AF331914
95
S. glaseri AF122015
S. cubanum AY230166
43
Steinernema sp isolate 15G JN808128
72
Steinernema sp. isolate 59F JN808127
S. arenarium AY230160
52
S. diaprepesi GU173997
80
S. longicaudum AY230177
90
S. bicornutum AY230163
83
S. ceratophorum AY230165
S. riobrave GU174001
99
S. carpocapsae HQ406729
S. scapterisci AY230183
29
S. intermedium AY230172
97
S. intermedium isolate 2B JN808124
59
S. affine AF331912
H. bacteriophora USA HQ225908
0.5
" feltiae -
" glaseri-group"
"bicornutum-
"carpocapsae-
"intermedium-

Fig. 3. Maximum likelihood ITS phylogenetic trees based on nucleotide sequences of *Steinernema* spp. and reference strains (alignment length 270 bp). Percentage bootstrap is indicated on internal branches (100 replicates); scale bar indicates number of substitutions per site. T92 model was used (Tamura and Nei, 1993).

ITS sequences indicate that all Portuguese *Heterorhabditis* isolates belong to the species *H. bacteriophora*. The eight Portuguese isolates of *H .bacteriophora* are 100% identical in sequence and all group together with foreign *H. bacteriophora* isolates (with bootstrap support of 98%), sharing identities of 98-100% (data not shown). *H. zealandica* also groups inside this cluster sharing 96-97% identity with *H. bacteriophora* isolates. The most distant species from *H. bacteriophora* are *H. indica, H. floridensis, H. amazonensis* and *H. baujardi,* sharing 70-72% identity with *H. bacteriophora* isolates (data not shown). *H. marelatus* and *H. megidis* are more similar to *H. bacteriophora* (sequence identity between 74-79%) than the previous species.

S. feltiae and H. bacteriophora D2D3 phylogenies

The D2D3 expansion region of the 28S rRNA gene sequences confirm the previous identification of EPN isolates, as *S. feltiae* and *H. bacteriophora*.

For twenty-seven *S. feltiae* and seven *H. bacteriophora* Portuguese isolates, a PCR product of approximately 1100 bp, containing the partial sequence of the D2D3 region of the 28S rRNA gene was obtained and sequenced. From the 27, only five representative isolates were used in the phylogenetic analysis, because the others share 100% sequence identity with these isolates (isolate 3C represents isolates 11A, 22A, 22F, 24A, 57F, 59A, 70A, 85F, 87A, C8, E2, F9, H1, H9, I2, I8, L6 and P6; and isolate 32A stands for isolates 86E, 93E, V1 and I1) (fig. 4). *Globodera pallida* was included as outgroup, considering that the D2D3 *H. bacteriophora* sequence was too close to species from the "*carpocapsae-group*" to serve this purpose.

Comparing the five Portuguese isolates of *S. feltiae* with foreign *S. feltiae* only a few polymorphisms were found. All Portuguese and foreign *S. feltiae* isolates group in the same cluster sharing 99-100% sequence identity with 99% bootstrap support, showing no genetic diversity between isolates (fig. 4). *S. feltiae* isolates group with species from the *"feltiae-group" (S. monticolum* and *S. kraussei)* sharing sequence identities of 92-98% (bootstrap support 77%).

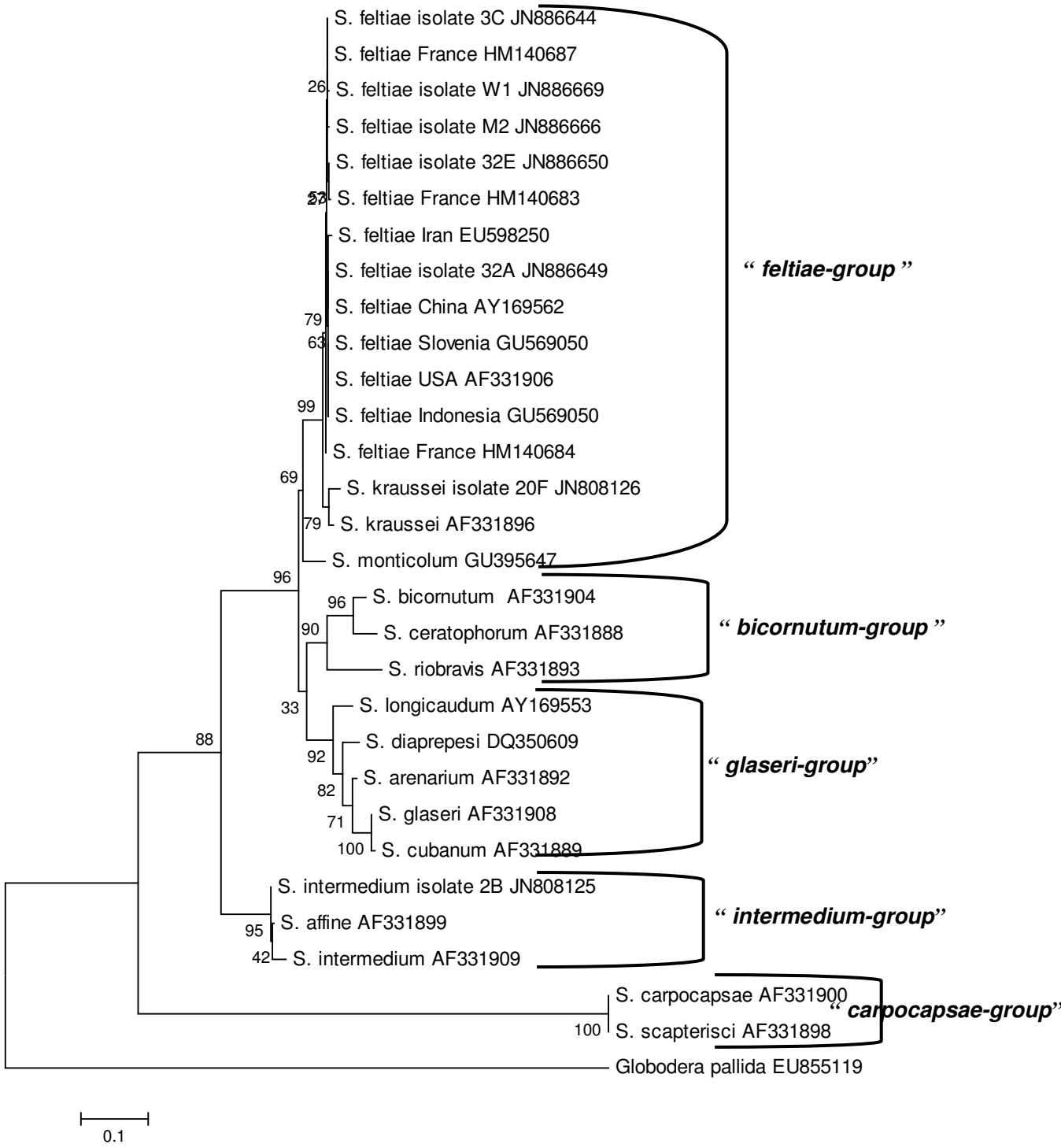


Fig. 4. Maximum likelihood D2D3 phylogenetic tree based on nucleotide sequences of *Steinernema* spp. and reference strains (alignment length 469 bp). Percentage bootstrap is indicated on internal branches (100 replicates); scale bar indicates 0.1 substitutions per site. K2 model was used (Kimura, 1980).

Regarding the D2D3 phylogeny, no polymorphisms were found among *H. bacteriophora* Portuguese isolates. One major cluster was obtained, which contains all Portuguese isolates and the reference strains of *H. bacteriophora* (bootstrap support 98%). All *H. bacteriophora* isolates are 100% identical in sequence (data not shown). The closest species to *H. bacteriophora* is *H. megidis* sharing a sequence identity between 91-93%; the most distant is *H. indica*, with a sequence identity of 90%.

S. feltiae COXI gene phylogeny

From the 23 Portuguese *S. feltiae* isolates, only 16 representative isolates were used for phylogenetic analysis (isolate 3C represents 87A; isolate 11A represents 22F and 70A; isolate H9 represents F9; isolate 24A represents P6; isolate I2 represents H1 and I8). *H. bacteriophora* was included as outgroup. A PCR product of approximately 1000 bp, containing the partial sequence of the COXI gene was obtained and sequenced for the Portuguese isolates.

There is a large cluster that includes all *S. feltiae* Portuguese isolates, together with the reference species of *S. feltiae*, sharing sequence identity values between 94-100% (bootstrap support of 99%) (fig. 5). Inside this major cluster there is some diversity between Portuguese isolates (sequence identity values of 97-100%), in some cases supported by bootstrap values that vary between 36-95% (fig. 5). For example isolates 59A and 85F form a group sharing a sequence identity of 99.5%, grouping also with isolate H9, with whom they share a sequence identity of 99%. All *S. feltiae* Portuguese isolates share high sequence identity values (98-100%). This major cluster groups with *S. kraussei* and *S. oregonensis*, both species of the "*feltiae-group*" sharing identities of 85-87% (fig. 5). The entire previous clade clusters together with species belonging to the *"intermedium-group",* the *"bicornutum-group",* the *"glaseri-group"* and the *"carpocapsae-group*" (fig. 5). The *Steinernema* sp. isolates 15G and 59F, group together sharing similarities of 92%. These isolates seem to represent a putative new species inside the "*glaseri-group"*, with whom they share 87-90% sequence identity (fig. 5).

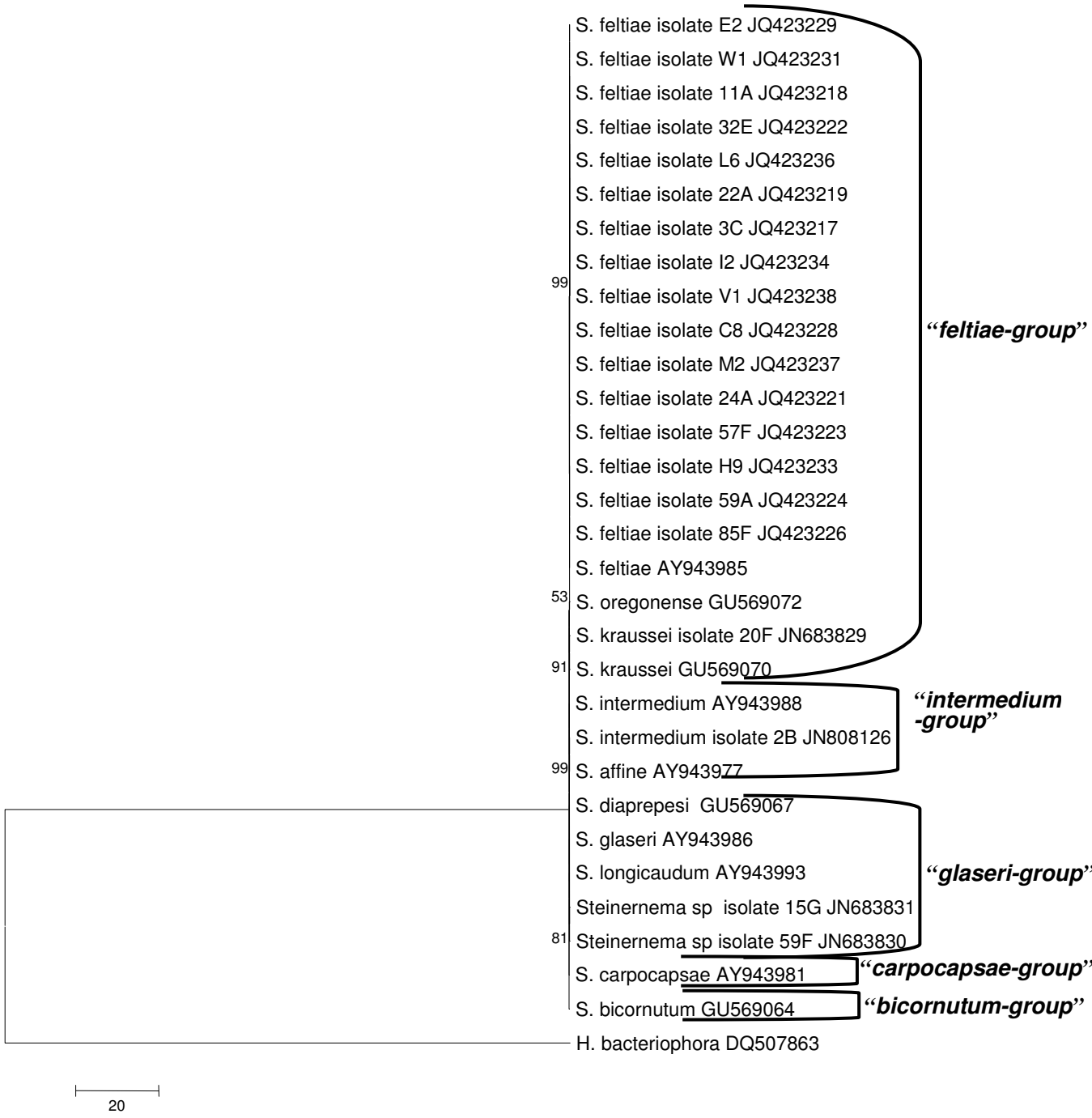


Fig. 5. Maximum likelihood COXI phylogenetic tree based on nucleotide sequences of *Steinernema* spp. Isolates and reference strains (alignment length 566 bp). Percentage bootstrap is indicated on internal branches (100 replicates); scale bar indicates 10 substitutions per site. HKY model was used (Hasegawa et al., 1985).

H. bacteriophora cytb gene phylogeny

For nine *H. bacteriophora* Portuguese isolates, a PCR product of approximately 1200 bp, containing a partial sequence of the *cytb* gene was obtained and sequenced. *S. carpocapsae,* was included as outgroup. No polymorphisms were found among Portuguese isolates (100% identical). One major cluster (bootstrap value of 68%) was obtained which contains all Portuguese isolates sharing 100% sequence identity (fig. 6). Portuguese isolates share 99.7% sequence identity with the reference strain of *H. bacteriophora* and 81.6% with *S. carpocapsae* (fig. 6).

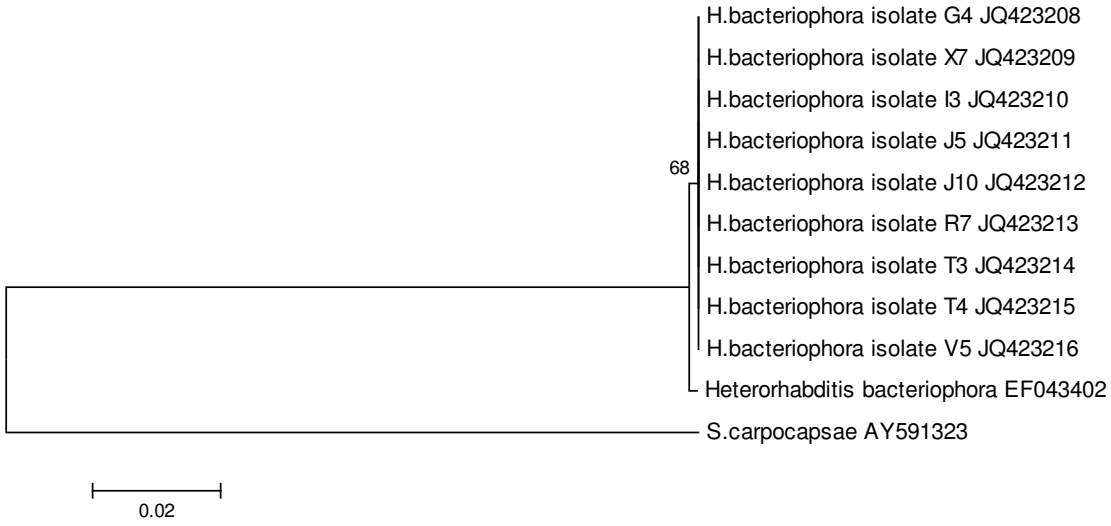


Fig. 6. Maximum likelihood *cytb* phylogenetic tree based on nucleotide sequences of *Heterorhabditis bacteriophora* isolates and reference strains (alignment length 749 bp). Percentage bootstrap is indicated on internal branches (100 replicates); scale bar indicates 0.05 substitutions per site. TN93 model was used (Tamura, 1992).

Discussion

Even though national surveys already undertaken provide valuable data on EPN distribution (Edgington *et al.,* 2010; García Del Pino & Palomo, 1996; Kary *et al*., 2009; Khatri-Chhetri *et al*, 2010; Ma *et al.,* 2010), species habitat preferences are still poorly understood. Nevertheless, surveys and the identification of new EPN species have a high potential for future efficient biological control of pests (Stock, 2009). The present study aimed at understanding the natural occurrence of EPN in continental

Portugal, representing the most systematic and extensive survey made for the first time in the country to evaluate indigenous species of EPN. The study involved a large diversity of habitats and ecosystems covering the entire territory of continental Portugal. Previous studies (Mráček *et al.,* 2005), showed that EPN distribution seems to depend on temperature and precipitation being closely related to vegetation and insect host, both affected by climate. However, in continental Portugal it was not possible to establish any association between temperature and precipitation and the distribution of EPN. The EPN abundance, distribution and habitat preference are related to host-parasite relationships, environmental conditions and soil characteristics (Campos-Herrera *et al*., 2007; Nielsen & Philipsen, 2003; Püza & Mráček, 2005). As with any survey, conclusions regarding diversity and biogeography of EPN must be made with some caution since the results in part reflect searching effort and sampling technique rather than actual numbers and/or habitat preferences of EPN.

The survey covered all NUTS, the different climatic regions and a wide variety of vegetation and soil types. Positive soil samples were analyzed with the additional information collected, namely temperature and precipitation values, altitude and soil type. Although EPN were recovered at a low rate (6.7% of sampling sites) in our study, five different species were isolated from the entire country: *S. feltiae* (75%), *H. bacteriophora* (17%), *S. intermedium* (1.9%), *S. kraussei* (1.9%), and *Steinernema* sp. (3.8%). *S. feltiae* and *H. bacteriophora* were the two most abundant species found in the country. One reason for the low recovery rate obtained in the present study, could be the fact that only one insect, *G. mellonela,* was used as trap insect, and it may not be the appropriate host for all EPN species (Kary *et al*., 2009). Also, the fact that just one temperature value (25ºC) was used for soil baiting samples may represent a limitation. The use of just one baiting temperature may not cover all the requirements for other EPN species (Mráček *et al*., 2005). Furthermore, the choice of sampling sites may contribute to differences in EPN recovery percentage (Mráček *et al*., 2005). However, this low recovery percentage is not unusual, and it has already been reported from other surveys (Hazir *et al*., 2003; Kary *et al*., 2009; Rosa *et al*., 2000).

Our results show that natural habitats present a higher percentage of positive samples, compared to agricultural ones, probably due to chemical control of insect pests in agricultural regions, which partially reduces the abundance of natural biocontrol agents.

Stock *et al.* (2008) also claimed a higher abundance of EPN in natural habitats such as forests. EPN were recovered from soils with high sand content, which favor their mobility and survival, such as cambisols, podzols, luvisols followed by lithosols and regosols. Observing the soils' physical and chemical characteristics, no clear relationship was found.

S. feltiae was the most common and widely distributed EPN in continental Portugal, followed by *H. bacteriophora*. *S. feltiae* is widely distributed in temperate regions, whilst *H. bacteriophora* typically occurs in regions with continental and mediterranean climates (Hominick, 2002). *S. feltiae* was present in almost all habitats sampled in our survey, including agriculture fields with natural vegetation, forest habitats, transitional woodland-shrub and non-irrigated land (table 3), presenting no distribution influenced by altitude or other chemical parameter from soil sample. The recovered percentage was higher in Alentejo, Centro, Algarve and Lisboa regions of continental Portugal (table 2). *H. bacteriophora* was present especially in forests habitats, transitional woodland-shrub, vineyards, agriculture fields with natural vegetation and permanently irrigated land (table 3). Abiotic factors, such as altitude, temperature or rainfall, do not influence the distribution of EPN species. Algarve and Alentejo regions represent the regions with the higher recovery percentage of *H. bacteriophora* (table 2). No correlation was observed between genetic diversity of *S. feltiae* isolates and any of the biotic or abiotic parameters that were analyzed for these isolates.

S. kraussei and *S. intermedium* were each found at only one site, in moors and heathland and mixed forests, respectively. The reason for the low recovery of these two species is not known. *S. kraussei* and *S. intermedium* are common in Europe, with *S. intermedium* showing a preference for tree habitats (Nguyen & Hunt, 2007). *Steinernema* sp. was recovered from only two sites, in mixed forests and broad-leaved forests habitats. Differences in nematode distribution may be related to differences in the distribution of suitable insect hosts and to the species of nematode involved (Akhurst & Bedding, 1986). Mráček *et al.* (2005) suggested that the occurrence of EPN depend on the presence of insect hosts. However, no information about the natural hosts of EPN in continental Portugal is available.

The species nature of the genus *Steinernema* is a result of its longer evolution history (Adams *et al.,* 2007) and its reproduction patterns (amphimictic and hermaphroditic),

making this genus more capable of occupying a wide range of habitats than *Heterorhabditis* (Edgington *et al.,* 2010). The geographical and habitat preferences of EPN species in continental Portugal may also reflect chance of dispersal events as well as feeding patterns. However, EPN diversity determined in this study is similar to the diversity reported in previous studies in northern Spain and southern France, which constitute the closest area already sampled and also most similar in terms of climate and soil (Campos Herrera *et al*., 2007; Emelianoff *et al*., 2008; García del Pino & Palomo, 1996). *S. feltiae* and *H. bacteriophora*, the two most abundant EPN species in continental Portugal, are also considered the two most common species in Europe, having also been found in southern France and northern Spain (Hominick, 2002).

The major objectives of this study were to determine the indigenous species present in continental Portugal and evaluate the genetic diversity of isolates. For *H. bacteriophora* and *S. feltiae*, the two most abundant species found in continental Portugal, genetic diversity was accessed based on different molecular markers: ITS, D2D3 expansion region of the 28S rRNA gene and two mitochondrial genes, COXI and *cytb*. There were no indications of a molecular and geographical intraspecific variation of both *S. feltiae* and *H. bacteriophora*. Sequences of the D2D3 expansion region of the 28S rRNA gene were used by some authors to characterize EPN populations (Stock *et al*., 2001) and may yield more information than the ITS region. Mitochondrial DNA sequences may be more useful in genetic diversity studies (Edgington *et al*., 2010).

According to our results, *H. bacteriophora* isolates show no differences concerning *cytb* gene, ITS and D2D3 expansion region of the 28S rRNA gene. *H. bacteriophora* is highly mobile, responding to chemical signals from the host, and being adapted to infect less mobile insects that are found in lower soil layers (Ishibashi, 2002). Since our samples were collected from the upper soil layer, this could explain the low recovery and genetic diversity of *H. bacteriophora* found in continental Portugal.

No genetic diversity was observed among *S. feltiae* isolates using the D2D3 expansion region of the 28S rRNA gene and ITS regions. However, COXI gene revealed some genetic diversity among *S. feltiae* isolates. The diversity found among *S. feltiae* isolates using COXI sequences has no correlation with the physical and chemical parameters that characterize soil samples, as well as NUTS, soil or vegetation type.

In conclusion, this survey shows that five EPN species are known to be present in Portuguese soils. Furthermore, the occurrence of *S. feltiae* and *H. bacteriophora* as the two most abundant species in continental Portugal, suggests the potential role of these nematodes in natural regulation of insect populations. Further research on host ranges and characterization of these nematodes in view of possible use in biological control should be undertaken to minimize the use of chemical pesticides.

Acknowledgments

The authors kindly thank Ms. Inácia Ferreira for technical assistance. We would also like to thank Mr. José Carlos Garcia dos Santos for helping with the ArcGIS software.

This communication is a portion of the PhD dissertation of the first author, who was supported by a doctoral scholarship from "Fundação para a Ciência e a Tecnologia" (SFHR\BD\22086\2005).

References

Adams, B.J., Peat, S.M. & Dillman, A.R. (2007) Phylogeny and evolution. pp. 693-733 in Nguyen, K.B. & Hunt, D.J. *(Eds) Entomopathogenic nematodes: systematics, phylogeny and bacterial symbionts.* Nematology Monographs and Perspectives 5. Leiden, The Netherlands, Brill.

Akhurst, R.J. & Bedding, R.A. (1986) Natural occurrence of insect pathogenic nematodes (Steinernematidae and Heterorhabditidae) in soil in Australia. *Journal of the Australian Entomological Society* **25**, 241–244.

Bedding, R.A. & Akhurst, R.J. (1975) A simple technique for the detection of insect parasitic nematodes in soil. *Nematologica* **21**, 109-110.

Bednarek, A. (1998) The agricultural system, as a complex factor, effects the population of entomopathogenic nematodes (Rhabditida: Steinernematidae) in the soil. *IOBC Bulletin* **21**, 155–216.

Boemare, N. (2002) Biology, taxonomy and systematics of *Photorhabdus* and *Xenorhabdus*. pp. 35–56 in Gaugler, R. *(Ed.) Entomopathogenic Nematology*. CABI Publishing, Wallinford, UK.

Caetano, M., Araújo, A., Nunes, A., Nunes, V. & Pereira, M. (2009) Accuracy assessment of the CORINE Land Cover 2006 map of Continental Portugal. *47 pp. Relatório Técnico, Instituto Geográfico Português.*

Campos-Herrera, R., Johnson, E.G., El-Borai, F.E., Stuart, R.J., Graham, J.H. & Duncan, L.W. (2011) Long-term stability of entomopathogenic nematode spatial patterns in soil as measured by sentinel insects and real-time PCR assays. *Annals of Applied Biology* **158**, 55-68.

Curran, J., Driver, F., Ballard, J.W.O. & Milner, R.J. (1994*)* Phylogeny of *Metarhizium*: analysis of ribosomal DNA sequence data. *Mycological Research* **98**, 547-552.

Edgington, S., Buddie, A.G., Moore, D., France, A., Merino, L., Tymo, L.M. &

Hunt, D.J. (2010) Diversity and distribution of entomopathogenic nematodes in Chile. *Nematology* **12 (6),** 915-928.

El-Borai, F.E., Stuart, R.J., Campos-Herrera, R., Pathak, E. & Duncan, L.W. (2012) Entomopathogenic nematodes, root weevil larvae, and dynamic interactions among soil texture, plant growth, herbivory, and predation. *Journal of Invertebrate Pathology* **109**, 134-142.

Emelianoff, V., Le Brun, N., Pages, S., Stock, P., Tailliez, P., Moulia, C. & Sicard, M. (2008) Isolation and identification of entomopathogenic nematodes and their symbiotic bacteria from Herault and Gard (Southern France). *Journal of Invertebrate Pathology* **98**, 211–217.

FAO (2006) *World reference base for soil resources 2006*. 145 pp. A framework for international classification, correlation and communication.

Felsenstein J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **34**, 783-791.

García del Pino, F. & Palomo, A. (1996) Natural occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Spanish soils. *Journal of Invertebrate Pathology* **68**, 84–90.

García del Pino, F. (2005) Natural occurrence of entomopathogenic nematodes in Spain. in *MC-Meeting and Working Group 4th Meeting: Natural occurrence and evolution of entomopathogenic nematodes and Management Committee Meeting*. Ceske Budejovice, The Czech Republic 14–17 Enero 2005.

Georgis, R.,Koppenhnhnh, A.M., Lacey, L.A., B A.M., G., Duncan, L.M., Grewal, P.S., Samish, M., Tan, L., Torr, P. & van Tol, R.W.H.M. (2006) Successes and failures in the use of parasitic nematodes for pest control. *Biological Control* **38**, 103-123.

Griffin, C.T., O´Callaghan, K.M. & Dix, I. (2001) A self-fertile species of *Steinernema* from Indonesia: further evidence of convergent evolution amongst entomopathogenic nematodes. *Parasitology* **122**, 181-186.

Gwynn, R.L. & Richardson, P.N. (1996) Incidence of entomopathogenic nematodes in soil samples collected from Scotland, England and Wales. *Fundamental and Applied Nematology* **19**, 427–431.

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

Hazir, S., Keskin, N., Stock, S.P., Kaya, H. & Özcan, S. (2003) Diversity and distribution of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Turkey. *Biodiversity and Conservation* **12**, 375–386.

Hominick W.M., Reid A.P., Bohan, D.A. & Briscoe B.R. (1996) Entomopathogenic nematodes: biodiversity, geographical distribution and the Convention on Biological Diversity. *Biocontrol Science and Technology* **6**, 317-331.

Hominick, W.M. (2002) Biogeography. pp. 115–143 in Gaugler, R. *(Ed.) Entomopathogenic Nematology*. CABI Publishing, Wallinford, UK.

Hozzank, A., Wegensteiner, R., Waitzbauer, W., Burnell, A., Mráček, Z. & Zimmermann, G. (2003) Investigations on the occurrence of entomopathogenic fungi and entomoparasitic nematodes in soils from lower Austria. *Bulletin OILB/SROP* **26**, 77–80.

Iraki, N., Salah, N., Sansour, M.A., Segal, D., Glazer, I., Johnigk, S.A. & Kepenekci, I. (2002) Entomopathogenic nematodes (Rhabditida) in the Mediterranean region of Turkey. *Nematologia Mediterranea* **30**, 13–15.

Ishibashi, N. (2002). Behaviour of entomopathogenic nematodes. pp. 511-529 in Lee, D.L. *(Eds) The Biology of Nematodes*. Taylor & Francis, London, CRC Press.

Ivanova, T.I., Danilov, L. & Ivakhnenko, O.A. (2000). Distribution of entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae in Russia and their morphological characteristics. *Parazitologiya* **34**, 323–334.

Kary, N.E., Niknam, G., Griffin, C.T., Mohammadi, S.A. & Moghaddam, M. (2009) A survey of entomopathogenic nematodes of the families Steinernematidae and

Heterorhabditidae (Nematoda: Rhabditida) in the Norte-west of Iran. *Nematology* **11**, 107–116.

Kaya, H.K. & Gaugler, R. (1993). Entomopathogenic nematodes. *Annual Review of Entomology* **38**, 181-206.

Kaya, H.K. & Stock, S.P. (1997) Techniques in insect nematology. pp. 281-324 in Lacey, L.A. *(Ed.) Manual of Techniques in Insect Pathology*. Academic Press, London, UK.

Khatri-Chhetri H.B., Waeyenberge L., Spiridonov S., Manandhar H.K. & Moens M. (2010) Two new species of *Steinernema* Travassos, 1927 with short infective juveniles from Nepal. *Russian Journal of Nematology* **19**, 53-74.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111-120.

Köppen, W. & Geiger, R. (1928) Klimate der Erde. Gotha: Verlag Justus Perthes. Wall-map 150cmx200cm.

Koppenhöfer, A.M. (2000) Nematodes. pp. 283–301 in Lacey, L.A., Kaya, H.K. *(Eds.) Field Manual of Techniques in Invertebrate Pathology*. Kluwer, Dordrecht, The Netherlands.

Liu, J. & Berry, R.E. (1996) *Steinernema oregonensis* n. sp. (Rhabditida: Steinernematidae) from Oregon, USA. *Fundamental and Applied Nematology* **19**, 375-380.

Ma, J., Shulong, C., Zou, Y., Xiuhua, L., Richou, H., De Clercq, P. & Moean, M. (2010) Natural occurrence of entomopathogenic nematodes in North China. *Russian Journal of Nematology* **18**, 117-126.

Menti, H., Wright, D.J. & Perry, R.N. (1997) Desiccation survival of populations of nematodes *Steinernema feltiae* and *Heterorhabditis megidis* from Greece and the UK. *Journal of Helminthology* **71**, 41–46.

Midituri, J.S., Waeyenberge, L. & Moens, M. (1997) Natural distribution of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) in Belgian soils. *Russian Journal of Nematology* **5**, 55–65.

Mráček Z., Bečvář S. & Kindlmann P. (1999) Survey of entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) in the Czech Republic. *Folia Parasitologica* **46**, 145-148.

Mráček Z., Bečvář S., Kindlmann P. & Jersáková J. (2005) Habitat preference for entomopathogenic nematodes, their insect hosts and new faunistic records for the Czech Republic. *Biological Control* **34**, 27-37.

Nadler, S.A., Bolotin, E. & Stock, S.P. (2006) Phylogenetic relationships of *Steinernema* Travassos, 1927 (Nematoda: Cephalobine: Steinernematidae) based on nuclear, mitochondrial and morphological data. *Systematic Parasitology* **63**, 161-181.

Nguyen, K.B., Maruniak, J. & Adams, B.J. (2001) The diagnostic and phylogenetic utility of the rDNA internal transcribed spacer sequences of *Steinernema*. *Journal of Nematology* **33**, 73–82.

Nguyen, K. B. & Hunt, D. (2007) *Entomopathogenic nematodes: Systematics, phylogeny and bacterial symbionts* 816 pp. Leiden: E. J. Brill.

Nielsen, O. & Philipsen, H. (2003) Danish surveys on insects naturally infected with entomopathogenic nematodes. *Bulletin OILB/SROP* **26**, 131–136.

Püza, V. & Mráček, Z. (2005) Seasonal dynamics of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* as a response to abiotic factors and abundance of insect hosts. *Journal of Invertebrate Pathology* **89**, 116–122.

Reid, A.P., Hominick, W.M. & Briscoe, B.R. (1997) Molecular taxonomy and phylogeny of entomopathogenic nematode species (Rhabditida: Steinernematidae) by RFLP analysis of ITS region of the ribosomal DNA repeat unit. *Systematic Parasitology* **37**, 187–193.

Rosa, J.S., Bonifassi, E., Amaral, J., Lacey, L.A., Simões, N. & Laumond, C. (2000) Natural occurrence of entomopathogenic nematodes (Rhabditida: *Steinernema*, *Heterorhabditis*) in Azores. *Journal of Nematology* **32**, 215–222.

Rosa, J.S. & Simões, N. (2004) Evaluation of twenty-eight strains of *Heterorhabditis bacteriophora* isolated in Azores for biocontrol of the armyworm, *Pseudaletia unipuncta* (Lepidoptera: Noctuidae). *Biological Control* **29**, 409-417.

Saitou, N. & Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–425.

Shamseldean, M.M. & Abd-Elgawad, M.M. (1994) Natural occurrence of insect pathogenic nematodes (Rhabditida: Heterorhabditidae) in Egyptian soils. *Afro-Asian Journal of Nematology* **4**, 151–154.

Shishiniova, M., Budurova, L. & Gradinarov, D. (2000) Entomopathogenic nematodes from Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) in Bulgaria. *Bulletin OILB/SROP* **23**, 75–78.

Simões, N., Laumond, C. & Bonifassi, E. (1994) Effectiveness of *Steinernema* spp. and *Heterorhabditis bacteriophora* against *Popillia japonica* in Azores. *Journal of Nematology* **25**, 480-485.

Spiridonov, S., Krasomil-Osterfeld, K. & Moens, M. (2004) *Steinernema jollieti* sp. n. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from the American Midwest. *Russian Journal of Nematology* **12**, 85-95.

Steiner, W. (1996) Distribution of entomopathogenic nematodes in the Swiss Alps. *Review Suisse Zoology* **103**, 439–452.

Stock, S.P., Campbell J.F. & Nadler S.A. (2001) Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology* **87**, 877-889.

Stock, S.P., Griffin, C.T. & Burnell, A.M. (2002) Morphological characterization of three isolates of *Heterorhabditis* Poinar, 1976 from the "Irish group" (Nematoda:

Rhabditida: Heterorhaditidae) and additional evidence supporting their recognition as a distinct species, *H. downesi* n. sp. *Systematic Parasitology* **51**, 95-106.

Stock, S.P., Al Banna, L., Darwish, R. & Katbeh, A. (2008) Diversity and distribution of entomopathogenic nematodes (Nematoda: Steinernematidae, Heterorhabditidae) and their bacterial symbionts (c-Proteobacteria: Enterobacteriaceae) in Jordan. *Journal of Invertebrate Pathology* **98**, 228–234.

Stock, S.P. (2009) Molecular approaches and the taxonomy of insect-parasitic and pathogenic nematodes. pp. 71-100 in Stock, S.P., Vandenburg, J., Glazer, I., Boemare, N. *(Eds) Insect pathogens: molecular approaches and techniques*. CAB International Press, USA.

Sturhan, D. & Liskova, M. (1999) Occurrence and distribution of entomopathogenic nematodes in the Slovak Republic. *Nematology* **1**, 273–277.

Sturhan, D. & Ruess, L. (1999) An undescribed *Steinernema* sp. (Nematoda: Steimernematidae) from Germany and the Scandinavian Subartic. *Russian Journal of Nematology* **7**, 43–47.

Tamura, K. & Nei, M. (1992) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**, 512-526.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* **28**, 2731-2739.

Triggiani, O. & Tarasco, E. (2000) Occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in pine and oak woods in Southern Italy. *Entomologica.* **34**, 23–32.

Valadas, V., Boyle, S., Vieira, P., Kakouli-Duarte T. & Mota, M. (2007) First report of an entomopathogenic nematode from continental Portugal. *Helminthologia* **44**, 226-229.

Valadas, V., Vieira, P., Oliveira, S. & Mota, M. (2009) First report of the genus *Heterorhabditis* (Nematoda: Heterorhabditidae) from continental Portugal. *Helminthologia* **46**, 45–48.

Valadas, V., Mráček, Z., Oliveira, S. & Mota, M. (2011) Three species of entomopathogenic nematodes of the family Steinernematidae (Nematoda: Rhabditida) new to Continental Portugal. *Nematologia Mediterranea* **39**, 169-178.

White, G.F. (1927) A method for obtaining infective juveniles from cultures. *Science.* **66**, 302-303.

Chapter V: State of the art of *Bursaphelenchus xylophilus*

V.1. Foreword

25% of the world´s forests (1 billion hectares) are in Europe, covering 44% of the total land area of Europe´s land (Anon, 2011). The most important industries depending on forests are located in temperate regions. These industries depend mainly on exploiting primary and derived forest products such as paper, pulp, lumber and chemical substances obtained from natural and planted forests. The global value of the planet´s forests is estimated at 3.5 x 10^{12} Euros / year, which corresponds to a global economic flux of 14.7% (Costanza *et al.,* 1997). Biogeographically, Portuguese forest is considered as being part of the "Mediterranean" type, in spite of some regions on the North and Northwest being typically atlantic/temperate. In Portugal, forests cover approximately 3.4 x 10^{6} ha (DGRF, 2007), and the industries that employ people working in these areas are responsible for 113 000 direct jobs (2% of the active population). About 80% of the Portuguese forestry is made up of pines, eucalyptus and oaks species (DGRF, 2007). Maritime pine, *Pinus pinaster*, is one of the most important forest species in Portugal, not only for occupying 27% of the national forest area, $8.8x10^{5}$ ha, but also for its economic relevance, with a production of "softwood sawn wood" of $9.2x10^{7}$ Euros in 2009. *P. pinaster* represents the most important pine production (62.5%) (Vicente *et al.,* 2012), industrial activity (production of wood and resin) and coastal protection associated with sand dunes and so, as in other countries, the global impact of pine wilt disease (PWD), promoted by the pinewood nematode (PWN), *Bursaphelenchus xylophilus,* has serious consequences and economical losses.

Forests around the world are threatened by factors such as climate change, desertification, pollution, fires, deforestation, pests and pathogens (FAO, 2007; http://www.un.org/en/events/iyof2011). Pests and pathogens attacking forests are insects, fungi, bacteria, viruses and nematodes, causing important tree decay, mortality and wood depreciation. Nematode tree pathogens have caused significant damage to many tree species in the last three decades (Mota and Vieira, 1993). Chemical substances such as nematicides are usually expensive and have toxic effects to other organisms and to the environment, for example,

through contamination of drinking water. Biological control is one area that is getting attention as an alternative use of pesticides, because their impact on the environment is reduced or nonexistent. This alternative is now very expensive but can represent a solution for solving major pest problems. It is important not to forget that the introduction of a species that does not belong to a particular ecosystem is dangerous and can disrupt its natural functioning. In Japan, previous studies have already evaluated the possibility of use natural enemies to control the insect vector of PWN, with promising results (Phan, 2008).

In an effort to control the spread of invasive pests, phytossanitary measures for treating wood packaging material in international trade (ISPM-15) were defined for Europe, and are currently being adopted by the European Union and several European countries (FAO, 2007). In 2011, new phytossanitary measures were addopted by Portugal to treat wood and wood products, named as NP 4487. This new measures consist in phytossanitary heat treatment for the elimination of the PWN in softwood sawn timber, pallets and other packages (IPQ, 2011).
In spite of these measures, successful biological invasions have established viable populations of exotic woody phytophagous insects pests and pathogens in Europe (Vanhanen, 2008).

Among nematodes, *B. xylophilus* (Steiner and Bührer, 1934) Nickle 1970 is considered the most important conifer forest pest in the world (Sutherland and Webster, 1993). For over one hundred years, the PWN has been introduced due to human activities into countries like Japan, China, Taiwan, South Korea and Portugal (Mota and Vieira, 2008). To avoid the proliferation of the disease, and by preventing and reducing the effects of this species, an approach combining biological, ecological and social sciences, economic policy analysis and engineering should be implemented (FAO, 2007). Further introduction should also be avoided. But to assure that these measures are properly applied, it is necessary to have specialized people in the field that can distinguish the symptoms caused by this nematode and by other pests.

B. xylophilus, is the causal agent of PWD, which affects primarily conifer species of the genus *Pinus*. *B. xylophilus* belongs to the order Rhabditida (Hunt, 2008), and to the genus *Bursaphelenchus* (Fuchs, 1937) that include

mycetophagous nematodes which establish phoretic relationships with insects and are found in association with decaying wood of conifers (Ryss *et al.,* 2005). This nematode is truly a plant parasitic nematode and the most important common insect vectors are long-hormed beetles of the genus *Monochamus*. *B. xylophilus* is indigenous from North America being widespread in natural coniferous forests in Canada and in the USA (Sutherland and Peterson, 1999). The worst fear of governments towards the PWN is its establishment into countries where conifer forests assume great importance and where PWD can cause devastating losses (Mamiya, 2004; Shin *et al.,* 2006). The introduction of the PWN into non-native areas (outside North America) is associated with trade and the global flow of forest products (Bergdahl and Halik, 1999; Webster, 2004). Many *Bursaphelenchus* species, including the PWN, have been routinely intercepted in packaging and wood products in several countries, e.g. Austria (Tomiczek *et al.,* 2003), China (Gu *et al,.* 2006), Finland (Tomminen, 1991), Germany (Braasch *et al.,* 2001) and Brazil (Oliveira *et al.,* 2011). Furthermore, the recent detections of PWN in packaging wood imported from countries considered free of this pest, due to the repeated use and circulation of this type of wood material, e.g. Brazil, Belgium, Italy and Spain (Gu *et al.,* 2006), stressed the importance of trade globalization for the potential entry/establishment of this pathogen into endemic forests worldwide. The introduction of this nematode into non-native areas has resulted in huge annual losses due to the effects on increased mortality and growth loss of the pine forest, and by the increased costs in management procedures and disease control (Mamiya, 2004; Shimazu, 2006). In addition, the introduction of this pest has resulted in vast and irreversible changes to native forest ecosystems including tree species conversions, wildlife habitat destruction, soil and water conservation and loss of biodiversity (Kiyohara and Bolla, 1990; Suzuki, 2002; Mota and Vieira, 2008). The PWN has already been established for more then 100 years in Japan (Yano, 1913), and in the past two decades the new reports of PWD came mainly from East Asia (Cheng *et al.,* 1983; Yi *et al.,* 1989) and Europe (Mota *et al.,* 1999; Abelleira *et al.,* 2011).

B. xylophilus was detected for the first time in Portugal in 1999 (Mota *et al.,* 1999), collected from dead *P. pinaster* trees located in the Setúbal Península,

30 km SE of Lisbon. Recently, it was detected in Spain as well (Abelleira *et al.,* 2011; Robertson *et al.,* 2011). During approximately 10 years, the PWN was thought to be confined to the Setúbal Peninsula. After its detection in 1999, a national program ("Programa Nacional de Luta Contra o Nemátode da Madeira do Pinheiro, PROLUNP"), has been implemented to ensure the restriction of the disease in Setúbal Peninsula, but despite efforts implemented by the Portuguese authorities in controlling and eradicating this quarantine nematode, in 2008 new outbreaks of the disease were established in the center part of the country (Rodrigues, 2008), and more recently in Madeira Island, 1000 km SW of continental Portugal (Fonseca *et al.,* 2012). It is fundamental to determine the origin of the new isolates and their spread routes, to prevent further spread of the disease in Europe.

B. xylophilus is listed as a harmful organism to plants and plant products by European Union (Annex II, Council Directive 2000/29/EC of May 2000) (Rodrigues, 2008). Due to the difficulties on morphological identification of nematodes that are found in trees and wood materials, it became of extreme importance to develop and determine the most suitable molecular marker to identify and discriminate nematodes species and *B. xylophilus* populations. For that purpose, and despite other actions, several molecular markers have been used in the last two decades with the goal of determining a suitable molecular marker that can be used to study intraspecific variability of different geographic isolates and compare them with foreign isolates to understand their spread routes and establish the infection origin(s). Sequence analysis of specific regions of genomic DNA has proven to be an effective approach for species identification and to determine inter and intraspecific variability in the genus *Bursaphelenchus* (Cheng *et al.,* 2008). With the release of the entire genome sequence of *B. xylophilus* it will be a challenge to discover novel and suitable markers to study intraspecific variability determine pathways of disease spread and follow the evolution of introduced isolates.

V.2. *Bursaphelenchus xylophilus*

V.2.1. PWN geographical distribution and hosts. Introduction in Portugal an the European Union

A few number of species have been reported in South Africa, associated with plantations of pine species, due to the worldwide distribution of the genus *Bursaphelenchus*, with the majority of the species being distributed in the northern hemisphere (Ryss *et al.,* 2005; Hunt, 2008; Braasch *et al.,* 2009, 2007). *B. xylophilus,* is native from North America where is distributed throughout Canada and USA (Sutherland and Peterson, 1999), with an additional report from Mexico (Dwinell, 1993) (Fig.VI1.). In native host species of North America the nematode does not cause PWD, since the pine species has co-evolved with the nematode becaming resistant/tolerant to its presence (Kiyohara and Bolla, 1990), except in some exotic *Pinus* spp. plantations (Evans *et al.,* 1996). The introduction and spread of this species in new countries outside its native origin, is associated with its high phenotypic plasticity connected with its excellent capacities to adapt to new environments, host trees (long starvation periods) and dispersion strategies (phoretic association with an insect) (Mamiya, 1984). The establishment and spread of PWN in non-native areas is associated with appropriate environmental conditions, such as mean summer temperature above 20ºC, the presence of host trees (mainly *Pinus* spp.), and the presence of an insect vector (usually a *Monochamus* species) (Evans *et al.,* 1996). Besides pines, the PWN can also be found associated with other conifers of the genera *Cedrus* Trew, *Larix* Philip Miller and *Picea* Link (Wingfield *et al.,* 1982a, 1982b; Dwinell, 1997).

The introduction of the PWN outside North America has been promoted through the years by human activity, as a consequence of export of untreated wood and wood products which are used for transportation (Webster, 2004; Mota and Vieira, 2008). In Japan, the disease is assumed to be present due to an accidental introduction by means of contaminated wood products from the USA in the beginning of the 20th century (Yano, 1913). However, only in 1971 PWN was associated with the high mortality of Japanese black pine (*P. thunbergii* Parl.) and the Japanese red pine (*P. densiflora* Sieb. and Zucc) (Kiyohara and

Tokushige, 1971) (Fig.VI1.). During the eighties it was found for the first time in China, associated with Japanese black pine, in Nanjing (Jiangsu Province) (Cheng *et al.,* 1983) (Fig.VI1.). The disease spread among different regions of the country mainly due to human factors (Yang, 2004). In Taiwan, the first report of PWN occurred in 1985, in luchu pine (*P. luchuensis* Mayr.) and in Japanese black pine (Fig.VI1.). In South Korea it was detected in 1989, also associated with Japanese black pine and Japanese red pine (Yi *et al.,* 1989) (Fig.VI1.). During the first years the disease was controlled, but in 1997 the infection spread all over the country becoming the major forest pest in the country (Shin *et al.,* 2006).

In 1999, *B. xylophilus* was found associated with *P. pinaster* in the Pegões region in Portugal (Mota *et al.,* 1999), the first detection of PWN in Europe (Fig.VI1.). The insect vector associated with the nematode was *M. galloprovinciallis*, a longhorn cerambycid beetle (Sousa *et al.,* 2001). After the initial detection, a national survey was carried out in pine forests, and a quarantine area was established in the Setúbal Peninsula (ca. 30km SE of Lisbon). Following evaluation of the situation in Portugal, several measures have been taken according to the recommendations of the European Comission´s Permanent Phytossanitary Committee. Procedures to avoid the dispersion of the disease have been adopted and implemented since 1999: elimination of declining symptomatic trees, surveys to evaluate and identify symptomatic pines, control of the insect vector population and control of movement of coniferous wood all over the country. *P. pinaster* is the only conifer species in Portugal that is so far known to be affected by the nematode and it covers $8.8x10^5$ ha of the entire country, representing an economically important asset in wood materials (DGRF, 2007). Phytossanitary measures conducted in the meantime have affected the industries that depended on wood material (Mota and Vieira, 2008) with a loss of 700 thousand maritime pine trees. During the national survey in the spring of 2008, *B. xylophilus* was detected outside the quarantine area, in the districts of Arganil and Lousã, in the center of Portugal. This new detection again led to new phytossanitary procedures (MADRP 2008a). In June 2008, an official publication from the Ministry of Agriculture confirmed the presence of PWN in some locations

outside the previous restriction area and defined the entire national territory as affected and restricted zone, concluding that phytossanitary measures had not been successful in prevention of the disease spread (MADRP, 2008b). Currently, PWN is present in a large area located in the center of the country, but also in Setúbal region. More recently, 2010, it was detected in Madeira Island (Fonseca *et al.,* 2012). In 2010, the presence of the disease was also confirmed in Northern Spain (Galicia) (Abeleira *et al.,* 2011) (Fig.VI1.). The current situation in Portugal assumes great importance because of the economical impact to the forest industry, but also due to the massive destruction of pine trees in the initial affected area – Setúbal Peninsula. At present, *B. xylophilus*, is listed as a harmful organism to plants or plant products by the European Union (Annex II, Council Directive 200/29/EC of 8 May 2000). Its introduction and spread within any member state must be avoided. Its presence in the territory of a member state obliges the country to notify the partners and to adopt immediate safeguard measures (Mota and Vieira*,* 2008).

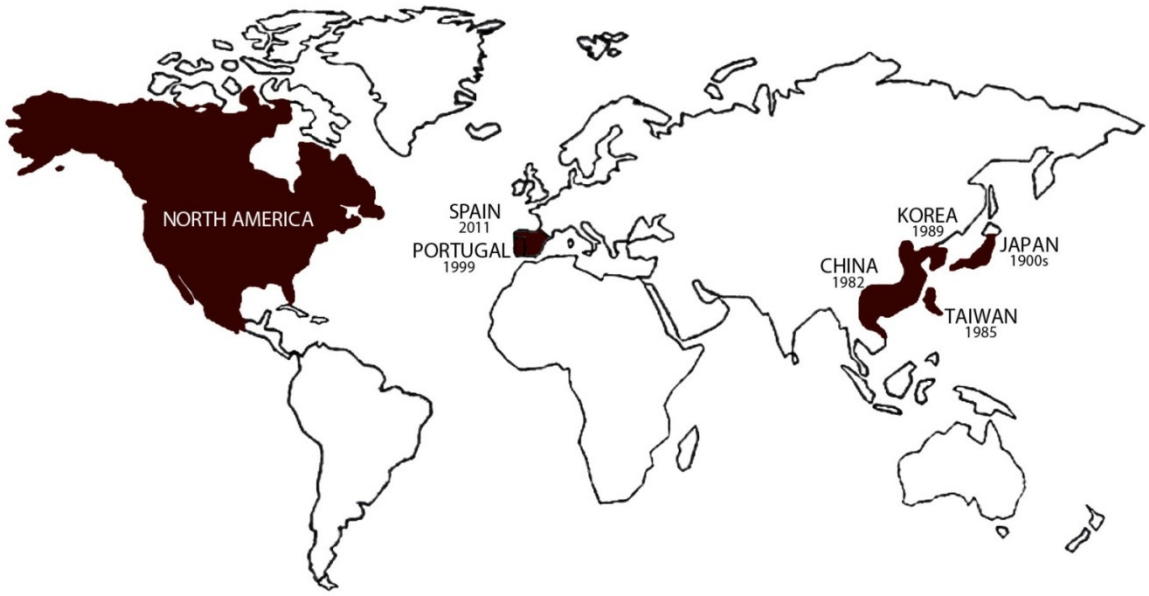


Figure.V1. Worldwide distribution of *Bursaphelenchus xylophilus* (Adapted by Vieira and Barbosa, from OEPP/ EPPO, 1996).

V.2.2. PWN bio-ecology

B. xylophilus has a complex life cycle, having both a phytophagous (transmission during feeding) and mycophagous (transmissions during insects oviposition) phase of development. The most important vectors of the PWN are beetles belonging to the genus *Monochamus* Dejean (order Coleoptera, family Cerambycidae). In Portugal, the only known vector is *Monochamus galloprovincialis* Olivier (Sousa *et al*., 2001, 2002) which has established with the nematode a phorectic association during its feeding on healthy trees and oviposition on dead pines.

The insect transports the nematode in its elytra (wing cases) and in the tracheae (breathing tubes). During maturation feeding on healthy pines (Fig.VI2.), the nematode is transmitted and spreads through the plant vascular system and resin canals, and begins to feed on epithelial cells and living parenchyma – phytophagus phase (Mamiya, 1984; Kishi, 1995). The nematode continues its life cycle, composed of four stages of propagative juveniles, which finally moult to an adult male and female stage (Fig.VI2.). The several stages of PWN includes a first juvenile stage (DL1) completed inside the egg, followed by hatching as second-stage juveniles (DL2) and proceeding through three moults prior to becoming adults (Mamiya, 1975). Under favourable climatic conditions, such as suitable temperatures (aprox. 20ºC), the nematodes multiply quickly and complete its life cycle from egg to adult in six days, each female can laying between 80 and 150 eggs during an oviposition period of 28-days (Mamiya, 1975). Nematodes block water conductance through the xylem inducing tracheid cavitation, thus contributing to plant death. Declining or dead trees, constitutes an attractive environment for insect oviposition during which nematodes enter the tree by oviposition slits in the bark. The trees needles progressively acquire a yellowish to brown coloration, due to the shut down of photosynthesis, and with tree death different species of fungi develop in the wood (Mamiya, 1984), becoming a major food source for the PWN. *B. xylophilus* can develop through two distinct pathways as “propagative” or “dispersal” life cycles, the first two juveniles stages (named L1 and L2) being common to both. Under suitable conditions, the nematode develops through the

propagative pathway, moults to the third (L3) and fourth (L4) juveniles stages and finally the adult stage (Fig.VI2.). When environmental conditions are not favourable (such as low temperatures, high or low moisture or absence of adequate food), the nematode switches to its dispersal pathway, the third and fourth stage juveniles now being referred to as DL3 and DL4.

Natural dispersion of the PWN is due to the insect vector which emerges, as young adults, from the dead tree carrying *B. xylophilus*. Prior to vector emergence, the nematodes DL3 begin to surround the pupal chambers in the Spring and eventually molt into DL4, which is a specialized non-feeding and dispersive stage - *dauer* juveniles or *dauer* larvae. The nematodes are attracted to the insects pupal chamber and enter into the insects natural openings such as the spiracles (Linit *et al*., 1983), in numbers which vary from a few hundred to as high as many thousands inside the body of each insect. The transmission of PWN to a new tree host can occur by maturation feeding or by oviposition activity of the adult *Monochamus* females (Linit, 1988). Transmission through maturation feeding occurs after emergence of the young adults, as the insects fly to feed on the branches of healthy pine trees and inadvertantly infect them with the nematode when the DL4 leave their body and enter the host through its feeding wounds (Mamiya, 1984; Linit, 1990). The mechanism involved and the chemical cues responsible for this are still largely unknown. Transmission through oviposition takes place when the female insects lay their eggs on dead or weakened trees, and nematodes (D4) leaves the insects body and entering the declining or dead host through the oviposition wounds.

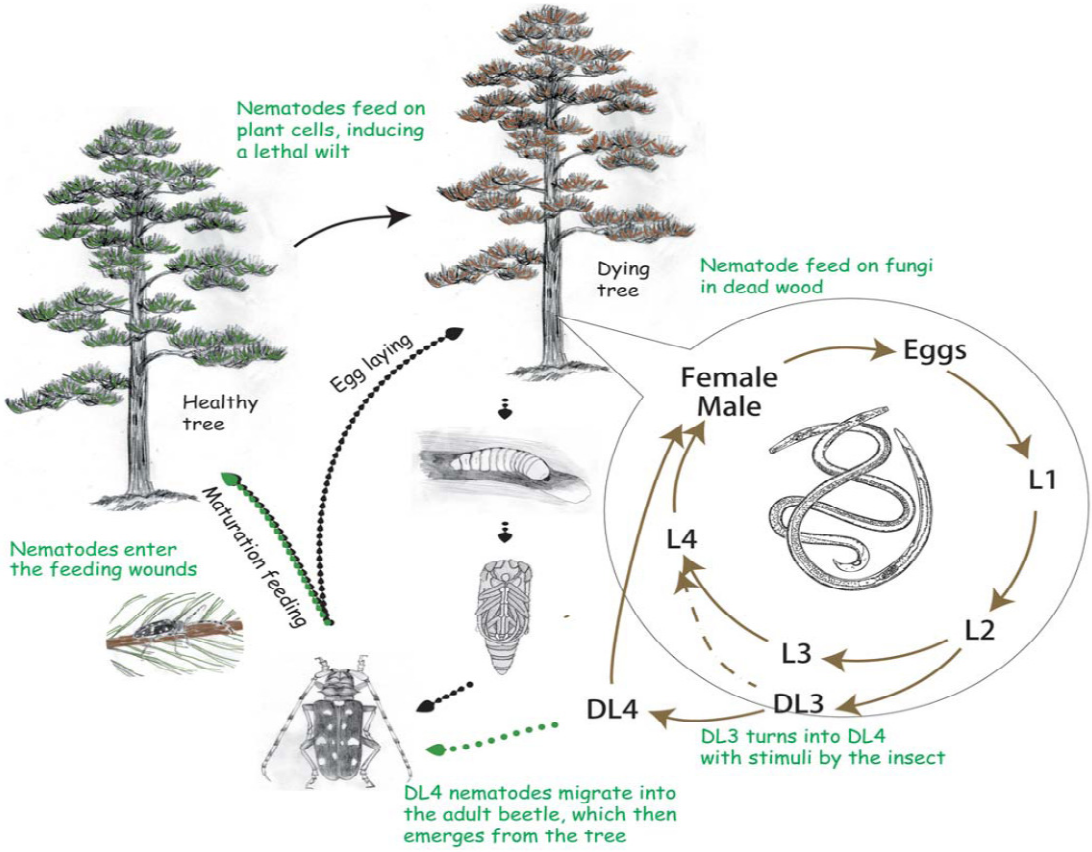


Figure.V2. Life cycle of the nematode, *Bursaphelenchus xylophilus,* and the pine wilt disease (From Kikuchi *et al.,* 2011).

VII.2.3. Taxonomy of *B. xylophilus*

Nematodes in the genus *Bursaphelenchus* are mycophagous or plant parasitic, or both, and have been considered a potential risk to cultivated plants, especially conifers, since the end of the 1970s. The genus contains two virulent plant pathogens, the PWN *B. xylophilus*, and the red-ring nematode *B. cocophilus*, causative agent of red-ring disease in palm trees. To date, almost 100 *Bursaphelenchus* species, usually associated with coleopteran beetles, have been described (Hunt, 2008; Ryss *et al.,* 2005), especially in Europe (Braasch, 2001) and the USA (Massey, 1974); however, because of the finding of the PWN in Portugal (Mota *et al.,* 1999), the practical importance of the taxonomy of this genus has been re-evaluated worldwide (Kanzaki, 2008).

Due to the large number of plant parasitic nematode species (over 3 000), a precise and rapid identification is crucial in applying correct and preventive measures in a timely manner and thus avoiding economical loss. Compared to other groups of plant pests, nematode identification has always been difficult because these organisms lack conspicuous and discriminating morphological characters for each species (De Ley and Blaxter, 2002). One other problem is that often we only have juveniles for identifying and they lack the morphological characteristics of the species.

Identification can be made by classical methods, with time consuming observations and measurements under a light microscope (Burrows, 1990). These observations require specialized taxonomists. However, observations can lead to misidentification; furthermore, species simply cannot be precisely identified due to the low resolution achieved by the light microscope, not sufficient to distinguish some important morphological characters for separating lower taxonomic levels. Despite their great importance, morphological characters do not have sufficient discriminatory power for detailed inter- or intraspecific taxonomy and, therefore, the use of molecular and biochemical techniques, based on differences in proteins, lipids, and nucleic acids, has become an important complement to traditional morphological studies. It has now been extensively and routinely used for over 30 years on nematode identification due to the revolutionising potential to establish inter and intraspecific relationships between nematode species and populations. Molecular methods are highly specific and capable of detecting and identifying closely related species, and also have the ability to determine particular genetic properties, establish infection pathways and spread routes of the disease. (Burrows, 1990; Metge *et al.,* 2006; Vieira *et al.,* 2007). Molecular techniques are also very important because with them we can establish phylogenetic studies and inferences, which may allow us to reconstruct the evolutionary pathways of the PWN and other economic important nematodes.

Reliable species identification and phylogenetic relationships among isolates within the genus *Bursaphelenchus* has been achieved using different molecular methods developed and applied by various nematologists: hybridization of

genomic DNA (Abad *et al.,* 1991; Bolla *et al.,* 1998), DNA sequencing (Beckenbach *et al.,* 1992; Iwahori *et al.,* 1998; Metge *et al.,* 2006; Ye *et al.,* 2007), RAPD (Irdani *et al.,* 1995; Metge and Burgermeister, 2006; Vieira *et al.,* 2007) and RFLP (Iwahori *et al,* 1998; Burgermeister *et al.,* 2005; Lange *et al.,* 2007).

Until recently, studies on relationships between species of *Bursaphelenchus* were made by using morphology of the male spicule, female tail shape and the number of incisures in the lateral field (Braasch, 2001; Ryss *et al.,* 2005). Recently, Metge *et al*., in 2006 inferred phylogenetic relationships among species of the genus *Bursaphelenchus* using molecular methods characterizing the ITS1, 5.8S and ITS2 sequences of rRNA. It is clear that using both approaches - morphological observation and molecular techniques - we can obtain a precise identification of the species and establish taxonomic relationships with other species and isolates (interspecific and intraspecific relationships).

For example, in the case of the two most similar species belonging to the "*xylophilus-group*", *B. xylophilus* and *B. mucronatus*, the species can only be distinguished by morphological characters when females with rounded tail are present, indicating the species as being *B. xylophilus* (Braasch, 2001). But often wood samples may only contain juveniles and males, or just juveniles, or females with a mucronated tail, and in that case we cannot distinguish if we have one or both species. In such cases, molecular studies are the only method that can make a precise diagnosis. Ideally, we should combine both methods, but it is clear that for a rapid and precise diagnosis, molecular techniques are the solution for the answers that we need: accurate and faster.

V.2.3.1. Taxonomy and morphological identification

The genus *Bursaphelenchus* was established in 1937 by Füchs, grouping nematodes previously included in the genus *Aphelenchoides* Fisher, 1984. However, taxonomic classification of this genus has not always been consistent, the majority of authors classifying *Bursaphelenchus* nematodes in the order

Tylenchida and suborder Aphelenchina (Nickle, 1970; Luc *et al.,* 1987; Maggenti *et al.,* 1987; Nickle and Hoper, 1991). According to Hunt (1993), the genus *Bursaphelenchus* is a member of the Family Parasitaphelenchidae, Superfamily Aphelenchoidoidea, Suborder Aphelenchina, Order Aphelenchida. In 2008, Hunt produced a checklist of the Aphelenchoidea and proposed the inclusion of these nematodes in the order Rhabditida, suborder Tylenchina, superfamily Aphelenchoidea, Family Aphelenchoididae and subfamily Parasitaphelenchinae. These changes in the higher taxa took into account new molecular data presented over the last decades, following the proposals of De Ley and Blaxter (2002).

B. xylophilus, was first described in 1934 from the United Stated and named *Aphelenchoides xylophilus* Steiner and Bührer, 1934. The species was transferred to the genus *Bursaphelenchus* by Nickle in 1970. The genus *Bursaphelenchus* includes nematodes that establish phoretic associations with insects and dead or dying trees, mainly conifers. The genus includes fungal feeders either transmitted to dead or dying trees during insect oviposition, or to healthy trees during insects maturation feeding (Hunt, 1993). Most *Bursaphelenchus* species are free living mycophagous species inhabiting soil or dead plant material, including dead wood. Many species are also known to be entomophilic (phoretic) nematodes.

Up to the date, the genus comprises at least 100 described species (Hunt, 2008). In Portugal and until 1999, no knowledge of this genus was available, 10 species now having been reported for the country, associated with maritime pine trees (Hunt, 2008; Penas *et al.,* 2004), including the description of a new species, *B. antoniae* (Penas *et al.,* 2006).

The economical impact of the PWN reinforced the need for an accurate identification of the species. Morphological studies remain the standard method for routine identification, being used to group species from the genus *Bursaphelenchus* into smaller and more convenient species groups. Despite the clear separation of the members of the "*xylophilus-group*" (*B. baujardi* Walia, Negi, Bajaj and Kalia, 2003; *B. conicaudatus* Kanzaki, Tsuda and Futai, 2000; *B. fraudulentus* Rhum, 1956; *B. doui* Braasch, Gu, Bürgermeister and Zhang,

2005; *B. crenati* Ruhm, 1956; *B. kolymensis* Korentchenko, 1980; *B. luxuriosae* Kanzaki and Futai, 2003; *B. mucronatus* Mamiya and Enda, 1979; *B. singaporensis* Gu, Zhang, Braasch and Bürgermeister 2005; *B. xylophilus* Steiner and Bührer, 1934) from other groups based on male spicule shape, several other taxonomic characters within some species of this group still have a difficult identification. Species belonging to "*xylophilus-group*" are characterised by: a narrow spicule; capitulum flattened; condylus small; lamina angular in posterior third; and presence of cucullus (except in *B. crenati*). Within the "*xylophilus-group*", *B. xylophilus* can be separated from the other species mainly by spicule rostrum-calomus junction angular, male tail terminus (lateral view) pointed and female tail terminus broadly rounded (Ryss *et al.,* 2005; Braasch *et al.,* 2009). Therefore, *B. xylophilus* can be distinguished morphologically from other species, using three morphological characters: a distinct vulval flap (Fig.VI3B.), a rounded tail terminus in the females (Fig.VI3D,F.) and the characteristic shape of the male spicule (Fig.VI3E.) (Mamiya, 1984). Specific identification can be difficult because of the existence of two distinct morphological forms of PWN, denominated the "r" (round) and the "m" (mucronate) forms, which can be separated by the shape of the female tail (Dwinell, 1997; Fonseca *et al.,* 2008). The taxonomic separation from its closest species, *B. mucronatus*, is made by the presence of a mucron in the tail of females of *B. mucronatus* (Mamiya and Enda, 1979; Webster *et al.* 1990). Because of the difficulties to distinguish these two species, the use of molecular techniques are of major importance (Bürgermeister *et al.,* 2005) and with this approach there are no difficulties in separating the two species.

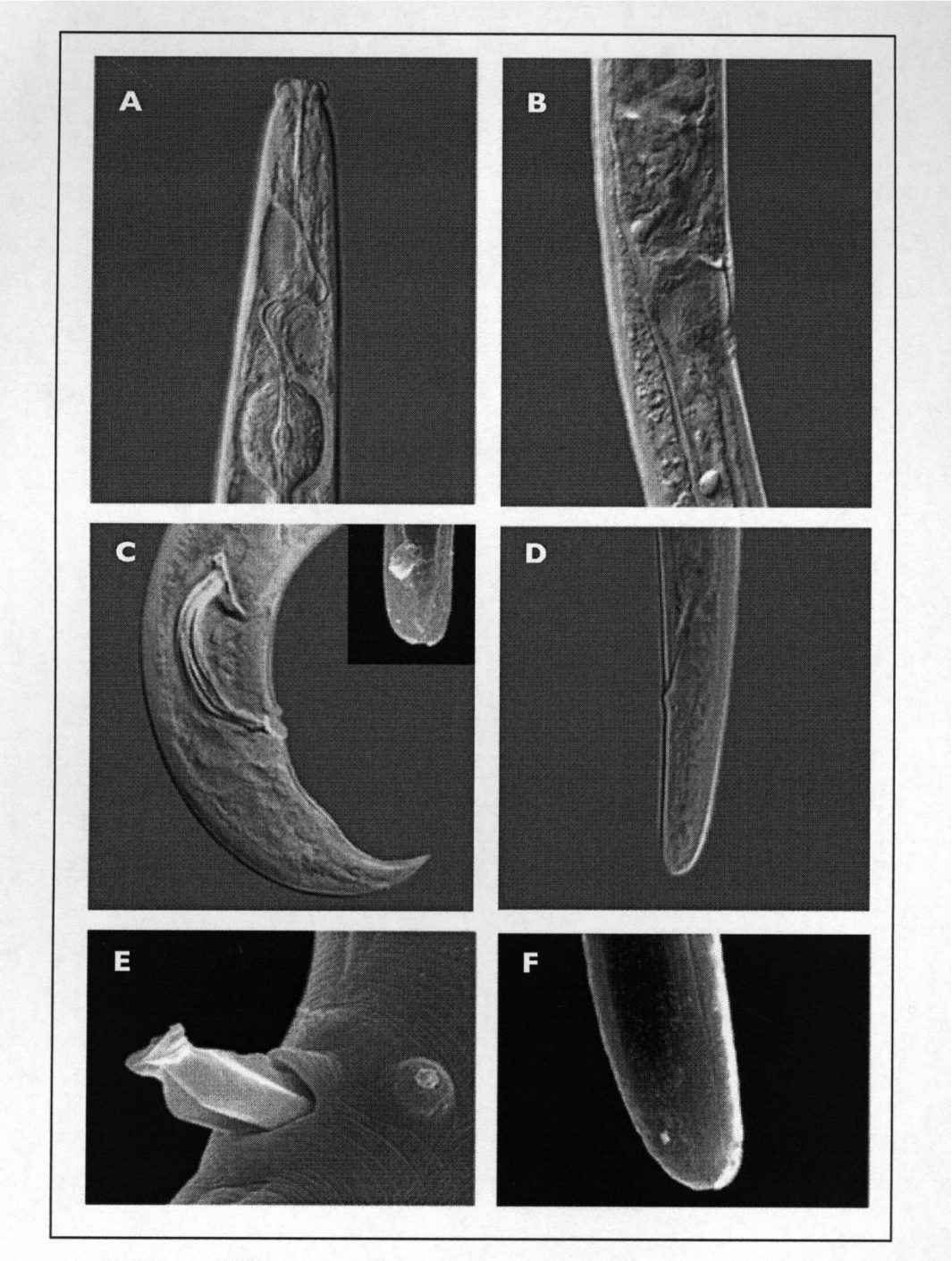


Figure.V3. *Bursaphlenchus xylophilus*. A, Anterior part; B, Female body (vulva with flap); C, Mail tail; D, Female tail; E, Spicules; F, Female tail. (From Penas *et al.,* 2004).

V.2.3.2. Molecular classification

The use of molecular tools became a valuable instrument for species and sub-specific separation of the genus *Bursaphelenchus*, due to the limitations of morphological observations mentioned above. Initially, molecular methods were used for species identification, but with the spread of the disease, the main goal has been to determine a molecular marker that allows the establishment of intraspecific variability between different geographic isolates and to determine infection points and spreading pathways. The first methods used for *Bursaphlenchus* species and isolates identification were based on protein profiles (Hotchkin and Giblin, 1984) and enzyme electrophoresis (Guiran *et al.,* 1985). Immunological approaches have also been used for specific-species identification, using polyclonal antibodies that could differentiate specific antigens of certain *B. xylophilus* isolates (Lawer and Harmey, 1993), as well as monoclonal phage antibodies (Fonseca *et al.,* 2006).

The study of genetic variation were first studied by Bolla *et al.* (1998), that try to differentiate *B. xylophilus* pathotypes using total genomic DNA restriction and hybridization. Abad *et al.* and Tàres *et al.,* studies showed that hybridization and satellite DNA (Abad *et al.,* 1991; Tàres *et al.,* 1994), offers a reliable characterization of the species, and the differentiation of specific and intraspecific groups. Following the objectives of determine genetic diversity of geographical isolates, the following molecular methods have been applied: sequence of heat shock protein genes hsp70 (Beckenbach *et al.,* 1992), sequence of rRNA ITS regions (Iwahori *et al.,* 1998, 2000; Beckenbach *et al.,* 1999; Zhang *et al.,* 2001; Kanzaki and Futai, 2002a,b), sequence of D2D3 region of the 28S rRNA gene (Zheng *et al.,* 2003; Ye *et al.,* 2007) and RAPD analysis (Kusano *et al.,* 1999; Zhang *et al.,* 2002; Zhang *et al*., 1999). Intraspecific variation study using DNA coding regions, such as D2D3 region of the 28S rRNA gene and cytochrome c oxidase from mtDNA, seems to be not a good choice, because they are conserved regions within the species. Intergenic spacer region (IGS) of the 5S rRNA, a region between the LSU and SSU (small subunit of ribosome) rRNA genes, is a highly variable sequence, even between closely related species (Jayasinghe and Wijesundera, 2003) and is commonly

used to study interspecific relations. More recently, ISSR have been used by Metge *et al*., (2006) to study intraspecific variation and determine genetic diversity. This method was introduced in 1994 (Zietkiewicz *et al.,* 1994), predominantly applied to plants (Reddy *et al*., 2002), but for similar studies in animals ISSR polymorphisms have already been demonstrated to be useful in analyzing the pathway of introduced species (Abbot, 2001; Metge *et al.,* 2006). Simple sequence repeats (SSRs) are short hypervariable sequences distributed across eukaryotic genomes that represent good markers for studies of populations of one species (intraspecific variability) (Abbot, 2001). ISSR provides informative markers because they are highly polymorphic. ISSR markers are typically highly reproducible, due to stringent annealing temperatures, long primers, and low primer-template mismatch (semi-arbitrary primers that anchor on SSR loci) (Jones *et al.,* 1997).

In recent years, due to a high copy number in individual cells, lack of recombination and strict maternal inheritance, mitochondrial genes (mtDNA) have been used as markers for diagnosis and to study intraspecific variation (Madani *et al.,* 2010). More recently, mtDNA has been used to study genetic relationships among Peruvian and Canadian populations of *Globodera pallida* and to identify the origin of this species (Madani *et al.,* 2010; Picard *et al.,* 2007; Plantard *et al.,* 2008*). Cytb* was used and proved to be a good marker to solve intraspecific variability between *G. pallida* populations (Plantard *et al.,* 2008).

PWN is a unique plant parasite that uses an insect vector, a cerambycid beetle, particularly *Monochamus* spp. (Akbult and Stamps, 2011), to be transmitted from one tree to another. Like all plant parasitic nematodes posses a structure called stylet, used to penetrate the wall of the plant cell. The stylet allows the nematode to remove cell contents during nematode feeding and to introduce secretions into plant tissue. Secretions of the nematode stylet are produced in the esophageal gland cells, and play an important function in plant parasitism. The glands developed and increased their size as nematodes evolved from bacterial feeders to parasites of plants. *B. xylophilus* is different from other plant parasitic nematodes, because it parasitizes the aboveground parts of the trees, migrating trougth plant tissues (Kikuchi, 2008). Furthermore, *B. xylophilus* is

basically a fungal feeder using the insect as a phoretic mean to reach the tree. All these factors imply that the PWN should have a set of parasitic genes, distinct from the major plant parasitic nematodes, such as cellulase genes. Cellulose is a carbohydrate polymer, being the major component of plant cell walls and consequently cellulases (endo-ß-1,4-glucanases) are produced by many plant pathogens including bacteria and fungi (Zhao *et al.,* 2005; Smant *et al.,* 1998). The ability to break down cellulose, is a major problem for plant parasitic nematodes as few animals can produce the required enzymes (cellulases). Previous work has shown that other plant parasitic nematodes, such as *Heterodera, Globodera* and *Meloidogyne* species have acquired cellulases from bacteria. However, in spite to be a plant parasitic nematode, *B. xylophilus* cellulases seems to have been acquired independently from fungi (Kikuchi *et al.,* 2004).

Endogenous cellulase genes have been identified in obligatory plant-parasitic nematodes, such as *Heterodera, Globodera* and *Meloidogyne* species (Smant *et al.,* 1998; Rosso *et al.,* 1999; Goellner *et al.,* 2000) being produced in esophageal gland cells and secreted through the nematode stylet during penetration in plant tissues (Zhao *et al.,* 2005, Smant *et al.,* 1998; Rosso *et al.,* 1999). The protein encoded by these genes belongs to the glycosyl hydrolase family (GHF), being similar to GHF5 cellulases from bacteria. Cell wall degradating enzymes found in plant parasitic nematodes are thought to have been acquired via lateral gene transfer (LGT) because similar genes are absent in almost other nematodes and because they are most similar to genes from bacteria and fungi (Jones *et al,* 2005). GHF5 has been found in plant parasitic nematodes such as *Meloidogyne, Globodera* and *Heterodera*. Recently, GHF45 has been detected in *Pristionchus pacificus* (Dieterich *et al,* 2009), but phylogenetic analysis showed that this GHF45 was not close to those found in *Meloidogyne, Heterodera* and *Globodera*. The GHF45 was detected in *B. xylophilus* and has not been found in any other nematode genus being more similar to those from fungi, this it has been hypothesized that they can have been acquired via LGT from fungi (Xie *et al.,* 2009; Kikuchi *et al.,* 2004). The absence of GHF5 genes in *B. xylophilus* and the absence of GHF45 in other plant parasitic nematodes support the hypothesis of HGT events playing an

important role in the evolution of plant parasitism in nematodes (Kikuchi *et al.,* 2011). Cellulase genes are parasitism genes of plant parasitic nematodes, important to *B. xylophilus* because it hydrolyses cellulose from the plant cell wall facilitating parasitic activity and penetration of the nematode inside the tree, but also influencing nematode feeding, development and propagation on fungal mats (Cheng *et al.,* 2010). This factor, together with the fact that *B. xylophilus* is also a fungal feeder, take investigators to consider that this nematode has a set of parasitic genes, distinct from the major plant parasitic nematodes, such as cellulase genes.

Previous studies of Ma *et al.* (2011) shows that when the gene that expresses cellulose is silenced, the dispersal activity and propagation of *B. xylophilus* inside the tree drecrease, and so, cellulose must be a potential marker to be considered in the analysis of genetic diversity of PWN. Sequence analysis of the cellulase genes allowed species identification as well as intraspecific variability evaluation within the genus *Bursaphelenchus* (Cheng *et al.,* 2008, 2010).

The recent release of the full *B. xylophilus* genome sequence (Kikuchi *et al.,* 2011) may unravel new molecular markers to study intraspecific variability to determine pathways of disease spread and follow the evolution of introduced isolates. Furthermore, recent studies on mitochondrial DNA sequencing (A. Amorim, personal communication) should be considered to evaluate the potential of mtDNA and its different genes for phylogenetic and biodiversity studies.

V.3. Literature cited

Abad, P., Tares, S., Bruier, N. and De Guiran, G. (1991). Characterization of the relationships in the nematode species complex (PWNSC) (*Bursaphelenchus* spp.) using a heterolous unc-22 DNA probe from *Caenorhabditis elegans*. Parasitology 102: 303-308.

Abbot, P. (2001). Individual and population variation in invertebrates revealed by inter-simple sequence repeats (ISSRs). 3pp. Journal of Insect Science, 1.8. Available online: insectscience.org/1.8.

Abelleira, A., Picoaga, A., Mansilla, J.P. and Aguin, O. (2011). Detection of *Bursaphelenchus xylophilus*, causal agent of pine wilt disease on *Pinus pinaster* in North-western Spain. EPPO Bulletin 39:344.

Akbult, S. and Stamps, W. T. (2011). Insect vectors of the nematode: a review of the biology and ecology of *Monochamus* species. Forest Pathology. doi: 10.1111/j.1439-0329.2011.00733.x.

Anon. (2011). Forests in Europe. FOREST EUROPE (The Ministerial Conference on the Protection of Forests in Europe) (http://www.foresteurope.org/).

Beckenbach, K., Blaxter, M, and Webster, J. (1999). Phylogeny of *Bursaphelenchus* species derived from analysis of ribosomal internal transcribed spacer DNA sequences. Nematology 1: 539-548.

Beckenbach, K., Smith, M.J., and Webster, J.M. (1992). Taxonomic affinities and intra- and interspecific variations in *Bursaphlenchus* spp. as determined by polymerase chain reaction. Journal of Nematology 24:140-147.

Bergdahl, D.R. and Halik, S. (1999). Inoculated *Pinus sylvestris* serve as long-term hosts for *Bursaphelenchus xylophilus*. In: Futai, K., Togashi, K. and Ikeda, T. (Eds). Sustainability of Pine Forest in Relation to Pine Wilt and Decline: proceedings of international symposium. Tokyo, Japan, Shokada, pp. 73-78.

Bolla, R.I., Weaver, C., and Winter, R.E.K. (1998). Genomic differences among pathotypes of *Bursaphelenchus xylophilus*. Journal of Nematology 20: 309-316.

Braasch, H., Burgermeister, W. and Gu, J. (2009). Revised intra-generic grouping of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae). Journal of Nematode Morphology and Systematics 12: 65-88.

Braasch, H., Gu, J., Burgermeister, W., Brandstetter, M. and Metge, K. (2007). *Bursaphelenchus africanus* sp. n. (Nematoda: Parasitaphelenchidae) – found in packaging wood exported from South Africa to Ningbo/ China. Journal of Nematode Morphology andSystematics 9: 71-82.

Braasch, H. (2001). *Bursaphelenchus* species in conifers in Europe: distribution and morphological relationships. European Plant Protection Organization Bulletin 31: 127-142.

Bürgermeister, W., Metge, K., Braasch, H. and Buchbach, E. (2005). ITS-RFLP patterns for differentiation of 26 *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) and observations on their distribution. Russian Journal of Nematology 13: 29-42.

Burrows, P.R. (1990). The use of DNA to identify plant parasitic nematodes. Nematological Abstracts 59: 1-8.

Cheng, X.Y., Dai, S.M., Xiao, L. and Xie, B.Y. (2010). Influence of cellulase gene knockdown by dsRNA interference on the development and reproduction of the pinewood nematode, *Bursaphelenchus xylophilus*. Nematology 12: 225-233.

Cheng, X.Y., Cheng, F.X., Xu, R.M. and Xie, B.Y. (2008). Genetic variation in the invasive process of *Bursaphelenchus xylophilus* (Aphelenchida: Aphelenchoididae) and its possible spread routes in China. Heredity 100: 356-365.

Cheng, H.R., Lin, M., Li, W. and Fang, Z. (1983). The occurrence of a pine wilting disease caused by a nematode found in Nanjing. Forest Pest and Disease 4: 1-5.

Costanza, R., d'Arge, R., de Groot, R., et al. (1997). The value of the world´s ecosystem services and natural capital. Nature 387: 253–260.

De Ley, P. and Blaxter, N. (2002). Systematic Position and Phylogeny. In: Donald L. Lee (Ed.). The Biology of Nematodes. Taylor and Francis Inc New York, pp. 1-30.

DGRF, (2007). Resultados do IFN 2005/06. (download as a pdf on May 2008 from: http://www.dgrf.min-agricultura.pt/portal).

Dieterich, C. and Sommer, R. J. (2009). How to become a parasite - lessons from the genomes of nematodes. Trends in Genetics 25: 203-209.

Dwinell, L.D. (1997). Nematode: regulation and mitigation. Annual Review of Phytopathology 35: 153-166.

Dwinell, L.D. (1993). First report of nematode (*Bursaphlenchus xylophilus)* in Mexico. Plant Disease 77: 846.

Evans, H., McNamara, D., Braasch, H., Chadoeuf, J. and Maggnusson, C. (1996). Pest Risk Analysis (PRA) for the territories of the European Union (as PRA area) on *Bursaphelenchus xylophilus* and its vectors in the genus *Monochamus*. European Plant Protection Organization Bulletin 26: 199-249.

FAO (2007). State of the World´s Forests 2007. Rome, pp. 157.

Fonseca, L., Cardoso, J.M.S., Lopes, A., Pestana, M., Abreu, F., Nunes, N., Mota, M. and Abrantes, I. (2012). The pinewood nematode, *Bursaphelenchus xylophilus*, in Madeira Island. *Helminthologia* 49(2): 96-103.

Fonseca, L., Santos, M. C. V., Santos, M. S., Curtis, R. H. C. and Abrantes, I. M. O. (2008). Morpho-biometrical characterisation of Portuguese *Bursaphelenchus xylophilus* isolates with mucronate, digitate or round tailed females. Phytopathology Mediterranea 47: 223 – 233.

Fonseca, L., Curtis, R., Halsey, K., Santos, M., Abrantes, M. and Santos, M. (2006). Morphological, molecular and serological characterization of *Bursaphelenchus xylophilus* isolates. Pine wilt disease: a worldwide threat to forest ecosystems, International symposium. 10-14 July, Lisbon: 63-64 (Abst.)

Goellner, M., Smant, G., De Boer, J.M., Baum, T. and Davis, E.L. (2000). Isolation of ß-1,4-endoglucanase genes *Globodera tabacum* and their expression during parasitism. Journal of Nematology 32: 154–165.

Guiran, G., Lee, M., Dalmasso, A. and Bongiovanni, M. (1985). Preliminary attempt to differentiate nematodes (*Bursaphelenchus* spp.) by enzyme electrophoresis. Revue de Nematologie 8: 88-90.

Gu, J, Braasch, H, Burgermeister, W, Brandstetter, M and Zhang, J (2006). Description of *Bursaphelenchus yongensis* sp. n. (Nematoda: Parasitaphelenchidae) isolated from *Pinus massoniana* in China. Russian Journal of Nematology 14: 91-99.

Gu, J., Zhang, J., Braasch, H. and Burgermeister, W. (2005). *Bursaphelenchus singaporensis* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Singapore -–a new species of the *B. xylophilus* group. Zootaxa 988: 1-12.

Hotchkin, P.G. and Giblin, R.M. (1984). Comporison of electrophoregrams from Bursaphelenchus spp. (Aphelenchoididae). Revue de Nématologie 7 (3): 319-320.

Huang, L., Ye, J. R.,Wu, X. Q., Xu, X. L., Sheng, J. M., and Zhou, Q. X. (2010). Detection of the pine wood nematode using a real-time PCR assay to target the DNA topoisomerase I gene. European Journal of Plant Pathology 127: 89–98.

Hunt, D.J. (2008). A checklist of the Aphelenchoidea (Nematoda: Tylenchina). Journal of Nematode Morphology and Systematics 10: 99-135.

Hunt, D.J. (1993). Aphelenchida, Longidoridae and Trichodoridae: Their systematics and bionomics. Wallingford, Oxon, UK, CAB International.

IPQ (2011). prDNP Guia 1. Caparica, pp. 12.

Irdani, T., Caroppo, S. and Ambrogioni, L. (1995). Molecular identification of pinewood *Bursaphelenchus* species. Nematologia Mediterranea 23 (Suppl.):99-106.

Iwahori, H., Tsuda, K., Kanzaki, N., Izui, K. and Futai, K. (1998). PCR RFLP and sequencing analysis of ribossonal DNA of *Bursaphelenchus* nematodes related to pine wilt disease. Fundamental and Applied Nematology 21: 656-666.

Jayasinghe, D.R. and Wijesundera, W.S.S. (2003). Differentiation of *Setaria digitata* and *Setaria labiatopapillosa* using molecular markers. The Veterinary Journal 165: 136-142.

Jones, J.T., Furlanetto, C. and Kikuchi, T. (2005). Horizontal gene transfer from bacteria and fungi as a driving force in the evolution of plant parasitism in nematodes. Nematology 7: 641–646.

Jones, C.J., Edwards, K.J., Castaglione, S., Winfield, M.O., Sala, F., Wiel, C., Bredemeijer, G., Vosman, B., Matthes, M., Daly, A., Brettschneider, R., Bettini, P., Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Volckaert, G., Rueda, J., Linacero, R., Vazquez, A. and Karp, A. (1997). Reprodicibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Molecular Breeding 3: 381-390.

Kanzaki, N. (2008). Taxonomy and systematic of the nematode genus *Bursaphelenchus* (Nematoda: Parasitaphelenchidae). In: Zhao B, Futai K, Sutherland JR, Takeuchi Y (Eds). Pine wilt disease Japan Springer, Japan, pp. 44-66.

Kanzaki, N. and Futai, K. (2003). Description and phylogeny of *Bursaphelenchus luxuriosae* n. sp. (Nematoda: Aphelenchoididae) isolated from *Acalolepta luxuriosa* (Coleoptera: Cerambycidae). Nematology 5: 565-572.

Kanzaki, N. and Futai, K. (2002a). Observation on the arrangement of caudal papillae of *Bursaphelenchus conicaudatus* and *B. fraudulentus*. Japanese Journal of Nematology 32: 21-24.

Kanzaki, N. and Futai, K. (2002b). A PCR primer set for determination of phylogenetic relationships of *Bursaphelenchus* species within the *xylophilus group*. Nematology 4: 35-41.

Kanzaki, N., Tsuda, K. and Futai, K. (2000). Description of *Bursaphelenchus conicaudatus* n. sp. (Nematoda: Aphelenchoididae), isolated from the yellow-spotted longicorn beetle, *Psacothea hilaris* (Coleoptera: Cerambycidae) and fig trees, *Ficus carica*. Nematology 2: 165-168.

Kikuchi, T. Cotton, J.A., Dalzell, J.J., Hasegawa, K., Kanzaki, N., McVeigh, P., Takanashi, T., Tsai, I.J., Assefa, S.A., Cock, P.J.A., Otto, T.D., Hunt, M., Reid, A.J., Sanchez-Flores, A., Tsuchihara, K., Yokoi, T., Larsson, M.C., Miwa, J., Maule, A.G., Sahashi, N., Jones, J.T. and Berriman, M. (2011). Genomic Insights into the Origin of Parasitism in the Emerging Plant Pathogen *Bursaphelenchus xylophilus*. PLOS Pathogens 7 (9): e10022.doi:10.1371/journal.ppat.1002219.

Kikuchi, T. (2008). Parasitism Genes of Pinewood Nematode. In: Zhao, B.G., Futai, K., Sutherland, J.R. and Takeuchi, Y. (Eds). Pine Wilt Disease. Japan, Springer Press. pp. 67- 80.

Kikuchi, T., Jones, J.T., Aikawa, T., Kosaka, H. and Ogura, N. (2004). A family of glycosyl hydrolase family 45 cellulases from the pinewood nematode *Bursaphelenchus xylophilus*. FEBS Letter 572: 201–205.

Kishi, Y. (1995). The pinewood nematode and the Japanese pine sawyer Thomas Company Limited. Tokyo, Japan, pp. 302.

Kiyohara, H. and Bolla, R.I. (1990). Pathogenic variability among populations of the nematode, *Bursaphelenchus xylophilus*. Forest Science 36: 1061-1076.

Kiyohara, T. and Tokushige, Y. (1971). Inoculation experiments of a nematode, *Bursaphelenchus* sp., onto pine trees. Journal of Japanese Forestry Science 53: 210-218.

Korentchenko, E.A. (1980). New species of nematodes from the family Aphelenchoididae, parasites of stem pests of the Dahurian Larch. Zoologicheskii Zhurnal 59: 1768-1780.

Kusano, T., Nakamura, K., Fujii, T., Sugawara, Y., Ichikawa, Y., Masuyama, E., Shinkawa, H., Okamatsu, M., Kitaura, Y., Tsubaki, K., Uchida, T., Togashi, K., Enoki, S., Jikumaru, S. and Ikeda, S. (1999). RAPD-PCR fingerprinting patterns of six *Bursaphelenchus xylophilus* isolates and a *B. mucronatus* isolate. In: Futai K, Togashi K, Ikeda T (Eds). Sustainability of pine forests in relation to pine wilt and decline. Proceedings of International Symposium, Tokyo, 27-28 Oct., 1998. Tokyo: Nakanishi Printing, pp. 52-56.

Lange, C., Burgermeister, W., Metge, K., and Braasch, H. (2007). Phylogenetic analysis of isolates of the *Bursaphelenchus sexdentati* group using ribosomal intergenic transcribed spacer DNA sequences. Journal of Nematode Morphology and Systematics 9: 95-109.

Lawer, C. and Harmey, A.M. (1993). Immunological differentiation between *Bursaphelenchus xylophilus* and *B. mucronatus*. Nematologica 39: 536-546.

Linit, M.J. (1990). Transmission of nematode through feeding wounds of *Monochamus carolinensis* (Coleoptera: Cerambycidae). Journal of Nematology 22: 231-236.

Linit, M. (1988). Nematode-vector relationships in the pine wilt disease system. Journal of Nematology 20: 227-235.

Linit, M.J., Kondo, E. and Smith, M.T. (1983). Insects associated with the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphlenchoididae), in Missouri. Enviromental Entomology 12: 467-470.

Luc, M.R., Fortuner, D.J. and Geraert E. (1987). A reappraisal of Tylenchina (Nemata). 1. For a new approach to the taxonomy of Tylenchina. Revue de Nematologie 10:127-134.

Ma, H.B., Lu, Q., Liang, J. and Zhang, X.Y. (2011). Functional analysis of the cellulose gene of the pinewood nematode, *Bursaphelenchus xylophilus*, using RNA interference. Genetics and Molecular Research 10 (3): 1931-1941.

MADRP (2008a). Portaria nº 305-A/2008 de 21 de Abril. Diário da República, 1º série – Nº 78 – 21 de Abril de 2008.

MADRP (2008b) Portaria nº 553-B/2008 de 27 de Junho. Diário da República, 1º série – Nº 123 – 27 de Junho de 2008.

Madani, M., Subbotin, S.A., Ward, L.J., Li, X. and 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.

Maggenti, A.R,, Luc, M., Raski, D.J., Fourtuner, R. and Geraert, E. (1987). A reappraisal of Tylenchina (Nemata) 2. Classification of the suborder Tylenchina (Nemata: Diplogasteria). Revue de Nématologie 10: 135-142.

Mamiya, Y. (2004). Pine wilt disease in Japan. In: Mota, M. and Vieira, P. (Eds). The nematode, *Bursaphelenchus xylophilus*. Nematology Monographs and Perspectives, vol. 1. Leiden-Boston, Brill Academic Publishers, pp 9-20.

Mamiya, Y. (1984). The pinewood nematode. In Nickle WR (Ed.). Plant and insect nematodes,. Marcel Dekker, New York, pp. 589-626.

Mamiya, Y. and Enda, N. (1979). *Bursaphelenchus mucronatus* n. sp. (Nematoda: Aphelenchoididade) from pinewood and its biology and pathogenicity to pine trees. Nematologica 25: 353-361.

Mamiya, Y. (1975). The life history of pinewood nematode, *Bursaphelenchus lignicolus*. Japanese Journal of Nematology 5: 16-25.

Massey, C.L. (1974). Biology and taxonomy of nematode parasites and associates of bark beetle in the United States. Washington DC, USA, US Government Printing Office, USDA Agriculture Handbook No. 446, pp. 233.

Metge, K. and Burgermeister, W. (2006), Intraspecific variation in provenances os *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididade) revealed by ISSR and RAPD fingerprints. Journal of Plant Disease and Protection 113: 1-8.

Metge, K., Braasch, H., Gu, J. and Burgermeister, W. (2006). Interspecific variation in ITS rDNA of *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) of different groups. Russian Journal of Nematology 14: 147-158.

Mota, M. and Vieira, P. (2008). Pine Wilt Disease: a worldwide threat to forest ecosystems. Springer, pp. 428.

Mota, M., Braasch, H., Bravo, M.A., Penas, A.C., Burgermeister, W., Metge, K. and Sousa, E. (1999). First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. Nematology 1: 727-734.

Nickle, W.R. and Hooper, D.J. (1991). The Aphelenchina: bud, leaf and insect nematodes. In: Nickle WR. (Ed.). Manual of Agricultural Nematology. Marcel Decker, New York, pp. 465-507.

Nickle, W.R. (1970). A taxonomic review of the genera of the Aphelenchoidea (Fuchs, 1937) Thorne, 1949 (Nematoda: Tylenchida). Journal of Nematology 2: 375-392.

Oliveira, C.M., Eulálio, J., Bessis, R. and Harakava, R. (2011). Caracterizações Morfológica e Molecular de *Bursaphelenchus fungivorus* (Nematoda: Aphelenchida), Detectado pela Primeira vez no Brasil. Nematologia Brasileira 35 (3-4): 63-70.

Penas, A.C., Bravo, M.A., Naves, P., Bonifácio, L., Sousa, E. and Mota, M. (2006). Species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Parasitaphelenchidae) and other nematode genera associated with insects from Pinus pinaster in Portugal. Annals of Applied Biology 148: 121-131.

Penas, A.C., Correia, P., Bravo, M.A., Mota, M. and Tenreiro, R. (2004). Species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Parasitaphelenchidae) associated with maritime pine in Portugal. Nematology 6: 437-453.

Phan, L.K. (2008). Potential of Entomopathogenic Nematodes for Controlling the Japanese Pine Sawyer, *Monochamus alternatus*. In: Zhao B, Futai K, Sutherland JR, Takeuchi Y (Eds). Pine wilt disease Japan Springer, Japan, pp. 371-381.

Picard, D., Sempere, T. and 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 Phylogenetic Evolution 42: 308-316.

Plantard, O., Picard, D., Valette, S., Scurrah, M., Greinier, E. and Mugniery, 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.

Reddy, M.P., Sarla, N. and Siddiq, E.A. (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128:-172.

Robertson, L., Arcos, C.S., Escuer, M., Merino, S.R. Eaparrago, G., Abelleira, A. and Navas, A. (2011). Incidence of the nematode *Bursaphelenchus xylophlius* Steiner and Buhrer, 1934 (Nickle, 1970) in Spain. Nematology 13 (6): 755-757.

Rodrigues, J. (2008). Eradication program for the nematode in Portugal. In Mota, M. and Vieira, P (Eds) Pine Wilt Disease: a Worldwide Threat to Forest Ecosystems. Springer Press. pp 5-14.

Rosso, M.N., Favery, B., Piotte, C., Arthaud, L., De Boer, J.M., Hussey, R.S., Bakker, J., Baum, T.J. and Abad, P. (1999). Isolation of a cDNA encoding a β-1,4-endoglucanase in the root-knot nematode *Meloidogyne incognita* and expression analysis during plant parasitism. Molecular Plant-Microbe Interactions 12: 585-591.

Rühm, W. (1956). Die Nematoden der Ipiden. Parasitologische Schriftenreihe 6: 1-435.

Ryss, A., Vieira, P., Mota, M. and Kulinich, O. (2005). A synopsis of the genus B*ursaphelenchus* Fuchs, 1937 (Aphelenchida: Parasitaphelenchidae) with keys to species. Nematology 7: 393-458.

Shimazu, M. (2006). Current status on research and management of pine wilt disease in Japan. Han, H.R., Koh, S.H., Jung, C.S., Chung, Y.J. and Shin, S.C. (Eds). Current Status on Research and Management of Pine Wilt Disease. Seoul, Korea, Korea Forest Research Institute, pp. 1-18.

Shin, S., Shokado, S. and Han, H. (2006). Current status on research and management of pine wilt disease in Korea. Current status on research and management of pine wilt disease, International Symposium, October 20. Korea Forest Research Institute, Seoul, Korea, 31-44.

Smant, G., Stokkermans, J.P., Yan, Y.T., Boer, J.M.D., Baum, T.J., Wang, X.H., Hussey, R.S., Gommers, F.J., Henrissat, B., Davis, E.L., Helder, J., Schots, A.

and Bakker, J. (1998). Endogenous cellulases in animals: isolation of ß-1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes. Proc. Natl. Acad. Sci. USA 95: 4906-4911.

Sousa, E., Naves, P., Bonifácio, L., Bravo, M., Penas, A.C., Pires, J. and Serrão, M. (2002). Preliminary survey for insects associated with the pinewood nematode *Bursaphelenchus xylophilus* in Portugal. European Plant Protection Organization Bulletin 32: 499-502.

Sousa, E., Bravo, M., Pires, J., Naves, P., Penas, A., Bonifácio, L. and Mota, M. (2001). *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididade) associated with *Monochamus galloprovincialis* (Coleoptera: Cerambycidae) in Portugal. Nematology 3: 89-91.

Steiner, G. and Buhrer, E.M. (1934) *Aphelenchoides xylophilus* n. sp., a nematode associated with blue-stain and other fungi in timber. Journal of Agricultural Research 48: 49-951.

Sutherland, J.R. and Peterson, M. (1999). The nematode in Canada: history, distribution, hosts, potential vectors and research. In: Futai K, Togashi K, Ikeda T (Eds). Sustainability of pine forests in relation to pine wilt and decline. Proccedings of the Symposium Shokado Shoten Kyoto, Japan, Shokado Shoten, pp. 247-253.

Sutherland, J.R. and Webster, J.M. (1993).Nematodes pests of Forest trees. In: Evans. K., Trudgill, D.L. and Webster, J.M. (Eds). Plant Parasitic Nematodes in Temperate Agriculture. CAB International, Wallingford, UK, pp. 351-380.

Suzuki, K. (2002). Pine wilt disease - a threat to pine forest in Europe. Dendrobiology 48: 71-74.

Tomiczek, C., Braasch, H., Burgermeister, W., Metge, K., Hoyer, U. and Brandstetter, M. (2003). Identification of *Bursaphelenchus* spp. isolated from Chinese packaging wood imported to Austria. Nematology 5: 573-581.

Tomminen, J., Halik, S. and Berglahl, D.R. (1991). Incubation temperature and time effects on life stages of *Bursaphelenchus xylophilus* in wood chips. Journal of Nematology 24(4): 477-484.

Vanhanen, H.M. (2008). Invasive insects in Europe – the role of climate change and global trade. Dissertationes Forestales 57, Faculty of Forest Sciences, University of Joensuu, Finland, pp.33.

Vicente, C., Espada, M., Vieira, P. and Mota, M. (2012). Pine Wilt Disease: a threat to European forestry. European Journal of Plant Pathology DOI 10.1007/s10658-011-9924-x.

Vieira, P., Burgermeister, W., Mota, M., Metge, K. and Silva, G. (2007). Lack of genetic variationof *Bursaphelenchus xylophilus* in Potugal revealed by RAPD-PCR analyses. Journal of Nematology 39: 118-126.

Walia, K.K., Negi, S., Bajaj, H.K. and Kalia, D.C. (2003). Two new species of B*ursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae) from pinewood and insect frass from India. Indian Journal of Nematology 33: 1-5.

Webster, J.M. (2004). The pinewood nematode: implications of factors past and present for pine wilt disease. In: Mota M, Vieira P (Eds). The nematode, Bursaphelenchus xylophilus. Nematology Monographs and Perspectives, vol 1.Leiden-Boston: Brill Academic Publishers, pp. 55-64.

Webster, J.M., Anderson, R.V., Baillie, D.L., Beckenbach, K., Curran, J. and Rutherford, T.A. (1990). DNA probes for differentiating isolates of the nematode species complex. Revue de Nématologie 13: 255-263.

Wingfield, M.J., Blanchette, R.A., Nicholls, T.H. and Robbins, K. (1982a). Association of pinewood nematode with stressed trees in Minnesota, Iwoa, and Wisconsin. Plant disease 66: 934-937.

Wingfield, M.J., Blanchette, R.A., Nicholls, T.H. and Robbins, K. (1982b). The pinewood nematode: a comparasion of the situation in the United States and Japan. Canadian Journal of Forest Research 12: 71-75.

Xie, B-Y., Cheng, X-Y., Shi, J., Zhang, Q-W., Dai, S-M., Cheng, F.X. and Luo, Y-Q. (2009). Mechanisms of invasive population establishment and spread of nematodes in China. Science in China Series C: Life Sciences 52 (6): 587-594.

Yano, M. (1913). Investigation on the cause of pine mortality in Nagasaki Prefecture. Sanrinkoho 4: 1-14.

Ye, W., Giblin-Davis, R.M., Braasch, H., Morris, K. and Thomas, W.K. (2007). Phylogenetic relationships among *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) inferred from nuclear ribosomal and mitochondrial DNA sequence data. Molecular Phylogenetics and Evolution 43: 1185-1197.

Yi, C., Byun, B., Park, J., Yang, S. and Chang, K. (1989). First finding of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle and its insect vector in Korea. Research Reports of the Forestry Research Institute Seoul 38: 141-149.

Zhang, L., Kong, F. and Yang, B. (2002). Intra and interspecific variation in *Bursaphelenchus xylophilus* and *B. mucronatus* revealed by mtDNA polymorphism. Forest Research 15: 7-12.

Zhang, L., Liao, J. and Feng, Z. (2001). Sequencing and PCR-SSCP analysis of ribosomal DNA of *Bursaphelenchus* nematodes. Acta Phytopathologica Sinica 31: 84-89.

Zhang, K., Lin, M., Wen, L. and Xu, W. (1999). Genetic variation of *Bursaphelenchus xylophilus* and *B. mucronatus* geographical isolates of China as shown by RAPD. In: Futai F Togashi K, Ikeda T (Eds). Sustainability of pine forests in relation to pine wilt and decline. Proceedings of International Symposium, Tokyo, 27-28 Oct., 1998. Nakanishi Printing,Tokyo, pp. 65-69.

Zhao, B.G., Liang, B., Zhao, L.G. and Xu, M. (2005). Inference of pinewood nematode on production of phytotoxins of an accompanying pathogenic bacterial strain (in Chinese with English abstract). Journal of Nanjing Forestry University 27: 71-75.

Zheng, J., Subbotin, S.A., He, S., Gu, J. and Moens, M. (2003). Molecular characterisation of some Asian isolates of *Bursaphelenchus xylophilus* and *B. mucronatus* using PCR-RFLPs and sequences of ribosomal DNA. Russian Journal of Nematology 11: 17–22.

Zietkiewicz, E., Rafalski, A. and Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20:176-183.

Chapter VI. Study of the genetic diversity of geographic populations from continental Portugal and Madeira Island

Sub-chapter VI.1.

VI.1. Valadas, V., Laranjo, M., Barbosa, P., Espada, M., Mota, M. and Oliveira, S. (2012). The nematode, *Bursaphelenchus xylophilus*, in Portugal: possible introductions and spread routes of a serious biological invasion revealed by molecular methods (*accepted in Nematology:* DOI:10.1163/156854112X632673).

Sub-chapter VI.2.

VI.2. Valadas, V., Laranjo, M., Mota, M. and Oliveira, S. (2012). Molecular characterization of Portuguese populations of the pinewood nematode *Bursaphelenchus xylophilus* using cytochrome b and cellulase genes (*accepted in Journal of Helminthology:* DOI:10.1017/S0022149X12000673).

Chapter VII. Final discussion and future perspectives

VII.1. Final discussion and future perspectives

For millions of years the distribution of the world´s biota depended on natural barriers. However, with the increase of globalization and the breaking down of geographic limits, new biological invasions emerged, becoming a global issue, due to the changes caused in the colonized environments and its economical impact.

The present thesis used to different biological models, one representing harmful/damaging nematodes - *Bursaphelenchus xylophilus*, and the other beneficial nematodes – entomopathogenic nematodes (EPN). Both nematodes belong to the Order Rhabditida, representing the large diversity and potential of this Phylum.

The pinewood nematode (PWN), *B. xylophilus*, is native from North America (Kanzaki and Futai, 2002) and widely distributed along Canada, USA and Mexico (CABI/EPPO, 1999), where it has co-evolved with the different North American pine species, during millions of years, therefore causing no disease. North America is probably the region where *B. xylophilus* populations have the highest genetic diversity (Vieira *et al*., 2007).

The spread of *B. xylophilus* to non-native areas occurs as a result of human activity, due to the exchange of wood products carrying the nematode and/or the insect vector, used in international trade. Once *B. xylophilus* is introduced into a non-native area, preventive measures have to be applied by removing dead or dying trees or by selecting resistant trees. Any uncut wilted tree and untreated dead wood can be a reservoir of nematodes for the following year (Evans *et al.,* 1996; Jones *et al.,* 2005; Gu *et al.,* 2006).

In forest ecosystems, *B. xylophilus* is considered one of the most important pests with a worldwide distribution. The main fear of PWN establishment into countries where conifers forests assume great importance is connected with the devastating effects of this nematode in pine trees (Shin and Han, 2006). The damages caused by this invasive species are well demonstrated in non-native

areas, where the disease is already established, e. g., Japan and China (Yang, 2004; Shimazu, 2006) causing huge annual losses in economy, but also irreversible changes to the native ecosystems (Suzuki, 2002).

In Portugal, the disease was firstly diagnosed in 1999 (Mota *et al.,* 1999), being restricted to Setúbal Peninsula until 2008. After 2008, new outbreaks in the north and center part of the country were detected, and more recently in Madeira Island. With this current scenario, the greatest fear is the danger of spread of the disease, which depends mainly on the efficacy of phytossanitary measures on the insect vector, *Monochamus galloprovinciallis*, and the host pine tree, *Pinus pinaster* (Rodrigues, 2008).

The research developed in this thesis contributed to the molecular characterization of Portuguese populations of *B. xylophilus* from 1999 until 2010, and to understand the possible origins of the new outbreaks of the disease since 2008 to avoid future infection and design new phytossanitary measures. These studies represent the first comprehensive analyses using Portuguese isolates from the different geographic locations including the initial affected area where the disease was detected in 1999 and restricted until 2008 (Setúbal Peninsula), as well as the new outbreaks in the north and center of continental Portugal, to where the disease spread after 2008. Isolates from Madeira Island were also included in the studies, representing the infection detected during 2010.

To achieve our purpose, *cytb* and cellulase genes, as well as IGS from rRNA and ISSR fingerprints were used as markers to evaluate the intraspecific variability of Portuguese isolates compared with USA, Japan, China and Korean isolates. Cellulase and *cytb* genes were used for the first time to determine genetic diversity among *B. xylophilus* populations.

Using IGS sequencing, intraspecific variability among Portuguese isolates of *B. xylophilus* was not detected, being not possible to group isolates according to their geographic origin. IGS markers, as well as ITS sequence analyses only allowed the confirmation of the Portuguese isolates as *B. xylophilus*.

In the dendrogram obtained from the ISSR fingerprint analysis, all Portuguese isolates collected until 2008 group together, indicating lack of genetic diversity and suggesting a single introduction from Asia, which is in agreement with the results of Vieira *et al.* (2007). Observing the distribution of isolates collected during 2009-2010 in continental Portugal, there were isolates grouping with those previously collected until 2008, suggesting a spread of the disease from the initial affected area to new outbreaks, which may have occurred with great probability considering the heavy traffic of vehicles carrying wood products from the Lisbon-Setúbal area to the center and north of Portugal, where a number of industries process pine wood for furniture. On the other hand, there are isolates that grouped apart, suggesting a new introduction in continental Portugal, possibly from a much diverse population than the one that infected Portugal in 1999. The bands shared between Portuguese isolates and North American, do not exclude the possibility of part of the new population in Portugal (2009-2010) have an North American origin.

The results obtained with the Madeira Island isolates suggest a high genetic diversity of *B. xylophilus* population in the island, suggesting the possibility of a new introduction in the island but also that at least, part of the Madeira population is related to the mainland population.

Using *cytb* and cellulase genes with the goal of characterizing Portuguese geographic isolates and understanding the spread routes of the PWD, our results agree with the results that we previously obtained with ISSR fingerprints (Valadas *et al.,* 2012a) and with previous studies of Vieira *et al*., 2007, in suggesting a possible Asian origin for Portuguese isolates. The clustering of Portuguese isolates with different Asian isolates may suggest more than one introduction of the PWN in Portugal from Asian origin. However, the low resolution obtained with *cytb*, prompted us to use the cellulase gene, in an attempt to find a better genetic marker. In the cellulase phylogenetic analysis, Portuguese and foreign isolates group together in the same large cluster (Valadas *et al.,* 2012b). It was possible to observe some genetic diversity among isolates showing that cellulase sequences are more discriminative to study intraspecific variations than *cytb* sequences. However, no clear grouping

of Portuguese isolates according to their geographic location or year of collection is observed (Valadas *et al.,* 2012b).

Although the results on PWN intraspecific diversity provided important information on the new outbreaks, more studies should be undertaken with more foreign isolates. This study used a limited number of PWN isolates, particularly from the foreign countries. Whenever possible, a higher number of isolates should be used, ideally representing the entire country, because the entrances of disease into non-native areas may have occur more than one time and in different locations. It should also be guaranteed that each isolate is collected from just one infected tree, and if possible, process the analysis, before isolates are maintained in culture to avoid the possibility of DNA changes.

On the other hand, the choice of the marker is very important. Fingerprints, such as ISSR seem to be a good choice to evaluate genetic diversity because the entire genome is analyzed. However, this is a time consuming technique and its results are difficult to exchange between different laboratories. The sequence of DNA regions exceed the limitation of fingerprints but a specific region of DNA that may not provide sufficient phylogenetic information. In the present work, however, ISSR fingerprint were the technique with better resolution in the study of intraspecific diversity, as well as the cellulose gene.

EPN are lethal parasites of insects and a potential alternative to insecticides as biocontrol agents (Köppenhofer *et al.,* 2000). With the increase of the use of chemical control in environment, and the development of resistance by insect pests, the search for an alternative way to eliminate pests and to preserve environmental conditions promote the development of EPN studies and biodiversity understanding. The study of their genetic diversity is extremely important because new species and/or isolates may be useful as biological control agents against agriculturally important pests and because they are worldwide distributed. The recovery and identification of indigenous EPN isolates is of major importance, due to differences in strain virulence, environmental conditions that may affect their survival, reproductive potential and virulence against natural and local insect pests (Stock, 2009).

The present study aimed to understanding the natural occurrence of EPN in continental Portugal, representing the most systematic and extensive survey made for the first time in continental Portugal to evaluate the indigenous species of EPN. The study involves a large diversity of habitats and ecosystems covering the whole territory of continental Portugal. The species identified provided the first information on the richness and diversity of the native EPN fauna in continental Portugal, thereby opening up the possibility of investigating the use these species as biocontrol agents.

A total of 791 soil samples were collected in continental Portugal, with the additional information of altitude, vegetation and soil type, as well as moisture and temperature from the closest meteorological stations for each sample.

The recovering rate of EPN in continental Portugal was 6.7% of sampling sites, with the identification of five different species from the entire country: *S. feltiae*, *H. bacteriophora*, as the two most abundant species in the country, together with *S. kraussei, S. intermedium* and *Steinernema* sp. The occurrence of *S. feltiae* and *H. bacteriophora* as the two most abundant species in continental Portugal, suggests the potential role of these nematodes in natural regulation of insect populations.

According to vegetation type, natural habitats present a higher percentage of positive samples, compared to agricultural ones, since in agricultural regions the chemical control to insect pests partially reduces the abundance of natural biocontrol agents, like EPN. Stock *et al*. (2008) also claimed the higher abundance of EPN in natural habitats like forests.

EPN recovery percentage does not present any relationship with soil type, temperature, moisture or physical and chemical characteristics.

S. feltiae has a wide distribution in temperate regions, whilst *H. bacteriophora* typically occurs in regions with continental and mediterranean climates (Hominick, 2002). *S. feltiae* is the most common species found in Europe, and in many other parts of the world (for a detailed EPN species distribution see Hominick, 2002). The present study shows that *S. feltiae* is the most common and widely distributed EPN in continental Portugal, followed by *H.*

bacteriophora. *S. feltiae* was found in all the five NUT of continental Portugal, associated with distinct types of habitats.

H. bacteriophora, the most geographically widespread species of this genus, is a common species in regions with continental and Mediterranean climates and it has been reported in different areas of Africa, Asia, Europe, and America (Hominick, 2002). In Southern Europe, this species has an abundant distribution, and it was found associated with several types of habitats in Northern Spain (Garcia del Pino and Palomo, 1996; Campos-Herrera *et al*., 2007) and in Southern France (Emelianoff *et al.,* 2008). Based on the biodiversity and distribution of EPN's from the Iberian Peninsula and other Mediterranean regions, the presence of *H. bacteriophora* and *S. feltiae* were expected in continental Portugal, specially representing the two most abundant species. The three additional species were also identified: *S*. *kraussei, S*. *intermedium* and *Steinernema* sp. *glaseri*-group. *S. intermedium* is a common species in Spain (Garcia del Pino, 2005), a neighbouring country with climatic conditions similar to continental Portugal. *S. intermedium* appears to have a global distribution, having been originally described in South Carolina, USA (Nguyen *et al*., 2007). In Europe this species is known from Germany (Sturhan, 1999), Czech Republic (Mráček *et al*., 1999), Spain (Garcia del Pino, 2005) and Switzerland (Steiner, 1996). *S. kraussei* is widely distributed in Europe, having been reported in Belgium, Czech Republic, Germany, Sweden, Netherlands, UK and Italy (Tarasco *et al*., 2009). This species is common in European woodlands and forest soils (Sturhan *et al*., 2005). Also, it has been found in Canada (Mráček and Webster, 1993) and USA (Stock *et al*., 2000); it is prevalent in woodland habitats (Mráček *et al*., 2005) and is rarely found in open habitats. *S. kraussei* and *S. intermedium* were each found at only one site, in moors and heathland and mixed forests, respectively. The reason for the low recovery of these two species is not known, because both species are common in Europe.

Differences in nematode distribution may be related to differences in the distribution of suitable insect hosts and to the species of nematode involved (Akhurst and Bedding, 1986). Mráček *et al.* (2005) suggested that the

occurrence of EPN depends on the presence of many insect hosts. However, no information about the natural hosts of EPN in continental Portugal is available.

Accurate identification of EPN has important implications in systematics and population genetics and is of major importance for selection of species for future use in biological control. The combination of molecular and morphological methods is necessary to solve a variety of issues in EPN taxonomy. A recent study of phylogenetic relationships among *Steinernema* species combined morphological and molecular methods and showed that most morphological features are not phylogenetically informative (Spiridonov *et al*., 2004) Stock *et al.* (2002) points to the difficulties in using just molecular identification for EPN. The species nature of the *Steinernema* genus is a result of its longer evolution history (Adams *et al.,* 2007) and its reproduction patterns of amphimictic and hermaphroditic reproduction, make this genus more capable of occupying a wide range of habitats than *Heterorhabditis* (Edgington *et al.,* 2010). The geographical and habitat preferences of EPN species in continental Portugal may also reflect chance of dispersal events as well as feeding patterns.

According to our studies, COXI seems to be a good marker to study intraspecific variability. In future both molecular data with morphological characteristics should be combine in the phylogenetic tree. However, these analyses will require a good knowledge of morphological characters and the understanding of which characteristics are phylogenetic informative or not.

As with any surveys, conclusions regarding diversity and biogeography of EPN were taken with caution, since the results in part reflect searching effort and sampling technique rather than actual numbers and/or habitat preferences of EPN. However, this study represents the most comprehensive survey for EPN so far carried out in continental Portugal and these results should be taken in account in future research, especially in experimental design (number of collected samples, prospected habitats, method to recover EPN from soil samples, sampling period, insects presence on the sampled habitats, among others).

In the future, surveys must be undertaken, especially in natural habitats, because agricultural ones are normally under the effect of pesticides. When soil samples are collected, several informations should be taken into account: vegetation, soil type, temperature and moisture from that place and the insect present in soil sample or associated with the vegetation. In laboratory, soil sample should be baited at least to two different temperatures that should vary between 15-22/25ºC (Mracek *et al.,* 2005), because each EPN species have different requirements, and if possible use other insect vector besides the *Galleria mellonella.* Direct extraction methods can also be used but they require a taxonomist expertise.

Previous studies on EPN have already demonstrated their potential for controlling, for example, the Japanese pine sawyer *M. alternatus* (Phan, 2008). However, EPN cannot migrate through the wood; however, when the beetles are feeding on the sapwood, they produce sawdust where the nematodes can migrate in search of the host (Phan, 2008). In the future it is necessary to screen for more effective EPN species and probably to combine control measures to obtain better results. It should be taken into account that, *M. alternatus*, associated with PWN, differs in nature, due to its association with the pinewood nematode, and must be regarded as an invasive species. This means that the pine wilt outbreak will never end without the use of artificial intervention (Yang, 2004).

Control measures for PWD pretend to reduce the application of insecticides targeted at larvae of the bark or wood borers inhabiting in dead pine trees. For that, some natural enemies have been examined as biological control agents against the vector beetle and PWN. Previous studies from Naves *et al.* 2005 provided some interesting information on the potential of certain parasitoid for the biological control of *M. galloprovinciallis*. Tapping fungi, applied on nematodes and entomopathogenic nematodes applied on the insect vector, have also been examined for their ability to control PWN and the vector beetle, respectively.

So far it has proved impossible to control *B. xylophilus* once it has been introduced into a tree. Infection can be prevented by a prophylactic chemical

treatment, that still not have efficient results. Several quarantine treatments for wood chips have been proposed by EPPO, but the only known effective treatment for wood already infected with *B. xylophilus* and its vectors appears to be heat treatment, in which all parts of the wood reach a temperature of 56℃ for at least 30 min (Evans *et al.,*1996).

It should be taking into account that, biological invasion is a process with several steps, involving, the transport of the colonizer organisms, its establishment, a lag time, a period of expansion followed by the spread of the populations. During this period, the invasive species may evolve during both its initial establishment but also during its subsequent range expansion, which in the case of Portugal can be represented by the period of 2009-2010. Pinewood introductions may involve multiple sources and large quantities of the pest, which allow considerable variation to be transferred from the colonizer population. For all these reasons, it is of extreme importance that in future studies, different markers be considered, but more importantly, a good representation of geographic isolates from all the countries where the nematode is established should be used, otherwise no conclusive results will be achieved.

Future research still continuing to try to find alternative means of control, such as biological control agents for both nematodes and vectors, and in that way, our studies on the natural diversity of EPN species in continental Portugal, should present an alternative way to control PWD, that in future studies should be evaluated.

The recent release of the full *B. xylophilus* genome sequence (Kikuchi *et al.,* 2011) is unravelling new molecular markers, like effectors, in a way that they are understanding as protein that suppresses host defense responses in order to facilitate infection, most of them evolved in parasitism (Haegeman *et al.,* 2011). Some of the effectors already identified are produced in esophageal gland cells being associated with *B. xylophilus* locomotion – calreticulin (Li *et al.,* 2011). Furthermore, recent studies on mitochondrial DNA sequencing (A. Amorim, personal communication) should be considered to evaluate the

potential of mtDNA and its different genes for phylogenetic and biodiversity studies.

VII.2. Literature cited

Adams, B.J., Peat, S.M. and Dillman, A.R. (2007). Phylogeny and evolution. In: Nguyen, K.B., Hunt, D.J. (Eds). Entomopathogenic nematodes: systematics, phylogeny and bacterial symbionts. Nematology Monographs and Perspectives 5. Leiden, The Netherlands, Brill, pp. 693-733.

Akhurst, R.J., Bedding, R.A. (1986). Natural occurrence of insect pathogenic nematodes (Steinernematidae and Heterorhabditidae) in soil in Australia. Journal of the Australian Entomological Society 25, 241–244.

CABI/EPPO (1999). Distribution Maps of Plant Diseases. CABI Publishing: Wallingford.

Campos-Herrera, R., Escuer, M., Labrador, S., Robertson, L., Barrios and L., Gutiérrez, C. (2007). Distribution of the entomopathogenic nematodes from La Rioja (Northern Spain). Journal of Invertebrate Pathology 95: 125–139.

Edgington, S., Buddie, A.G., Moore, D., France, A., Merino, L., Tymo, L.M. and Hunt, D.J. (2010). Diversity and distribution of entomopathogenic nematodes in Chile. Nematology 12 (6): 915-928.

Emelianoff, V., Le Brun, N., Pages, S., Stock, P., Tailliez, P., Moulia, C. and Sicard, M. (2008). Isolation and identification of entomopathogenic nematodes and their symbiotic bacteria from Herault and Gard (Southern France). Journal of Invertebrate Pathology 98: 211–217.

Evans, H.F., McNamara, D.G., Braasch, H., Chadouef, J. and Magnusson, C. (1996). Pest risk analysis (PRA) for the territories of the European Union (as PRA area) on *Bursaphelenchus xylophilus* and its vectors in the genus Monochamus. EPPO Bulletin 26: 199-249.

García del Pino, F. (2005). Natural occurrence of entomopathogenic nematodes in Spain. In: MC-Meeting and Working Group 4th Meeting: Natural occurrence and evolution of entomopathogenic nematodes and Management Committee Meeting. Ceske Budejovice, The Czech Republic 14–17 Enero 2005.

García del Pino, F. and Palomo, A. (1996). Natural occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Spanish soils. Journal of Invertebrate Pathology 68: 84–90.

Gu, J, Braasch, H, Burgermeister, W, Brandstetter, M and Zhang, J (2006). Description of *Bursaphelenchus yongensis* sp. n. (Nematoda: Parasitaphelenchidae) isolated from Pinus massoniana in China. Russian Journal of Nematology 14: 91-99.

Jones, J.T., Furlanetto, C. and Kikuchi, T. (2005). Horizontal gene transfer from bacteria and fungi as a driving force in the evolution of plant parasitism in nematodes. Nematology 7: 641–646.

Kanzaki, N. and Futai, K. (2002). A PCR primer set for determination of phylogenetic relationships of *Bursaphelenchus* species within the xylophilus group. Nematology 4: 35–41.

Kikuchi, T. Cotton, J.A., Dalzell, J.J., Hasegawa, K., Kanzaki, N., McVeigh, P., Takanashi, T., Tsai, I.J., Assefa, S.A., Cock, P.J.A., Otto, T.D., Hunt, M., Reid, A.J., Sanchez-Flores, A., Tsuchihara, K., Yokoi, T., Larsson, M.C., Miwa, J., Maule, A.G., Sahashi, N., Jones, J.T. and Berriman, M. (2011). Genomic Insights into the Origin of Parasitism in the Emerging Plant Pathogen *Bursaphelenchus xylophilus*. PLOS Pathogens 7 (9): e10022.doi:10.1371/journal.ppat.1002219.

Koppenhofer, A.M. (2000). Nematodes. In: Lacey, L.A., Kaya, H.K. (Eds.), Field Manual of Techniques in Invertebrate Pathology. Kluwer, Dordrecht, The Netherlands, pp. 283–301.

Haegeman, A., Mantelin, S., Jones, J and Gheysen, G. (2011). Functional roles of effectors of plant-parasitic nematodes. Gene 492: 19-31.

Hominick, W.M. (2002). Biogeography. In: Gaugler, R. (Ed.). Entomopathogenic Nematology. CABI Publishing, Wallinford, UK, pp. 115–143.

Li, X., Zhuo, K., Luo, M., Sun, L. And Liao, J. (2011). Molecular cloning and characterization of a calreticulin cDNA from the pinewood nematode *Bursaphelenchus xylophilus*. Experimental Parasitology 128: 121-126.

Mota, M., Braasch, H., Bravo, M.A., Penas, A.C., Burgermeister, W., Metge, K. and Sousa, E. (1999). First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. Nematology 1: 727-734.

Mráček Z., Bečvář S., Kindlmann P. and Jersáková J. (2005). Habitat preference for entomopathogenic nematodes, their insect hosts and new faunistic records for the Czech Republic. Biological Control 34: 27-37.

Mráček Z., Bečvář S. and Kindlmann P. (1999). Survey of entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) in the Czech Republic. Folia Parasitologica 46: 145-148.

Mráček Z. and Webster J. (1993). Survey of Heterorhabditidae and Steinernematidae (Rhabditida, Nematoda) in Western Canada. Journal of Nematology 25: 710-717.

Nguyen K.B., Hunt D. J. and Mráček Z. (2007). Steinernematidae: species description. In: Nguyen, K.B. and Hunt, D.J. (Eds). Entomopathogenic nematodes: Systematics, Phylogeny and Bacterial symbionts Nematology Monographs and Perspectives No. 5, Hunt D.J. and Perry R.N. series editors. Brill, Leiden, The Netherlands. pp. 121-609.

Phan, L.K. (2008). Potential of Entomopathogenic Nematodes for Controlling the Japanese Pine Sawyer, *Monochamus alternatus*. In: Zhao B, Futai K, Sutherland JR, Takeuchi Y (Eds). Pine wilt disease Japan Springer, Japan, pp. 371-381.

Rodrigues, J. (2008). Eradication program for the nematode in Portugal. In: Mota, M. and Vieira (Eds). Pine Wilt Disease: A Worldwide Threat to Forest Ecosystems. Springer, pp. 5-14.

Shimazu, M. (2006). Current status on research and management of pine wilt disease in Japan. Han, H.R., Koh, S.H., Jung, C.S., Chung, Y.J. and Shin, S.C. (Eds). Current Status on Research and Management of Pine Wilt Disease. Seoul, Korea, Korea Forest Research Institute, pp. 1-18.

Shin, S., Shokado, S. and Han, H. (2006). Current status on research and management of pine wilt disease in Korea. Current status on research and management of pine wilt disease, International Symposium, October 20. Korea Forest Research Institute, Seoul, Korea, 31-44.

Spiridonov, S., Krasomil-Osterfeld, K. and Moens, M. (2004). *Steinernema jollieti* sp. n. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from the American Midwest. Russian Journal of Nematology 12: 85-95.

Steiner, W. (1996). Distribution of entomopathogenic nematodes in the Swiss Alps. Revue Suisse Zoologie 103: 439–452.

Stock, S.P., (2009). Molecular approaches and the taxonomy of insect-parasitic and pathogenic nematodes. In: Stock, S.P., Vandenburg, J., Glazer, I., Boemare, N. (Eds), Insect pathogens: molecular approaches and techniques. CAB International Press, USA, pp. 71-100.

Stock, S.P., Al Banna, L., Darwish, R. and Katbeh, A. (2008). Diversity and distribution of entomopathogenic nematodes (Nematoda: Steinernematidae, Heterorhabditidae) and their bacterial symbionts (c-Proteobacteria: Enterobacteriaceae) in Jordan. Journal of Invertebrate Pathology 98: 228–234.

Stock S.P., Griffin, C.T. and Burnell, A.M. (2002). Morphological characterization of three isolates of Heterorhabditis Poinar, 1976 from the “Irish group” (Nematoda: Rhabditida: Heterorhaditidae) and additional evidence supporting their recognition as a distinct species, *H. downesi* n. sp. Systematic Parasitology 51: 95-106.

Stock, P., Mráček, Z. and Webster, J.M. (2000). Morphological variation among allopatric populations of *Steinernema kraussei* (Steiner, 1923) (Rhabditida: Steinernematidae). Nematology 2: 143-152.

Sturhan, D., Spiridonov, S.E. and Mráček, Z. (2005). *Steinernema silvaticum* sp. n. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from Europe. Nematology 7: 227-241.

Sturhan, D., Ruess, L. (1999). An undescribed *Steinernema* sp. (Nematoda: Steimernematidae) from Germany and the Scandinavian Subartic. Russian Journal of Nematology 7. 43–47.

Suzuki, K. (2002). Pine wilt disease - a threat to pine forest in Europe. Dendrobiology 48: 71-74.

Tarasco, E., Clausi, M., Rappazzo, G., Vinciguerra, M., Longo, A. and Triggiani, O. (2009). Could be Italy considered a favorite place in Europe for EPN biodiversity? IOBC/wprs Bulletin Vol. 45: 387-389. Working Group "Insect Pathogens and Insect Parasitic Nematodes". Proceedings of the 12th European Meeting at Pamplona, Spain, June 22-26, 2009 (Ehlers R.-U., Crickmore N., Enkerli J., Glazer I., Lopez Ferber M. and C. Tkaczuk C., eds). Bern, Switzerland.

Valadas, V., Laranjo, M., Barbosa, P., Espada, M., Mota, M. and Oliveira, S. (2012)a. The nematode, *Bursaphelenchus xylophilus*, in Portugal: possible introductions and spread routes of a serious biological invasion revealed by molecular methods. (DOI:10.1163/156854112X632673).

Valadas, V., Laranjo, M., Mota, M. and Oliveira, S. (2012)b. Molecular characterization of Portuguese populations of the pinewood nematode *Bursaphelenchus xylophilus* using cytochrome b and cellulase genes (DOI:10.1017/S0022149X12000673).

Vieira, P., Burgermeister, W., Mota, M., Metge, K. and Silva, G. (2007). Lack of genetic variation of *Bursaphelenchus xylophilus* in Portugal revealed by RAPD-PCR analyses. Journal of Nematology 39: 118-126.

Yang, B. (2004). The history, dispersal and potential threat of pinewood nematode in China. In: Mota M, Vieira P (Eds). The nematode, *Bursaphelenchus xylophilus*. Nematology Monographs and Perspectives, vol. 1. Leiden-Boston: Brill Academic Publishers, pp. 21-24.

Contactos:
Universidade de Évora
Instituto de Investigação e Formação Avançada - IIFA
Palácio do Vimioso | Largo Marquês de Marialva, Apart. 94
7002-554 Évora | Portugal
Tel: (+351) 266 706 581
Fax: (+351) 266 744 677
email: iifa@uevora.pt