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CRYOPRESERVATION OF ENTOMOPATHOGENIC NEMATODES

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Ai miei genitori, al mio compagno e a mio figlio…

ABSTRACT

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* have received the most attention among all nematodes studied for the control of insects because they possess many of the attributes of effective biological control agents. Recently, also same *Oscheius* species have been identified as potential insect pest.

The overall aims of this study were (i) to characterize and describe entomopathogenic nematodes isolated from the soils collected during different surveys; (ii) to develop a technique for long-term storage of nematodes; and (ii) to determine the virulence and efficacy of the *Steinernema carpocapsae* ItS-CAO1, as well as to determine its stability and potential for mass production.

In this study a cryopreservation protocol has been tuned for the long-term storage of *S. carpocapsae* (ItS-CAO1), a new strain isolated in a lagoon plain in Veneto region (Northern Italy). An optimum survival rate of 91% was obtained using the following protocol: (i) storage of IJs in distilled water for 15 days at 12 °C after the emergence from *Galleria mellonella* larvae, (ii) 48 h incubation in 18% glycerol at 24 °C, (iii) 10 min in 70% methanol at 0 °C, (iv) several minutes in liquid nitrogen and finally (v) storage in mechanical freezer at -140 °C.

Starting with the protocol developed for *S. carpocapsae*, some experiments were performed in order to cryopreserve also other EPNs, because different species vary in their adaptability of cryogenic storage and the protocols must be established empirically case by case. All species tested were successfully cryopreserved although with different

survival rates: *Heterorhabditis bacteriophora* (71.2%), *S. feltiae* (56.5%), *S. affine* (45.5%) and *S. apuliae* (25.0%). The thawed nematodes of all species have infected *G. mellonella* larvae after cryopreservation and were able to complete their life cycles.

Four types of bioassays, namely: Penetration assay, Exposure time assay, Sand column assay and One-on-one assay, were carried out against *G. mellonella* larvae, to determine the infectivity and stability of *S. carpocapsae* ItS-CAO1 before (control) and after cryopreservation (cryopreserved). Insect mortality of this strain is very high in Sand column assay (100% for nematodes cryopreserved and 93% for the control) and Penetration (100% in both cases). In the first assay, penetration rate was higher for the IJs cryopreserved, while in the second one the highest ability of IJs to penetrate in the host was recorded for the control. Larval mortality in One-on-one assay was 50% for the control and 66.6% for nematodes cryopreserved. In Exposure time assay, the exposure time of nematodes to insects that are required to achieve 50% insect mortality was estimated in both cases ET_{50}<20 min. With the results of infectivity assays we can evaluate the possibility to use this new strain in biological control programs.

RIASSUNTO

I nematodi entomopatogeni (EPNs) del genere *Steinernema* e *Heterorhabditis* hanno ricevuto la maggiore attenzione tra tutti i nematodi studiati per il controllo degli insetti, poiché possiedono molte caratteristiche che li rendono efficaci come agenti per il controllo biologico. Recentemente, però, anche alcuni nematodi appartenenti al genere *Oscheius* sono stati identificati come potenziali parassiti di insetti.

Gli scopi di questo lavoro erano (i) caratterizzare e descrivere i nematodi entomopatogeni isolati dai suoli raccolti durante vari rilevamenti, (ii) sviluppare una tecnica per lo stoccaggio a lungo termine di tali nematodi e (iii) valutare la virulenza del ceppo *Steinernema carpocapsae* ItS-CAO1, nonchè la sua stabilità e il potenziale per la produzione massale.

In questo studio è stato messo a punto un protocollo di crioconservazione per lo stoccaggio a lungo tempo dei un nuovo ceppo di *S. carpocapsae* (ItS-CAO1), isolato da un terreno nella regione Veneto (Nord Italia).

Un alto tasso di sopravvivenza degli individui (91%) è stato ottenuto seguendo il seguente protocollo: i nematodi al loro stadio infettivo (IJs), una volta fuoriusciti dalle larve di *Galleria mellonella* sono stati stoccati in acqua distillata per 15 giorni a 12 °C prima di essere incubati a 24 °C per 48 ore in glicerolo (18%) e successivamente a 0 °C per 10 minuti in metanolo al 70%. IJs sono stati immersi per alcuni minuti in azoto liquido e infine stoccati in freezer a -140 °C.

Partendo da questo protocollo messo a punto per *S. carpocapsae*, sono stati effettuati diversi esperimenti per cercare di crioconservare anche altri EPNs. Dato che ogni specie si adatta in maniera diversa alle condizioni di stoccaggio criogenico, i

protocolli devo essere stabiliti empiricamente a seconda della specie in esame. Nel presente studio, tutte le specie testate sono state crioconservate con successo, con tassi differenti di sopravvivenza: *Heterorhabditis bacteriophora* (71.2%), *S. feltiae* (56.5%), *S. affine* (45.5%) and *S. apuliae* (25.0%). I nematodi sopravvissuti, dopo la fase dello scongelamento, sono stati in grado di infettare nuovamente le larve di *G. mellonella* e di portare a termine il loro ciclo vitale all'interno dell'ospite.

Per valutare l'infettività e la stabilità di *S. carpocapsae* ItS-CAO1 prima (controllo) e dopo la crioconservazione (crioconservati) sono stati condotti quattro biosaggi su larve di *G. mellonella*: Saggio di penetrazione, Saggio del tempo di esposizione, Saggio della colonna di sabbia e Saggio uno-a-uno.

Questo nuovo ceppo ha fatto registrare un tasso di mortalità dell'insetto ospite molto alto sia nel Saggio della colonna di sabbia (100% per i nematodi crioconservati e 93% per il controllo) sia in quello di penetrazione (100% in entrambi i casi). Nel primo biosaggio, il tasso di penetrazione più elevato è stato registrato nelle tesi con i nematodi crioconservati, mentre nel secondo biosaggio, le IJs del controllo hanno avuto un'abilità maggiore nel penetrare l'ospite. Il tasso di mortalità nel biosaggio uno-a-uno è stato del 50% per il controllo e del 66,6% per i nematodi crioconservati. Nell'ultimo biosaggio, il tempo di esposizione dei nematodi alle larve di *Galleria* necessario affinché il 50% di quest'ultime sia portato a morte è stato stimato essere inferiore a 20 minuti, sia nel controllo che per i nematodi crioconservati. Esaminando i risultati dei diversi saggi di infettività, si valuta la possibilità dell'impiego di questo nuovo ceppo come agente di controllo biologico.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
bp	Base pair(s)
ca	Circa
$CaCl_2$	Calcium chloride
cm	Centimeter(s)
df	Degrees of freedom
diam.	Diameter
DNA	Deoxyribonucleic acid
e.g.	exempli gratia
ET	Exposure Time
et al.	et alii
F	F-ratio
F/M	Female/Male
Fig.	Figure
g	Gram(s)
h	Hour
ha	Hectare
i.e.	Id est
IJ(s)	Infective Juvenile(s)
ITS	Internal transcribed spacer
J	Juvenile
kb	Kilobase(s)
KCl	Potassium chloride
Kg	Kilograms
l	Litres
LD	Lethal dose
LT	Lethal time
m	Meter

min	Minute(s)
ml	Millilitres
mm	Millimetres
n	Number
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
No	Number
P	P-level
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic acid
SD	Standard Deviation
s	Second(s)
SE	Standard Error
sec	Second(s)
s.l.	sea level
spp.	Species
sp.	Species
Tab.	Table
v/v	volume/volume
µ	Micro
°C	Degrees Celsius
%	Percentage

Chapter 1

INTRODUCTION

1.1 ENTOMOPATHOGENIC NEMATODES

1.1.1 History of entomopathogenic nematology

Entomophathogenic nematodes (EPNs) are lethal insect parasite. They share a mutualistic relationship with bacteria that are capable of killing host quickly. *Steinernema* is symbiotically associated with bacteria of the genus *Xenorhabdus* (Thomas & Poinar, 1979), while the bacterial symbiont of *Heterorhabditis* are placed in the genus *Photorhabdus* (Boemare *et al*., 1993). The bacteria of both genera are Gram-negative motile rods and belong to Enterobacteriaceae.

The first entomopathogenic nematode was described by Steiner as *Aplectana kraussei* (now *Steinernema kraussei*) in 1923, but its systematic position was problematic (Poinar & Grewal, 2012). Only in the 1930s began to seriously consider the use of these nematodes for the control of insects. In 1929, Glaser and Fox found a nematode infecting grubs of the Japanese beetle *Popillia japonica* in New Jersey (Glaser & Fox, 1930) and Steiner described the nematode as *Neoaplectana* (= *Steinernema*) *glaseri*. Glaser was not aware of the symbiotic bacterium, but he achieved mass-production of this nematode on artificial media and he conducted field trials to control population of Japanese beetle (Glaser & Farrell, 1935).

In 1955 Jaroslav Weiser described a European population of *Neoaplectana* (= *Steinernema*) *carpocapsae* from larvae of codling moth, *Carpocapsa pomonella* (Weiser, 1955). Coincidentally, in the same year Dutky and Hough, working in the USA, independently found the same nematode in the same host species (Dutky & Hough, 1955). The isolate that they found was named DD-136. The use and application of this nematode and *S. glaseri* marked the beginning of a new era for the implementation of entomopathogenic nematodes in biological control. For many decades the only genus of entomopathogenic nematode described was *Steinernema* (= *Neoaplectana*). It was not until 1976 that Poinar described *Heterorhabditis* Poinar, 1976.

Historically, this definition of EPNs has referred to the families Steinernematidae and Heterorhabditidae. Recently, the definition of EPNs was expanded to include other nematodes, such as certain species of the genus *Oscheius.* Three *Oscheius* species, *O. chongmingensis* (Zang *et al.*, 2008), *O. carolinensis* (Ye *et al.*, 2010), *O. onirici* (Torrini *et al*., 2015) and *Caenorhabditis briggsae* (Abebe *et al*., 2010) have been identified as potential insect pathogens by baiting soil for nematodes using insect larvae as prey, a common approach used for finding EPNs (Dillman *et al.*, 2012).

1.1.2 Biology and life cycle

The life cycle of EPNs has several developmental stages: egg, four juveniles stages (denoted as J1, J2, J3 and J4) and adult stage (male, female and hermaphrodite in the case of *Heterorhabditis* spp.). The developmentally arrested non-feeding third stage

infective juvenile (J3), in both *Steinernema* spp. and *Heterorhabditis* spp. is the only freeliving stage formed as a response to depleting food sources and adverse conditions. The J3 infective juveniles (IJs) also known as the dauer juveniles, are adapted to long term survival in the soil for extended periods of time. They are non-feeding with a non-functional alimentary canal; the lumen and digestive tract are greatly reduced while the mouth and anus are closed (Poinar, 1990). This stage survive without any exogenous source of energy for weeks or even months, and they depend exclusively on their accumulated energy reserves (Johnigk & Ehlers, 1999). IJs contain their bacterial symbiont in the intestinal tract. *Photorhabdus* mainly colonizes the anterior region of the intestine of *Heterorhabditis,* just posterior to the basal bulb, while *Xenorhabdus* colonizes a specialized bilobed intestinal vescicle in *Steinernema*.

Where conditions are otherwise favourable, the duration of survival is determined by the amount and rate of utilization of the reserves. In the soil IJs locate their hosts by means of two strategies, ambushing and cruising (Gaugler *et al*., 1989). Ambusher species, such as *S. carpocapsae*, tend to remain stationary at or near the soil surface and locate host insects by direct contact, while cruisers are highly mobile and able to move throughout the soil profile. They locate their host by sensing carbon dioxide or other volatiles released by the host. EPNs that use the cruising strategy include *Heterorhabditis bacteriophora* and *S. glaseri,* while *S. affine* and *S. feltiae* do a bit of both ambushing and cruising (Campbell *et al*., 2003).

IJs invade the insect host through the mouth, anus, spiracles, or through soft cuticular parts (Bedding & Molyneux, 1982; Poinar, 1990). Once in the host haemolymph, the nematodes encounter optimal conditions for reproduction. They respond to yet unknown food signals (Strauch & Ehlers, 1998), which induce the exit

from the developmentally arrested dauer juvenile stage. The IJs moults to initiate growth to the J4 stage; pharynx, digestive tract and excretory metabolism are activated. During this process, the J3 release the symbiont cells into the insect's haemocoel. The bacteria produce toxins and other metabolites (Burman, 1982), which contribute to overcome the insect's defence mechanisms and kill the insect within approximately 24 to 48 hours after nematode invasion (Dowds & Peters, 2002). The bacteria proliferate and produce suitable conditions for nematode reproduction. Feeding on the symbiont cells, they develop into adults and produce offspring. The first generation inside the host consists of both males and females for *Steinernema* spp. whereas the *Heterorhabditis* spp. develop into self-fertilizing hermaphrodites, with later generations producing two sexes (Kaya & Gaugler, 1993; Johnigk & Ehlers, 1999). As long as abundant nutrients are available, additional adult generations develop. When food resources in the host become scarce, the adults produce new IJs which retain the symbiotic bacteria in the intestine and leave the insect cadaver in a search for other hosts (Fig. 1.1).

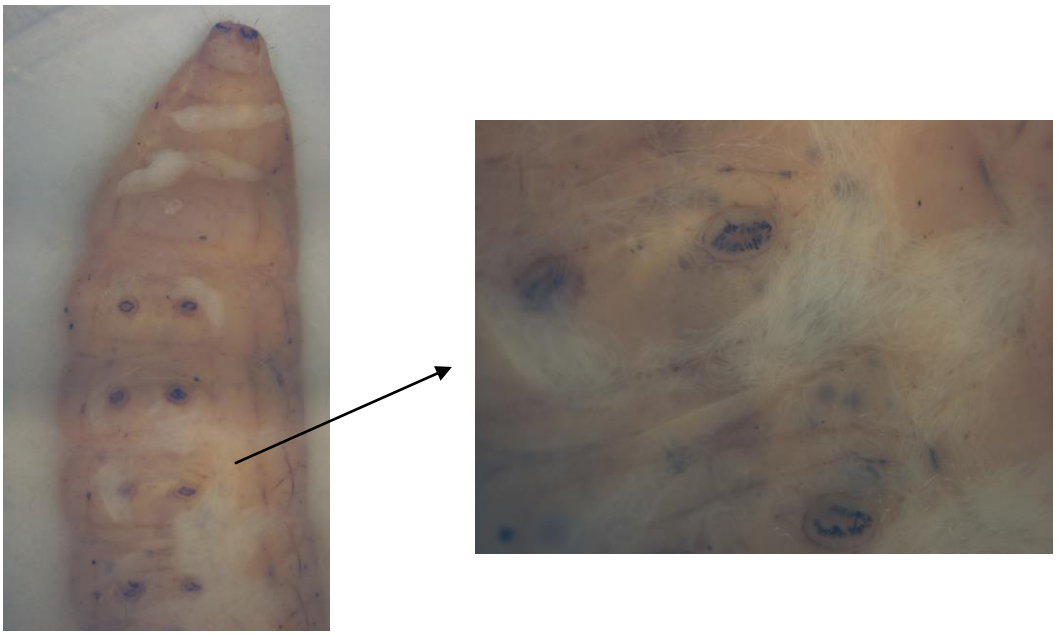

Fig. 1.1- Insective juveniles of entomopathogenic nematode *Steinernema carpocapsae* spilling out of a *Galleria mellonella* larva.

The life cycle (Fig. 1.2), from the entry of the IJs into a host, to their emergence from the host, depends on the temperature and amount of food available, and varies for different species and isolates. It generally takes approximately 6-18 days at temperatures ranging from 18-28 °C in *Galleria mellonella* L. (Lepidoptera: Pyralidae) (Poinar, 1990). The length of time that the IJs survive in the soil in the absence of an insect host is dependent on factors such as temperature, humidity, natural enemies and soil type.

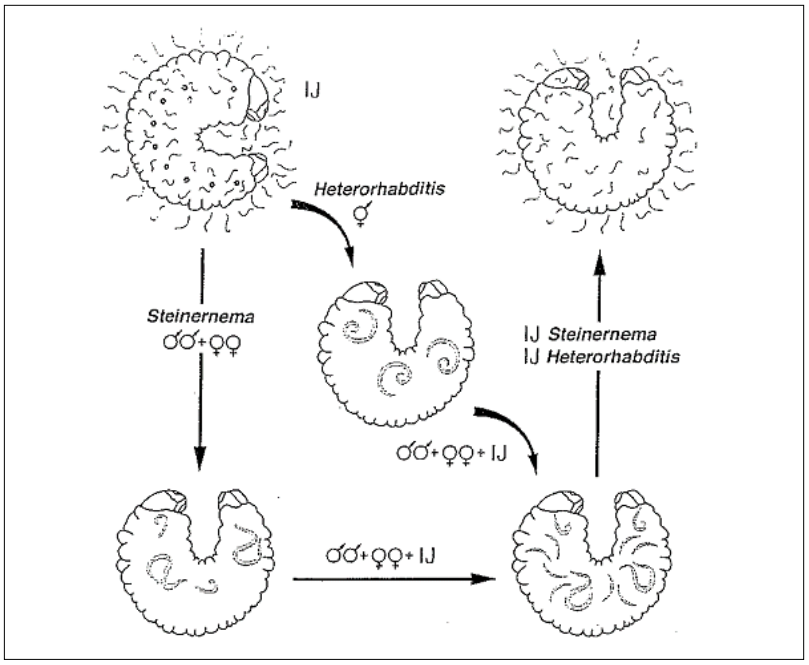

Fig. 1.2 - Life cycle of *Steinernema* and *Heterorhabditis.*
Taken from: Clausi *et al.*, 2014.

The nematodes and bacteria have a mutualistic relationship. Nematodes depend on the bacteria for (i) killing the insect host, (ii) creating a suitable environments for its development by producing antibiotics that suppress competing microorganisms, (iii) transforming the host tissues into food source, and (iv) serving as a food resource

(Boemare *et al*., 1997). On the other hand, the bacteria need the nematodes for (i) protection from the external environment, (ii) penetration into the host's haemocoel, and (iii) inhibition of the host's antibacterial proteins (Kaya & Gaugler, 1993).

1.1.3 EPNs and pests control

Entompathogenic nematodes possess many attributes of an optimal biological control, to protect vegetation in natural and agro- ecosystems from invasive insect pest species. The efficacy of EPNs in biological control depends on certain beneficial traits such as host-finding and virulence (Shapiro-Ilan *et al*., 2002). EPNs are soil-inhabiting organisms and can be used effectively to control soilborne insects pest, killing them within 24 to 48 hours of application (Gaugler & Kaya, 1990; Ehlers, 1996; Ramos-Rodriguez *et al.,* 2006). Furthermore, they can be applied with conventional pesticide equipment (Hayes *et al*., 1999) and they appear to be compatible with many chemical pesticides (Koppenhofer *et al*., 2000; Nishimatsu & Jackson, 1998; Rovesti & Deseö, 1990). In addition, they are environmentally safe and they have not effects to non-target organisms (Bathon, 1996; Gaugler & Kaya, 1990; Georgis *et al*., 1991; Kaya & Gaugler, 1993; Smart, 1995). EPNs and their associated bacteria cause no detrimental effect to mammals or to human beings (Akhurst & Smith, 2002).

A joint workshop supported by the EU COST Action 819 "Entomopathogenic Nematodes" and the OECD (Organisation for Economic Co-operation and Development) Research Programme "Biological Resource Management for Sustainable Agriculture Systems", which met in 1995 to discuss potential risks related with the use

of EPNs in biological control, concluded that EPNs are safe to production and application personnel and the consumers of agriculture products treated with EPNs (Ehlers & Hokkanen, 1996). The expert group could not identify any risk for the general public related to the use of EPNs.

Moreover, they also find their host either actively or passively (cruise foraging or ambush strategy). Their recycling inside the host insect, causes long-term, sustainable effects on the pest population, but due to their sensitivity to ultraviolet light and desiccation, EPNs are most effective against insects that occur in moist, dark locations. Many insect pests spend part of their life cycle in the soil, as a result; the potential to use EPNs is very promising.

The environmental has benefits of using these organisms, as no residues and groundwater contamination. Today some species of EPNs are mainly used against a variety of pests, mostly where chemical compounds fail, i.e. in the soil, in the galleries of boring insects or in cases where resistance to insecticides has developed (Ehlers, 2001).

1.1.4 Biodiversity of EPNs in Italy

Results of many surveys all over the word have revealed that EPNs are widespread and they have been recovered from soils of all continents except Antarctica (Griffin *et al*., 1990; Hominick, 2002). *Steinernema* and *Heterorhabditis* are ubiquitous in distribution and they have been found in a wide range of diverse soil habitats, such as cultivated fields, woodlands, grasslands, deserts and even ocean beaches.

In Italy, the first survey on the biodiversity of EPNs was carried out in 1988, in the Emilia-Romagna region (Deseö *et al*., 1988). Recently the diversity and distribution of EPNs in the families Steinernematidae and Heterorhabditidae were assessed throughout an extensive soil survey during the period 1990-2010 (Tarasco *et al.* 2014).

In order to sample ecologically different habitats, several samples were taken in natural and cultivated ecosystems, from 11 different habitats: uncultivated land, orchard, field, sea coast, pinewood, broadleaf wood, grassland, river and lake borders, cave, salt-pan borders and wetland.

A mapping of EPNs distribution in Italy showed 133 indigenous EPN strains belonging to 12 species: *Heterorhabditis bacteriophora*; *H. downesi* Stock, Griffin & Burnell, 2002; *H. megidis* Poinar, Jackson & Klein, 1987; *Steinernema feltiae* Filipjev, 1934; *S. affine* Bovien, 1937; *S. kraussei* Steiner, 1923; *S. apuliae* Triggiani, Mráček & Reid, 2004; *S. ichnusae* Tarasco, Mráček, Nguyen & Triggiani 2008; *S. carpocapsae*, *S. vulcanicum* Clausi, Longo, Rappazzo, Tarasco & Vinciguerra, 2011; *Steinernema* 'isolate S.sp.MY7' of '*S. intermedium* group' and *S. arenarium* Artyukhovsky, 1967.

Steinernematids are more widespread than heterorhabditids and the most common entomopathogenic nematode species isolated was *S. feltiae* followed by *H. bacteriophora.* Three of the species recently described – *S. apuliae*, *S. ichnusae* and *S. vulcanicum* – are known only from Italy and seem to be endemic.

The EPNs biodiversity data does not cover all geographical areas and habitats, so it is important to investigate in regions where the nematodes are unknown. Different species or strains of nematode may possess different biological, ecological, or physiological characteristics that affect the field efficacy of the nematode-based biopesticides, such as high host range, behaviour, and tolerances to high or low

temperature. Therefore, accumulation and correct identification of entomopathogenic nematode species are critical for success in using them as biopesticides for controlling insect pests (Qiu *et al*., 2004).

1.2 AIMS AND OBJECTIVES

Underlined the importance of EPNs as biological control agents, this project focuses on the possibility to discover indigenous nematodes isolated from different areas and evaluate their potential to replace synthetic chemical pesticides in field. In this investigation several techniques including soil baiting, White trap method, DNA isolation, Polymerase chain reactions and DNA sequencing, morphological studies were employed in the identification process of EPNs isolated from local soils collected in Italy regions.

The main focus of this project was increase the knowledge on the procedures for long-term storage at ultra-low temperature in order to develop cryopreservation protocols for each EPN species and to optimally cryopreserve nematodes and associated bacteria. The final objective is to create a genetic bank (EPN-CRYO-BANK) of these useful organisms, so as to prevent routine subculturing of EPNs, which is labour intensive and can lead to trait deterioration.

The specific objectives were:

- Isolate, identify and characterise indigenous EPNs from collected soil samples;
- Develop protocols for the long-term storage of *Steinernema carpocapsae* and others different EPNs;
- Evaluated efficacy and pathogenicity of *S. carpocapsae* using laboratory bioassays before and after cryopreservation.

This dissertation has been structured as follows: The previous sections of this Chapter 1, was a literature review covering some of the important aspects surrounding a background on EPNs, their history, the life cycle, the possibility to use these organisms in biological control programs and their biodiversity in Italy. The chapter concluded with the objectives of the study. Chapter 2 presents the techniques for the isolation and identification of EPNs used in this study. Chapter 3 presents the development of a cryopreservation protocols for *Steinernema carpocapsae* and others four EPN species. Chapter 4 describes a series of bioassays that were carried out to evaluate the virulence and efficacy of *S. carpocapsae* before and after cryopreservation, as well as to determine their stability and potential for mass production. Chapter 5 presents the conclusion. This project concluded with the Appendix A, where a new entomopathogenic nematode, isolated from a karst cave soil of Tuscany (Central Italy) was described as *Oscheius onirici* sp.n. and the Appendix B, where there is the article about *S. carpocapsae* ItS-CAO1.

Chapter 2

IDENTIFICATION AND CHARACTERISATION OF EPNs

2.1 INTRODUCTION

EPNs are found under diverse ecological conditions (Hominick, 2002; Kaya *et al*., 2006). Many species and strains of both genera *Steinernema* and *Heterorhabditis* have been isolated worldwide, and it has often proven that these nematodes possess many attributes of ideal biological control agent against economically important insect pests. One strain of a specific nematode species can be used to control various insect pests in agricultural industries, forestry and other fields where needed but no one species of EPNs is the best control agent for all or even most insect species. For this reason, it is preferable to examine different species rather than various strains of one or more species because species usually exhibit greater differences in infectivity.

Italy represents a fertile field for EPNs exploration because only some regions have been surveyed (Tarasco & Triggiani, 1997; Tarasco *et al.*, 2009; Tarasco *et al.*, 2014). In contrast to human modified areas, natural habitats are likely uncontaminated by introduced nematodes and offer increased opportunities for finding native nematode species (Stock *et al*., 1999). In this respect, the isolation of native species of EPNs provides a valuable source not only from a biodiversity perspective but this contributes to the expansion of the use of native isolates in future biological control programs in these areas (Stock *et al.*, 1999).

At present, mainly non-native strains have been used in nematode applications, but these strains may not be well adapted to environmental conditions, hence, their efficacy might be reduced (Campos-Herrera & Gutierrez, 2009), whereas indigenous nematodes are adapted to local climatic conditions and have therefore more likely to survive after application. The effectiveness of EPNs typically depends on a combination of nematode foraging strategy, insect host species, host location, soil conditions (soil type, pH, soil moisture, etc.), climate (Glazer & Lewis, 2000) and application methods (Shapiro-Ilan *et al*., 2006) Therefore, ecological studies are recommended to develop hypotheses about the abilities and limitations of new EPN species (Shapiro-Ilan *et al.*, 2006).

During different surveys soil samples were collected to determine the occurrence and distribution of EPNs in Italian regions. The surveys resulted in the recovery of more than 10 isolates of insect-parasitic nematodes, including *Oscheius onirici* sp. n. (Appendix A), a new entompathogenic nematode, isolated from a karst cave in Tuscany region (Central Italy) and a new strain *Steinernema carpocapsae* (Appendix B), the isolate described in the current chapter. Others EPNs are under study for morphological and molecular identification.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of EPNs from soil

A survey was conducted on September 2013 in a site located at farm Vallevecchia of Veneto Agricoltura (45°37'51.21"N-12°58'10.29"E), in Caorle (Venezia) in lagoon plain at 1 m s.l. The mean annual air temperature of the site is 13.5 °C with average maximum temperatures in July-August (28.4 °C) and average minimum temperatures in January (-0.8 °C). The mean annual precipitation is 806 mm concentrated in October (109 mm). The soil texture was classified as silt-loam with 19.5% (standard error 0.38) of sand, 63.4% (0.19) of silt and 17.1% (0.23) of clay.

Until 2008, a rotation based on maize and sorghum was applied. Three different set-aside regimes were established in 2008. The total area of three plots was 1.5 ha (0.5 ha per plot) with ecotonal zone on one side for each. The fields of the three set-aside managements were covered by mowing on May, on July or none, respectively.

In each plot, 6 scores were randomly sampled and then mixed to form one composite sample of approximately 2 Kg each. The soil was transported in sterile polythene bags to the laboratory and 3 *G. mellonella* larvae were inserted in a long-handled tea infuser in the middle of each sample (Tarasco & Triggiani, 1997).

Then the samples were incubated at 25 °C for 7 days. Observations were done daily to monitor the dead larvae. Then died, *Galleria* larvae were placed individually in modified White traps to allow the emergence of nematodes (Kaya & Stock, 1997). The modified White trap consists of two Petri dishes of different sizes. A small plastic watch dish was inverted and covered with a moist filter paper and then placed within a Petri

dish of 90 mm diameter. The outer Petri dish was filled with sterile distilled water up to 2 mm and each dead insect larva was placed on top of the moist filter paper (Fig. 2.1).

White traps were then placed at 24 °C. When IJs emerged from the cadavers, they were collected from water and established in a laboratory culture in 50 ml plastic tubes. Some of them were used for molecular characterization and some IJs were used to infect fresh *G. mellonella* larvae to produce nematodes (IJs and males) used for morphological identification.

Fig. 2.1 – Illustration of modified White traps used to collect IJs from one EPNs infected *Galleria mellonella* cadaver.

2.2.2 Morphological identification of EPNs

For morphological studies, 5 *G. mellonella* larvae were placed singly in a Petri dish (3.5 cm diam.) with two layers of filter paper (Whatman No. 1) and a distilled water suspension of 250 µl containing about 400 IJs was inoculated into each Petri dish.

The first generation males and females were obtained by dissecting infected insects in Ringer's solution 2-4 days after the *Galleria* died. Second-generation adults were observed after 5-7 days. Third-stage IJs were obtained when they emerge from the cadavers after 7-10 days. The observation of the specimens has been performed within a week after collection.

For light microscope observation, temporary mounts of these specimens were made by heat killing nematodes on glass slides in a drop of water, and then a cover glass was applied for observation. Permanent mounts of holotype, paratype IJs and adults were made, so they could be used further for more observation. Nematodes were heat-killed in warm water at 60 °C and fixed in triethanol-amine-formalin (TAF) (Courtney *et al*., 1955), processed to glycerin by a modification of a glycerin-ethanol series of Seinhorst's (1959) rapid method and then permanently mounted in anhydrous glycerin on microscope slides.

Specimens were examined and measured with a Leitz ORTHOPLAN light microscope at up to 1000 magnification and photographed using CANON Power Shot S50. IJs and males were identified using morphological and morphometric characters based on Nguyen & Smart (1996), Nguyen & Hunt (2007) and many articles.

2.2.3 Molecular identification

Specimens for molecular analysis were collected from *Galleria* larvae and directly processed. Genomic DNA was extracted from fifteen individual nematodes as

described by De Luca *et al.* (2004). The crude DNA isolated from each individual nematode was directly amplified by using the forward primer TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and the reverse primer AB28 (5'-ATATGCTTAAGTTCAGCGGGT -3') (Joyce *et al*., 1994) for the ITS region, and by using the 18SnF (5'-TGGATAACTGTGGTAATTCTAGAGC-3') and 18SnR (5'-TTACGACTTTTGCCCGGTTC-3') (Zeng *et al*. 2007) for the 18S rRNA gene. PCR cycling conditions used for amplification were:

- Initial denaturation at 94°C for 5 min,
- 35 cycles of denaturation at 94°C for 50s,
- Annealing at 55°C for 50s,
- Extension at 72°C for 1 min,
- Final step at 72°C for 7 min

The size of the amplification products was determined by comparison with the molecular weight marker ladder 100 (Fermentas, St. Leon-Rot, Germany) following electrophoresis of 10 µl on a 1% agarose gel.

PCR products of the ITS containing region and the 18S rRNA gene from three individual nematodes for each marker were purified for sequencing using the protocol listed by manufacturer (High Pure PCR elution kit, Roche, Germany). Purified DNA fragments were cloned and sequenced in both directions.

A BLAST (Basic Local Alignment Search Tool) search at NCBI (National Center for Biotechnology Information) was performed in order to confirm their nematode origins and species (Altschul *et al*., 1997). The newly obtained sequences for

the ITS containing region and the 18S rRNA gene, were aligned using ClustalW (Larkin *et al*., 2007) with default parameters with corresponding published gene sequences of *Steinernema.* Sequence alignments were manually edited using BioEdit. Outgroup taxa for each dataset were chosen according to the results of previously published data. Phylogenetic trees, obtained for both ITS and 18S datasets, were constructed with Neighbour-Joining (NJ), Minimum Evolution (ME) and Maximum Parsimony (MP) methods using MEGA version 6 software (Tamura *et al*., 2013). The phylograms were bootstrapped 1,000 times to assess the degree of support for the phylogenetic branching indicated by the optimal tree for each method.

The newly obtained sequences were submitted to GenBank with the following accession numbers LN624756-LN624757 for the 18S rRNA gene, LN624758-LN624759 for the ITS region.

For Restriction Fragment Length Polymorphism (RFLP) analysis, ten µl of each product, containing the ITS region, from two individual nematodes from *S. carpocapsae* isolate were digested with the following restriction enzymes: *Alu* I (Roche), *Ava* II (Roche), *Bam* HI (Roche), *Dde* I (Roche), *Hinf* I (Roche), and *Rsa* I (Roche) (5 U of enzyme for each digestion) at 37 °C overnight.

The digested DNA fragments were loaded onto 2.5% agarose gel and visualized by red staining gel according to the manufacturer.

2.3 RESULTS

2.3.1 Isolation of EPNs from soil

Only from the plot in set-aside no mowing regime (Fig. 2.2) was isolated a new strain of *S. carpocapsae*, named ItS-CAO1.

Fig. 2.2 – Area of the farm Vallevecchia where *Steinernema carpocapsae* ItS-CAO1 was isolated

2.3.2 Morphological identification

Morphometric data of first generation males and infective juveniles are in table 1.

Males (first generation): Body C-shaped when heat-relaxed. Cuticle smooth. Stoma shallow (Fig. 2.3D). Pharynx with cylindrical procorpus, slightly enlarged metacarpus, followed by an isthmus and a basal bulb with valve (Fig. 2.3E). Nerve ring surrounding isthmus just anterior to basal bulb. Excretory pore anterior to

nerve ring. Reproductive system monorchic, testis reflexed. Spicules paired, symmetrical, slightly curved (Fig. 2.3G). Gubernaculum length *ca* 65% of spicule, boat-shaped in lateral view. Tail short, tip with a mucron and *ca* 70% of anal body diam. long (Fig. 2.3F). Bursa absent.

Females (first generation): Variable in size (n=10, L: 6493.7 ± 971). Body in form of spiral on heat-relaxation. Anterior region similar to male. Reproductive system amphidelphic, reflexed. Vulva in form of transverse slit (Fig. 2.3H). Tail short and conical

Infective juveniles: Body slender, when newly formed, always enclosed in cuticle of second-stage juvenile as a sheath (Fig. 2.3A). Pharynx long and narrow, often degenerate, with valvate basal bulb. Excretory pore and nerve ring localized at *ca* 31 and 68% of pharyngeal length respectively (Fig. 2.3B). Tail elongate and pointed (Fig. 2.3C). Lateral fields distinct with six ridges

Males and *females* (second generation): Second-generation male and female similar to the first generation except body length shorter and body width less.

Table 2.1 – Morphometrics of Infective juveniles and first generation males of *Steinernema carpocapsae* (ItS-CAO1). All measurements are in µm: mean ± s.d. (range).

Character	Male	Infective Juveniles
n	20	20
L	1608.96 ± 143.3 (1339.5-1845.1)	559.0 ± 23.8 (505.8-597.0)
a	13.8 ± 1.1 (11.3-15.5)	21.3 ± 2.0 18.7-27.1)
b	11.4 ± 1.0 (9.1-13.9)	4.7 ± 0.4 (4.2-5.5)
c	49.9 ± 4.4 (42.1-59.0)	10.7 ± 0.4 (10.2-11.5)
Max. body diam.	117.2 ± 12.5 (102.3-156.5)	26.4 ± 2.6 (20.1-30.4)
Pharynx	141.9 ± 5.8 (130.9-151.1)	118.5 ± 9.0 (99.7-139.0)
Excretory pore	68.0 ± 5.5 (60.2-78.2)	36.2 ± 3.4 (28.2-40.3)
Nerve ring	101.0 ± 6.2 (93.3-114.4)	81.3 ± 5.5 (70.0-90.0)
Tail length	32.3 ± 2.0 (29.5-36.1)	52.5 ± 2.7 (48.8-57.0)
Anal body diam.	47.1 ± 3.0 (41.8-54.2)	13.4 ± 0.5 (12.7-14.2)
Testis reflex	525.9 ± 59.3 (396.0-624.0)	–
Spicule length	69.4 ± 4.3 (54.8-75.2)	–
Spicule width	13.8 ± 1.3 (11.4-15.1)	–
Gubernaculum length	45.4 ± 1.8 (42.1-48.2)	–
SW	1.5 ± 0.1 (1.3-1.6)	–
GS%	65.7 ± 5.3 (58.3-79.6)	–
D%	48.0 ± 4.2 (41.7-55.1)	30.7 ± 3.7 (23.9-40.1)
E%	211.6 ± 24.1 (166.8-265.1)	69.1 ± 6.4 (53.6-79.0)

[a] L = body length, W = max. body diam, ES = pharynx, EP = excretory pore, NR = nerve ring, T = tail length, ABD = anal body diam., SL = spicule length, GL = gubernaculum length. Ratios: a = L/W, b = L/ES, c = L/T, SW = SL/ABD, GS% = GL/SL x 100, D% = EP/ES x 100, E% = EP/T x 100

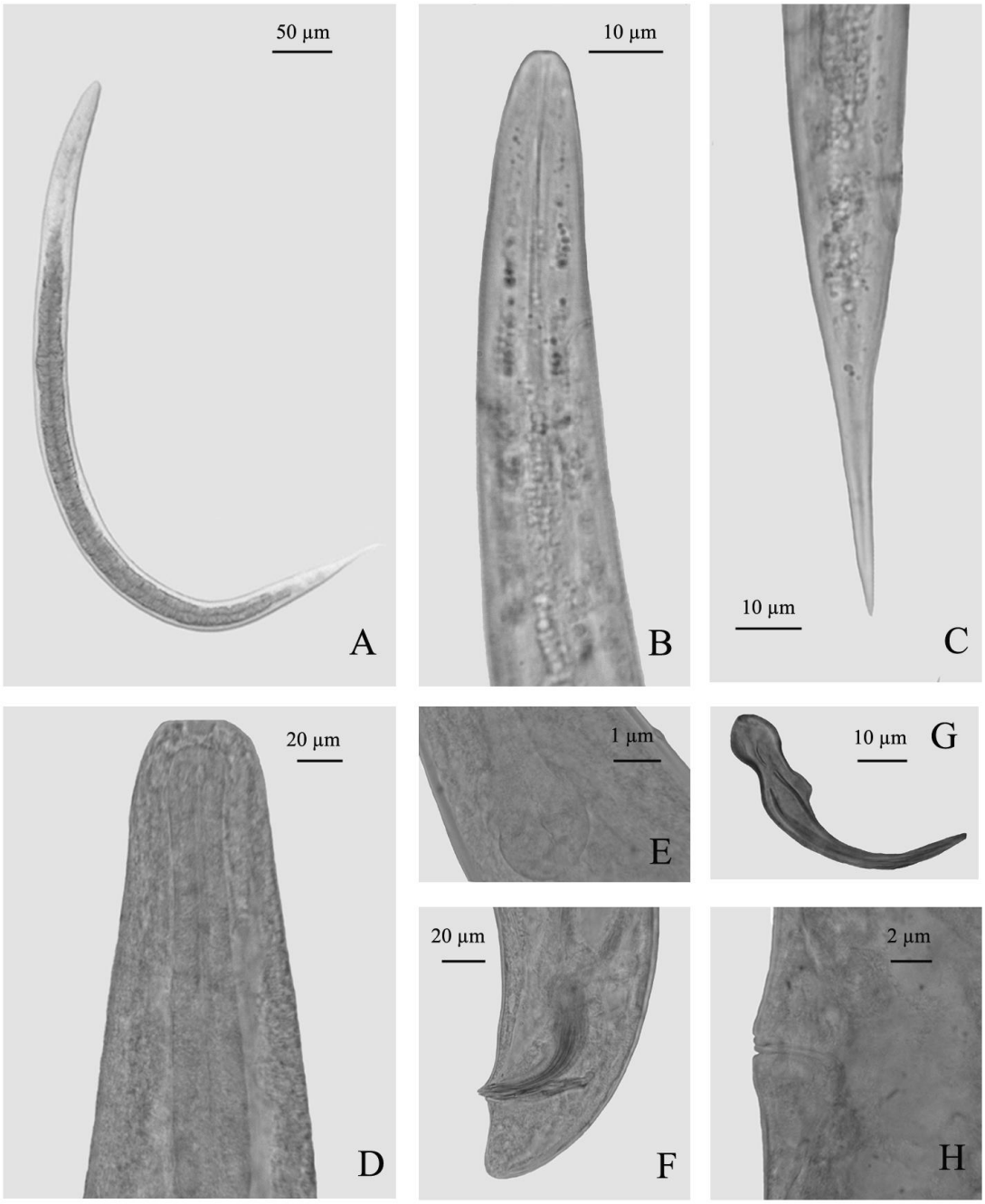

Fig. 2.3 - Light micrographs of *Steinernema carpocapsae* ItS-CAO1. A-C: Infective juveniles; A: Entire body, B: Pharyngeal region, C: Tail region. D-G: Male (first generation); D: Head region, E: Basal bulb, F: Tail region, lateral view, G: Spicule, lateral view. H: Female (first generation), vulva.

2.3.3 Molecular identification

Amplification of the ITS region in *S. carpocapsae* from Italy produced a fragment of approximately 0.8 kb in length for all specimens. A restriction profile of the ITS region from the Italian isolate of *S. carpocapsae* is shown in Fig. 2.4.

The restriction patterns with *Alu* I, *Dde* I , *Hinf* I and *Rsa* I enzymes resulted the same of those reported by Reid *et al*. (1997) and Susurluk & Toprak (2006). *Alu* I enzyme yielded two polymorphic bands that were not present in the restriction profiles of other *S. carpocapsae* isolates identifying specifically the Italian isolate of *S. carpocapsae* (Hominick *et al*., 1997; Nguyen, 2003).

The sequences of the entire ITS of *S. carpocapsae* were determined for 2 specimens for the Italian population. No intraspecific differences were found in the sequences of these specimens. The ITS sequences of *S. carpocapsae* from Italy showed an intra-specific variability with the corresponding sequences of *S. carpocapsae* present in the database ranging from 0 to 2% (5-16 bp different).

The sequencing of the 18S rRNA gene produced a sequence of 1633 bp. The partial 18S sequence was 99% identical to *S. carpocapsae* present in the database, 1627/1630 identities and 0 gap and 99% similar to *S. monticolum* Stock, Choo & Kaya, 1997 (FJ040423), 1585/1589 identities and 1 gap.

For phylogenetic analysis of the ITS region, the corresponding sequences of *S. carpocapsae*, *S. feltiae*, *S. surkhetense* Khatri-Chhetri, Waeyenberge, Spiridonov, Manandhar & Moens, 2011, *S. nepalense* Khatri-Chhetri, Waeyenberge, Spiridonov, Manandhar & Moens, 2011, *S. tami* Luc, Nguyen, Reid & Spiridonov, 2000, *S. siamkayai* Stock, Somsook & Reid, 1998, *S. minutum* Maneesakorn, Grewal &

Chandrapatya, 2010 and *Steinernema* sp. from the database were aligned along with the ITS sequences obtained in this study.

Phylogenetic trees generated by Neighbour-Joining, Maximum Parsimony and Minimum Evolution methods showed no significant conflict in branching order and support level, so only ML tree is shown (Fig. 2.5). ML tree supported five clades within *Steinernema*. The Italian isolate of *S. carpocapsae* formed a well supported clade (90% support) with all sequences of *S. carpocapsae*, despite the high sequence variability as reported by Spiridonov *et al.* (2004). The *S. carpocapsae* cluster resulted closely related to *S. surketense*, *S. nepalense* and *Steinernema* sp. (clade II). Clade III included only one taxon, *S. scapterisci* Nguyen & Smart, 1990. Clade IV grouped *S. tami*, *S. siamkayai* and *S. minutum*. Clade V contained only one taxon, *S. feltiae* from the database, located at the basal position of the tree. Most clades were supported by high bootstrap values giving strong support to these associations.

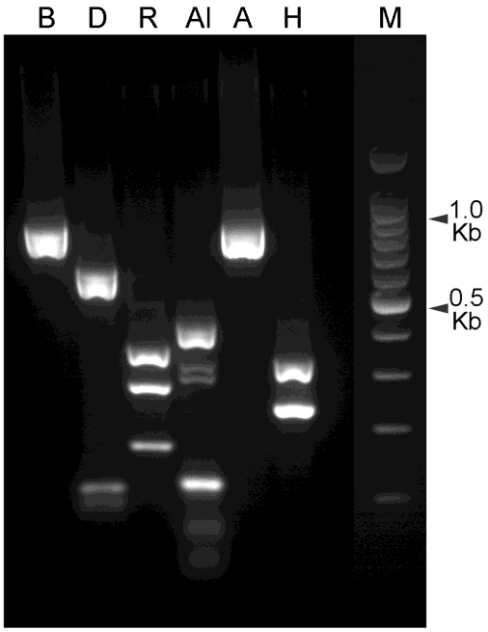

Fig. 2.4 - Restriction fragments of amplified ITS containing region of *Steinernema carpocapsae*. AI: *Alu*I, A: *Ava* II, B: *Bam* HI, D: *Dde*I, H: *Hinf* I, R: *Rsa* I and M:100 bp ladder.

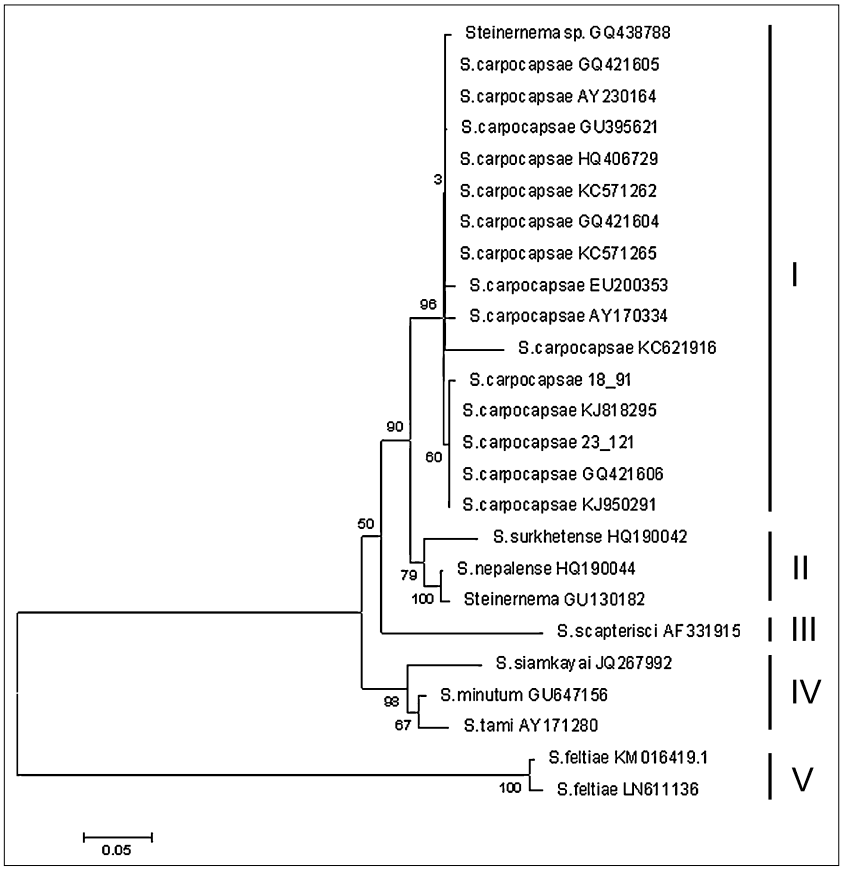

Fig. 2.5 - Phylogenetic trees of ITS containing region of *Steinernema carpocapsae* and the closest species. Sequences were analysed using Maximum Likelihood method. Numbers at nodes indicate bootstrap values.

2.4 DISCUSSION

Steinernema carpocapsae was previously isolated from soil sample in Veneto region (Ehlers *et al*., 1991) but it was not morphologically or molecularly characterized. Morphological and molecular identification of *Steinernema* species is a prerequisite for proper classification, biodiversity study and their potential use in biological control programs.

DNA sequence and phylogenetic analyses of entomopathogenic nematodes provide additional criteria for identifying and delimiting species within *Steinernema*. Sequence analyses of the ITS and the 18S rRNA gene well supported the identification

of *S. carpocapsae* isolate from Italy. In particular, sequence analyses of the ITS containing region has allowed to assess the heterogeneity among isolates of *S. carpocapsae*, and among *Steinernema* species, even for those that are closely related (Hominick *et al.*, 1997). This result was also confirmed by RFLP profiles that were in accordance with the patterns reported previously for *S. carpocapsae* (Spiridonov *et al*., 2004; Susurluk & Toprak, 2006). The alignment of the optimized ITS sequences from different species of *Steinernema* spp. revealed multiple insertion and deletion events (indels) among taxa of varying size (1-20 bp) and the longest indels were localized in the ITS1 (data not shown). Furthermore, the multiple alignment constructed revealed several conserved sequence motifs characteristic for each *Steinernema* species.

The phylogenetic analyses with ML, NJ and MP methods yielded congruent phylogenetic trees. Notably, one highly supported group was evident in both analyses, consisting of all *S. carpocapsae* sequences closely related to *S. surkhetense, S. nepalense, Steinernema* sp. and *S. scapterisci*.

In conclusion, the ITS sequences allowed clear separation of *Steinernema* species in spite of the high intra-specific variability. The alignment revealed small species-specific DNA sequences suitable for the construction of potentially useful species-specific primers or for a more promising approach for DNA barcoding of entomopathogenic nematodes.

Chapter 3

CRYOPRESERVATION OF ENTOMOPATHOGENIC NEMATODES

3.1 INTRODUCTION

3.1.1 Long-term storage by cryopreservation

Entomopathogenic nematodes are used for the biological control of a wide spectrum of insect pests and interest in these nematodes as biocontrol agents is growing. Large-scale application of these nematodes requires multiple generations to be reared in mass quantitates and stored for variable periods of time. Routine subculturing of EPNs is labour intensive and can lead to trait deterioration, due to genetic factors (e.g. inbreeding, drift, inadvertent selection) or non-genetic factors (e.g. disease or nutrition) and a decrease in pest control abilities (Shapiro *et al*., 1996; Wang & Grewal, 2002).

Trait deterioration has been observed during laboratory culturing of EPNs; relatively rapid loss of various traits was reported: virulence, environmental tolerance, reproductive capacity and host findings (Shapiro *et al*., 1996; Bai *et al*., 2005; Bilgrami *et al*., 2006). One approach to minimize loss of traits of nematodes is to maintain genetic diversity by storing isolates at low temperature and rear them only when necessary.

Cryopreservation can be considered a method for long-term preservation of cells, tissues or even whole organisms at ultra-low temperature such as -196 °C (the

boiling point of liquid nitrogen), since under these conditions, biochemical and most physical process are completely arrested. Cells must survive freezing and thawing in the successful cryopreservation method. It is possible only if intra-cellular ice crystal formation is avoided, since this causes irreversible damage to cell membranes thus destroying their semi-permeability (Mazur, 2004; Panis & Lambardi, 2005). The method in which not only cells but also the whole solution is solidified is vitrification. It is a physical process of transition of an aqueous solution from liquid into an amorphous glassy solid, at the glass transition temperature, while avoiding crystallization. This technique relies on treatment of the samples with a concentrated vitrification solution (cryoprotectant) for variable periods of time, followed by a direct plunge into liquid nitrogen (Panis & Lambardi, 2005). Cryoprotectants help to minimise intracellular and intercellular crystal formation, and to increase the glass formation of the samples being frozen. Glycerol and Dimethyl sulfoxide (DMSO) are the most commonly used cryoprotective agents (Panis & Lambardi, 2005). The general properties of cryoprotectants are that have low molecular weight, are non-toxic and cheap. They also need to provide high levels of cell viability post thaw and have no other influence on the cells.

Finally, after storage at ultra-low temperature, an appropriate thawing rate has to be used, because crystallization can easily occur also in this step, if the thawing is slow (Nugent *et al.*, 1996). Other factors affect the survival of nematodes following cryopreservation, such as the age and strength of the nematodes prior to freezing; the cryoprotectant concentration; the exposure time of nematodes in the cryoprotectant and the nematode concentration before and after freezing (Bai *et al*., 2004; Bridge & Ham 1985; Curran *et al.*, 1992; Nugent *et al.*, 1996).

The growing interest in nematodes as biological control agents has made it necessary to develop a successful technique for their long-term storage. Cryopreservation has many benefits over the normal maintenance of cultures, because it is less expensive, as it does not require the same amount of labour and space, or the maintenance of controlled environmental conditions (Bridge & Ham, 1985). Cryopreservation offers the possibility of preserving such isolates free from contamination by other strains, changes in genotype resulting from genetic drift and from adaptive selection in response to laboratory culturing (Nugent *et al*., 1996). Nonetheless, few EPNs are storage in liquid nitrogen, because different species vary in their adaptability cryogenic storage and the protocols are established empirically case by case (James, 2004; Nugent *et al*., 1996).

Prior studies have demonstrated that cryoprotectant concentrations, incubation time and the speed of thawing can be altered to optimize the survival of different species and isolates of nematodes following cryopreservation (Curran *et al*., 1992; Nugent *et al*., 1996).

3.1.2. Previous works about nematodes cryopreservation

Successful cryopreservation has been reported for some EPNs. Smith *et al.* (1990) conducted an extensive series of test on cryoprotectant toxicity and freezing regimes for *Steinernema feltiae* first stage and IJs. Only IJs appeared to have been successfully cryopreserved, however no long term data is given. Popiel and Vasquez (1991) reported a protocol for the cryopreservation of *S. carpocapsae* and

Heterorhabditis bacteriophora, using a two-stage procedure of IJs incubation in a glycerol solution (22% and 14% respectively) for 24 hours followed by incubation in 70% methanol at 0 ºC to 1 ºC for 10 min before immersion in liquid nitrogen. In 1992, Curran *et al.* modified this protocol and obtained a mean survival rate of 69% for isolates of *Steinernema* spp. and 68% for isolates of *Heterorhabditis* spp. They noted that the speed of thawing was critical, and that even slight delays in thawing resulted in a significant drop in the survival rate.

A study by Nugent *et al.* (1996) showed that while *S. carpocapsae* could be successfully cryopreserved in 0.5 ml volumes of 70% methanol, post-cryopreservation survival was considerably higher for *H. bacteriophora* when the pre-treated IJs were frozen on filter paper strips rather than in liquid suspension. Bai *et al.* (2004) demonstrated in *S. carpocapsae* and *H. bacteriophora* that the cryo-survival rates of IJs are positively correlated with worm concentration during the cryoprotectant glycerol incubation step. The survival rates ranged from about 20% to 100%, which was proportional to worm concentration of 120-12,000 per ml. IJs indeed produce cryoprotecting molecules such as trehalose and glyce in response to thermal and other environmental stresses (Jagdale & Grew, 2003). Production of natural cryoprotectants by the animal itself could, therefore, be the key to good animal survival in this cryopreservation procedure.

Cosi *et al.* (2008) tested the efficacy of two different procedures to cryopreserve *S. carpocapsae*, using in the first treatment the protocol applied to several *Bursaphelenchus* species (Irdani *et al.*, 2006), and in the second treatment a modification of Bai's protocol (Bai *et al*., 2004).

Cryopreservation has been demonstrated as a method for long-term storage not only for EPNs but also with free-living nematodes (i.e. Masakazu *et al.*, 2013), animal- (Jaekson-Gegan & James, 1988; Van Wyk & Gerber, 2000) and plant-parasitic nematodes.

In 1970 Hwang described the cyopreservation of *Aphelenchoides sacchari* Hooper, 1958, using DMSO as a cryoprotectant. The same cryoprotectant was not as successful with juveniles of the plant-parasitic nematode *Ditylenchus dipsaci* Filipjev, 1857 (Sayre & Hwang, 1975). In 1985 Bridge & Ham cryopreserved J2 of the root-knot nematode, *Meloidogyne graminicola* Golden & Birchfield, 1965 and their results clearly demonstrated that the exposure time of nematodes to the cryoprotectant is crucial. Triantaphyllou & McCabe (1989) observed that J2 of several *Meloidogyne* and *Heterodera* species start moving within one hour of thawing and can be used immediately as inoculum.

Riga & Webster (1991) discovered that isolates of the pinewood nematode, *Bursaphelenchus* spp. can be best cryopreserved in 15% glycerol respect other cryoprotectant and that after thawing they reproduced rapidly over several generations onto *Botrytis cinerea* (Pers., 1794). Results of a Galway & Curran (1995) study showed that better survival of *Pratylenchus* spp. was obtained when fresh nematodes were used and that juveniles survived cryopreservation better than adults.

In 1996 a procedure for long-term preservation in liquid nitrogen of *M. hapla* Chitwood, 1949 and *M. chitwoodi* (Golden, O'Bannon, Santo, and Finley) was described (Van der Beek *et al.*, 1996). Juveniles after freezing were able to reproduce on plants, but infectivity was significantly lower than of non-frozen juveniles. Carneiro *et al.* (2001) discovered that, although the number of *M. javanica* (Treub, 1885)

Chitwood, 1949 larvae is reduced post cryopreservation, those collected after thawing infected tomato roots and produced a large number of eggs.

Irdani *et al.* (2006) describe a cryopreservation method for several species of *Bursaphelenchus* employing a high concentration of ethylene glycol (EG). In 2007, *Radopholus similes* (Cobb, 1893) Thorne, 1949, a plant-parasitic nematode from the humid tropics was cryopreserved, using a two-step treatment consisting of an incubation in glycerol followed by the application of a vitrifying mixture of methanol, glycerol and glucose (Elsen *et al.*, 2007). Recently Irdani *et al.* (2011) developed protocols for successfully cryopreserving the plant parasitic nematodes *Globodera tabacum tabacum* Lownsbery et Lownsbery, Skarbilovich, *Heterodera schachtii* Schmidt and *M. incognita* Kofoid et White, Chitwood, based on low, non-toxic concentrations of cryoprotectants combined with a rapid cooling rate in the liquid nitrogen phase.

3.2 MATERIALS AND METHODS

3.2.1 Source and maintenance of nematodes

Nematodes used in the current study were obtained from field survey (*Steinernema carpocapsae* ItS-CAO1) and from the collection of the Department of Soil, Plant and Food Sciences, Section of Entomology and Zoology (DISSPA), - University of Bari (*S. affine* ItS-FO1, *S. apuliae* ItS-CS3, *S. feltiae* ItS-OT15 and *Heterorhabditis bacteriophora* ItH-LU1). They were cultured in laboratory, *in vivo* in last instar larvae of the wax moth larvae *G. mellonella*. IJs were harvested daily for one

week after their emergence from modified White traps. Nematode suspension was cleaned from dead IJs and *Galleria* tissues, by sedimentation on the bottom of the container and washing in sterile distilled water for several times. They were stored horizontally in 50 ml plastic tubes at 12 ºC.

3.2.2 Adjusting nematode concentrations

A defined number of nematodes was needed for bioassays. Counting large numbers of nematodes is impractical, so serial dilutions were used. The nematode suspension was shaken well with an agitator. A 50 µl aliquot was withdrawn with a micropipette and transferred to a nematode counting dish. Ten millilitres of water were then added. The EPNs in the dish were counted under dissecting microscope. Nematode concentration per millilitre was calculated by multiply the number of EPNs in 50 µl by 15. For a better precision three 50 µl aliquot were taken and the results was the average number.

As such, to adjust to any concentration, the following formula (Glazer & Lewis, 2000) was used:

$$[(i/c) - 1] \times V = Va \quad (1)$$

Where i = initial concentration 50 μl^{-1}, c = final concentration 50 μl^{-1}, V = volume of the suspension (ml), and Va = the amount of water (ml) to be added if (positive) or removed (if negative) from the suspension. If a higher concentration was needed, the nematodes

were allowed to settle to the bottom of the container for at least 30 min, after which excess water (determined by the above formula) was removed. The final concentration was checked by repeating the initial counting. The final count was usually within ± 10% of the needed concentration.

3.2.3 Cryopreservation protocol

About 12,000 nematodes/ml were suspended in Petri dishes (6 cm diameter) containing glycerol solution as cryoprotectant at 24 ±1 °C. After incubation, IJs were filtered (Whatman filter paper No.1) through a vacuum filtration system. The nematodes on the filter paper were dipped into 15 ml 70% methanol (pre-chilled at -10 °C) for 10 min on the ice. Following the incubation, the cold nematode suspension was filtered onto chromatography paper (nylon membranes, Roche), which was rolled and placed in pre-chilled cryogenic vials and immediately plunged in liquid nitrogen at -196 °C for several minutes before being transferred to a mechanical freezer at -140 °C.

Thawing was carried out transferring the nylon membrane from the vials to plastic tubes containing 20 ml of Ringer's solution (8 g NaCl, 0.2 g $CaCl_2$, 0.2 g KCl and $NaHCO_3$ per litre of distilled water) at 24 °C. Removal from mechanical freezer and thawing of the nematodes was completed as rapidly as possible (within 10 seconds).

The tubes were horizontally incubated at 24 °C for 4 hours. After this period, survival was measured by determining the percentage of motile nematodes (average of three counts of 1 ml of total suspension). This protocol was used for every experiment.

3.2.4 Tuning of a cryopreservation protocol for *Steinernema carpocapsae*

In order to achieve a successful cryopreservation protocol for *S. carpocapsae*, a sequence of experiments was performed.

3.2.4.1 Experiment 1 – Effect of storage days before cryopreservation

An initial experiment was performed to determine an optimum time for cryopreserve EPNs after the emergence of IJs from *G. mellonella* larvae. IJs were stored at 12 °C for different days (1 – 7 – 15 – 30 – 90) and then cryopreserved. In this experiment IJs were incubated in 18% (v/v) glycerol solution for 24 h before freezing treatment. Thawing was carried out after 24 h in mechanical freezer at -140 °C.

3.2.4.2 Experiment 2 – Different incubation periods in cryoprotectant

This experiment was performed to check if nematode survival was influenced by increasing the length of the incubation period in cryoprotectant before their immersion in liquid nitrogen. In this experiment IJs, which were stored 15 days at 12 °C, were incubated in 18% (v/v) glycerol solution for 24 h and 48 h. Thawing was carried out after 24 h.

3.2.4.3 Experiment 3 – Different periods of preservation in mechanical freezer

This experiment was performed to evaluate the effect of long term storage at -140 °C on nematode survival. IJs, which were stored 15 days at 12 °C, were incubated in 18% (v/v) glycerol solution for 48 h and then cryopreserved. Thawing was carried out after 1, 7, 15, 30 days to verify the survival rate.

3.2.4.4 Virulence assay

An additional assay was conducted to determine if the level of IJs virulence was retained in the cryopreservation process. IJs surviving at cryopreservation and thawed after 24 h and 30 days in mechanical freezer was compared with IJs originating from the same culture that had been kept at 12 ºC (Control).

A Petri dish (3.5 cm diam.) with two layers of filter paper (Whatman No. 1) containing one last instar larva of *G. mellonella* was used as experimental unit. A distilled water suspension of 250 µl containing about 200 IJs was inoculated into each Petri dish. Petri dishes were stored at 24 °C in darkness (Fig. 3.1). Mortality of larvae was evaluated every 24 h for a week. Fifteen larvae/host insect were used for each assay. Dead larvae were transferred to a modified White traps. The time to emergence, i.e., number of days after infection to the moment when IJs started to emerge from the infested host cadaver, was determined by checking all the modified White traps daily until the first IJs started to come out from the cadaver.

Fig. 3.1 – Virulence assay set up in Petri dishes. A: Control, B: Cryopreserved nematodes

3.2.4.5 Reproductive potential after cryopreservation

Reproductive potential after cryopreservation was determined for *S. carpocapsae* after the "Experiment 1" described above. IJs stored at 12 °C for different periods (1 – 7 – 15 – 30 – 90 days) and thawed after cryopreservation was used for this assay. They were compared with IJs originating from the same culture that had been kept at 12 ºC (control).

A Petri dish (3.5 cm diam.) with two layers of filter paper (Whatman No. 1) containing one last instar larva of *G. mellonella* was used as experimental unit. A distilled water suspension of 250 µl containing about 200 IJs was inoculated into every Petri dishes. They were stored at 24 °C in darkness. Mortality of larvae was evaluated every 24 h for a week. Six larvae were used for each thesis and for the control. Each cadaver was transferred into individual White trap. The progeny IJs (first progeny) that emerged and migrated to the water were harvested every 48 h for 20 days after initial infection and stored in 50 ml plastic tubes.

The total number of nematodes (dead and alive) that emerged from a single host was estimated by counting (as described in section 3.2.2). This assay was repeated a second time using IJs of the first progeny to inoculate *G. mellonella* larvae.

3.2.4.6 Observation of the freezing damages

Nematodes thawed and incubated at 24 °C for 4 hours in Ringer's solution were temporary mounted in tap water on a glass slide with cover glass support and examined with a Leitz ORTHOPLAN light microscope at up to 1000×magnification to assess the freezing damage. Living and dead IJs were photographed using CANON Power Shot S50.

3.2.5 Cryopreservation protocol adjustment in others EPNs

3.2.5.1 Cryopreservation experiments

Starting with the protocol developed for *S. carpocapsae*, some experiments were performed in order to develop a protocol for each of the other EPN species of the Collection. Different cryoprotectant concentrations were tested for:

- *Steinernema affine:* 15 and 18% (v/v) glycerol solution
- *Steinernema feltiae:* 15 and 18% (v/v) glycerol solution
- *Heterorhabditis bacteriophora:* 13 and 15% (v/v) glycerol solution

In these experiments IJs, after 15 days of storage at 12 °C, were incubated for 48 h in different glycerol solution and thawing was carried out after 24 h in mechanical freezer.

For *S. apuliae*, IJs stored 15 days at 12 °C, were incubated in 18% (v/v) glycerol solution for 24 h and 48 h before cryopreservation.

3.2.5.2 Virulence assays

Following the same procedure used for *S. carpocapsae* (as described in section 3.2.4.4), virulence assays were also performed for *S. affine*, *S. feltiae* and *H. bacteriophora*.

3.2.6 Data analysis

All percentage data were an average of 6 replications ± SE. Mean percentage nematode survival following cryopreservation among treatments was analysed by using a one-way ANOVA. The differences were compared using Tukey HDS test (CoStat statistical software package (http://www.cohort.com/costat.html). In virulence assays lethal time to 50% and 90% mortality (LT_{50} and LT_{90}) were estimated.

Percentage data were arcsine-transformed prior to analysis; non-transformed means are presented in tables and figures.

3.3 RESULTS

3.3.1 Tuning of a cryopreservation protocol for *Steinernema carpocapsae*

3.3.1.1 Experiment 1 – Effect of storage days on cryopreservation

The percentage survival of *S. carpocapsae* IJs, stored at 12 °C for different days (1–7–15–30–90) before cryopreservation for the control in water and cryoprotectant (18% glycerol) was assessed. Significant differences were obtained (F= 120.239, df=50, P=0.00001).

Figure 3.2 shows the effect of 18% glycerol solution on the IJs survival after 24 h incubation, compared with the nematode population that was active in water before the glycerol incubation. Only in the case of the nematodes storage 15 days at 12 °C before cryopreservation there was no significant difference. Moreover this is the highest

level of *S. carpocapsae* survival (85.8%), attended by nematodes storage 7 days (84.3%). The lowest percentages of survival were obtained with the nematode stored for long periods before cryopreservation, e.g. 90 days (46.8%).

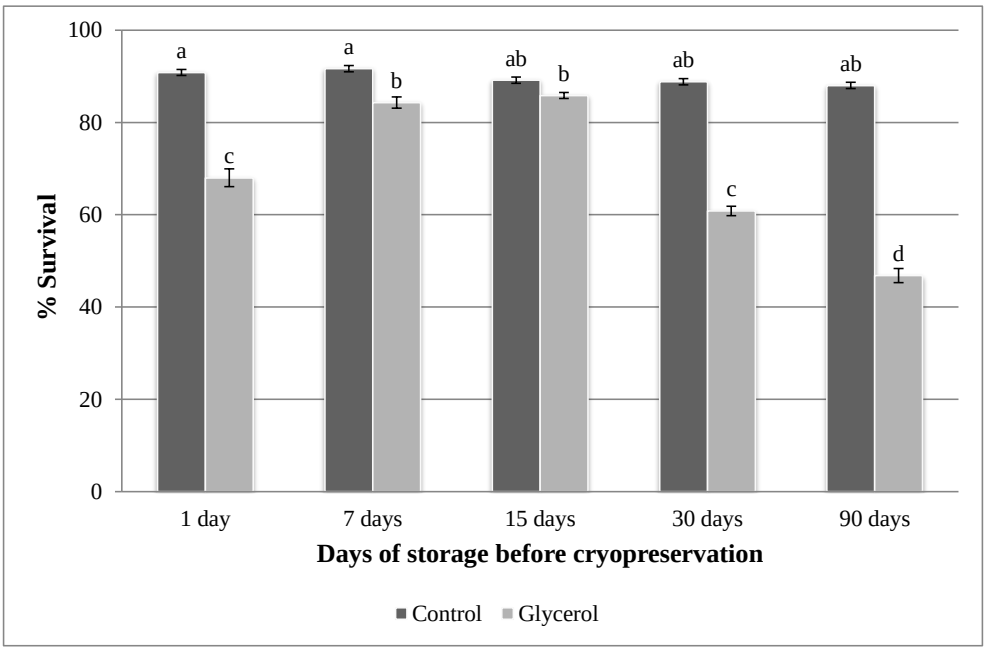

Fig. 3.2 – Mean percentage *Steinernema carpocapsae* (ItS-CAO1) survival before cryopreservation (95% Confidence interval) as influenced by different period of storage at 12 °C (one-way ANOVA and Tukey HDS test).

Significant differences in *S. carpocapsae* survival after cryopreservation were detected among nematodes stored at 12 °C for different period (F=44.75, df=25, P=0.00001) (Fig. 3.3). The trend of the percentage of survival is similar to the survival results after 24 h in glycerol. Nematodes, which after the emergence from the cadaver of *G. mellonella* larvae in White traps, were storage 15 days at 12 °C, have been showed the highest level of survival (71.5%). The lowest number of active nematodes (44.8%) occurred in the treatment with nematodes which have been stored for longer times.

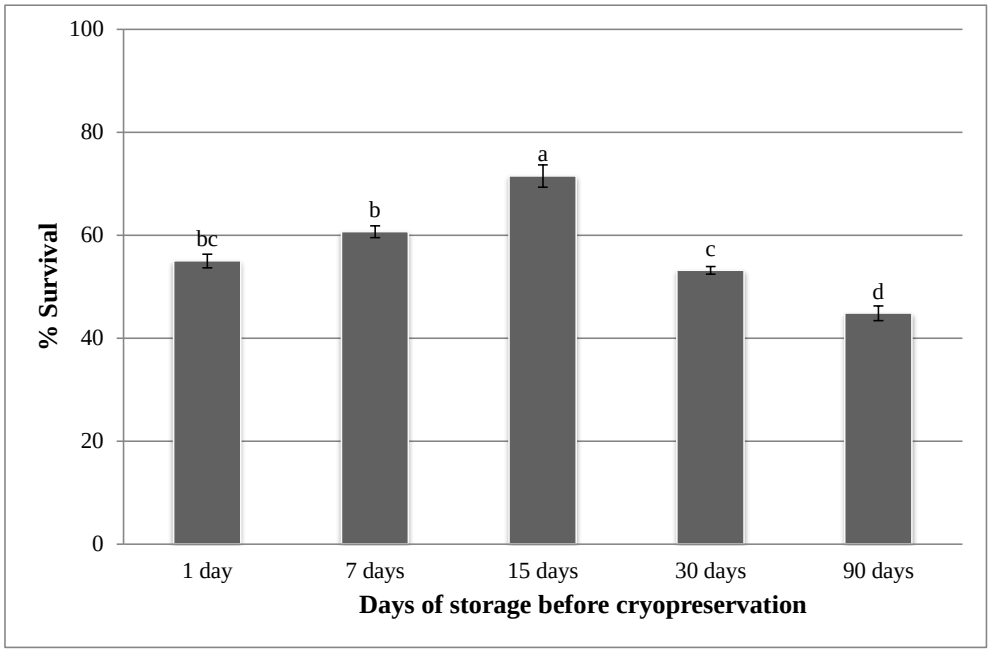

Fig. 3.3 – Mean percentage *Steinernema carpocapsae* (ItS-CAO1) survival after cryopreservation (95% Confidence interval) as influenced by different period of storage at 12 °C (one-way ANOVA and Tukey HDS test).

3.3.1.2 Experiment 2 – Different incubation periods in cryoprotectant

Results of the length of incubation in glycerol solution were analysed with significant differences. The survival percentage increased with the time exposed to 18% glycerol: after 24 h of incubation, survival rate of 89.2% was obtained and after 48 h this percentage increased (91.0%).

After cryopreservation the two treatments were significant different (F=25.80, df=30, P=0.00001). In both case the nematode survival percentage was high, but when IJs were incubated for 48 h in cryoprotectant this rate was higher (86.8%) than 24 h of incubation (71.5%) (Fig.3.4).

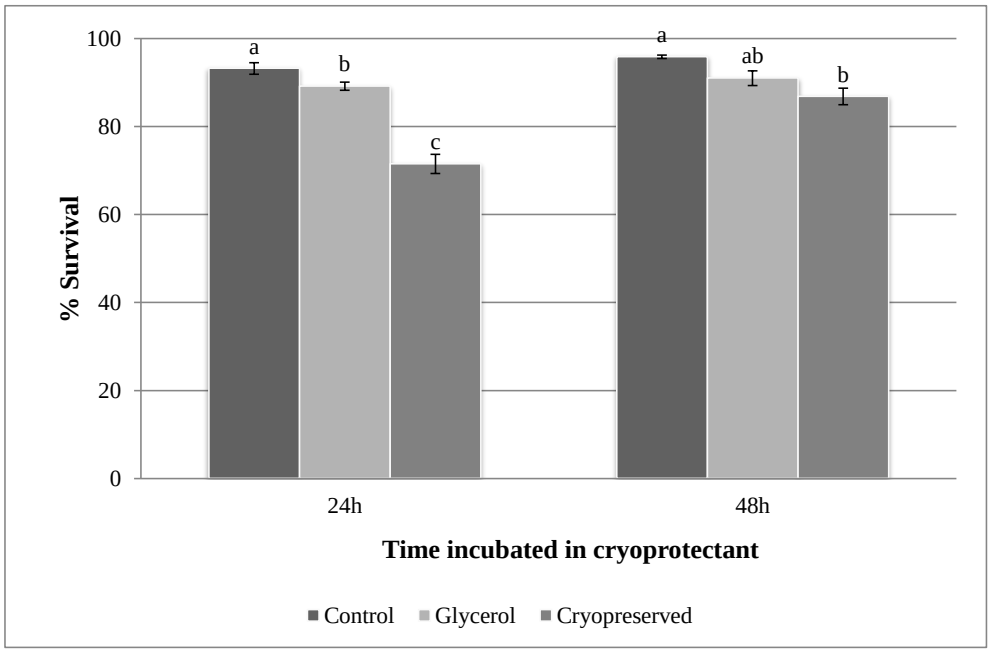

Fig. 3.4 – Mean percentage *Steinernema carpocapsae* (ItS-CAO1) survival (95% Confidence interval) after incubation for 24 and 48 hours in glycerol 18% (one-way ANOVA and Tukey HDS test).

3.3.1.3 Experiment 3 – Different periods of preservation in mechanical freezer

Significant differences were detected (F=37.23, df=25, P=0.00001) between the control in water and nematodes cryopreserved and thawed (Fig. 3.5). After one day at -140 °C the survival percentage was high (86.8%); in subsequent thawing this percentage decreased, remaining constant after 7, 15 and 30 days of storage at ultra-low temperature. The IJs survival was still high (about 74-75%).

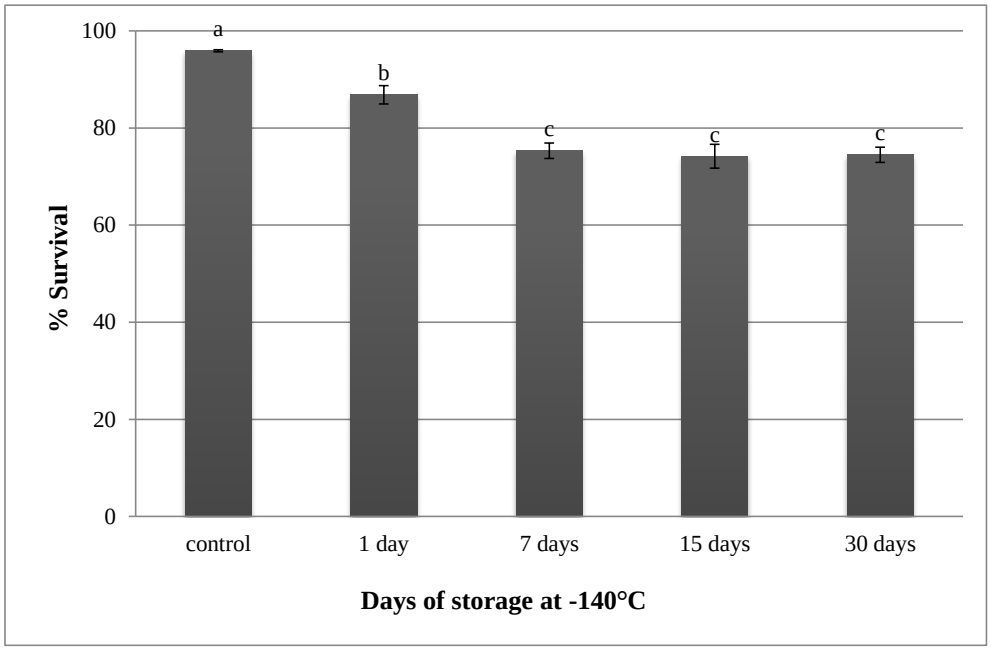

Fig. 3.5 – Mean percentage *Steinernema carpocapsae* (ItS-CAO1) survival (95% Confidence interval) after different periods of storage in mechanical freezer (one-way ANOVA and Tukey HDS test).

3.3.1.4 Virulence assay

The surviving IJs retained their original virulence against *G. mellonella* larvae. No differences in *S. carpocapsae* virulence were detected between nematodes subjected to cryopreservation and those from the controls (kept at 12 °C). Both groups of nematodes infected 100% of *Galleria* larvae in 48 h, with the similar LT_{50} and LT_{90} (Tab. 3.1).

Table 3.1 – Results of Virulence assay of *Steinernema carpocapsae.*

EPNs treatment	Mortality (%)	LT_{50} (days)	LT_{90} (days)	Emergence from cadavers	Time of emergence
Control	100%	1.4	1.9	100%	10 days
Thawed after 1 day	100%	1.4	1.9	100%	10 days
Thawed after 30 days	100%	0.9	1.8	93.3%	10 days

3.3.1.5 Reproductive potential after cryopreservation

Figure 3.6 shows the estimates of the mean number of *S. carpocapsae* IJs produced per cadaver, both for the control and for IJs stored at 12 °C for different periods (1 – 7 – 15 – 30 – 90 days), cryopreserved and thawed. Nematodes were reproduced in all larvae inoculated, but significant differences in the number of progeny IJs (first progeny) emerged from insect-larva were observed (F=6.78, df=33; P=0.0002). The highest mean IJs count was obtained from the control (739,938), followed by nematodes stored at 12 °C for 15 days before cryopreservation (670,500).

There were no differences in the mean of progeny IJs between nematodes storage for 1 day, 7 days and 30 days. The lowest mean number of IJs was obtained with the nematode stored for long period before cryopreservation (303,920). This value is less than half of the value of the control and the nematodes stored 15 days before cryopreservation.

Regarding the second progeny no significant differences (F=1.61, df=33, P=0.182) were detected in the mean number of IJs emerged from *G. mellonella* larvae between the control and nematodes of the first progeny (Fig. 3.7).

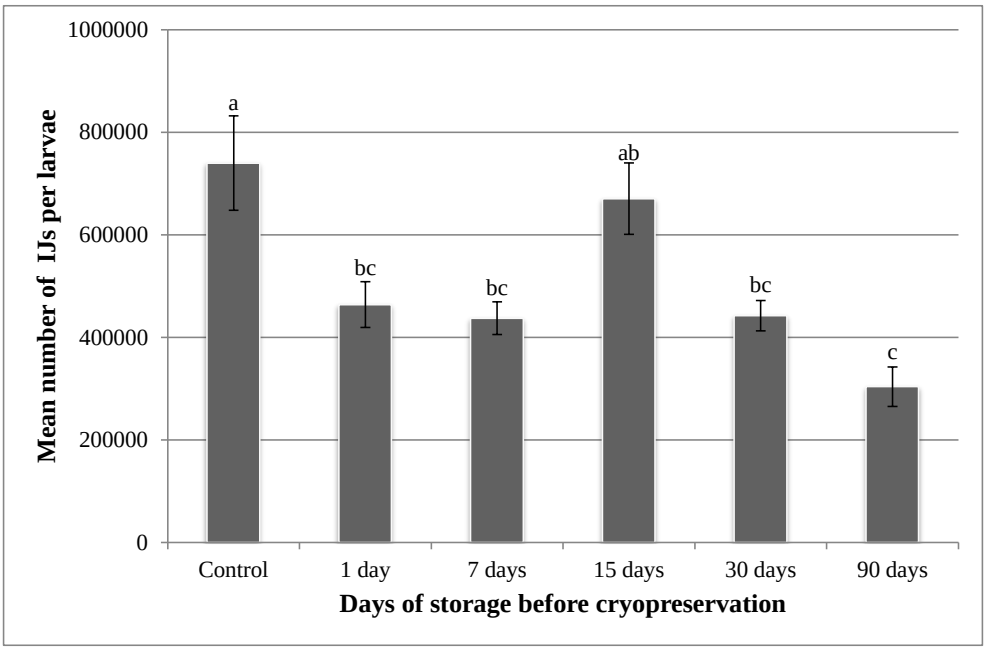

Fig. 3.6 - Estimates of the mean number of IJs *Steinernema carpocapsae* (ItS-CAO1) produced per cadaver of first progeny (95% Confidence interval - one-way ANOVA and Tukey HDS test).

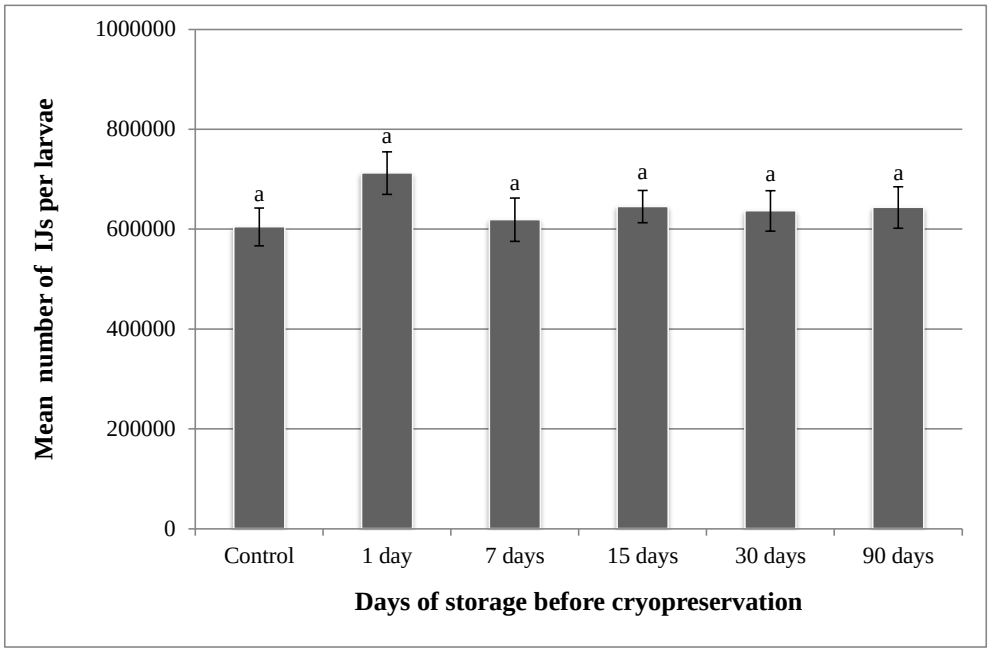

Fig. 3.7 - Estimates of the mean number of IJs *Steinernema carpocapsae* (ItS-CAO1) produced per cadaver of second progeny (95% Confidence interval - one-way ANOVA and Tukey HDS test).

3.3.1.6 Observation of the freezing damages

Examining *S. carpocapsae* specimens under optical microscope it was possible to photograph and compare surviving and died IJs after cryopreservation. Living and moving nematodes showed the same shape and the same internal structures to nematodes not cryopreserved, while the majority of dead IJs appeared with an irregular content and show organs damage and vacuolization in all body (Fig. 3.8). No severe rupturing or cut nematodes, as described to Elsen *et al*., 2007 and Brawn & Gaugler (1998) were observed.

Fig. 3.8 – *Steinernema carpocapsae* IJs after cryopreservation. A-B: Living nematode. A: Entire body; B: Higher magnification of the middle part of the body. C-D: Dead nematode. C: Entire body; D: Higher magnification of the middle part of the body.

3.3.2 Cryopreservation protocol adjustment in others EPNs

3.3.2.1 Results of cryopreservation experiments

For *S. affine*, two glycerol concentrations were tested. In this experiment, the survival percentage decreased respect to the control after 48 h incubation in both treatments: 68.6% in 15% glycerol and 71.3% in 18% glycerol. Significant differences (F=179.80, df=30, P=0.00001) were found after cropreservation, as presented in Figure 3.9. The better survival rate was occurred with 18% glycerol concentration (45.5%).

Similar results were obtained for *S. feltiae* but the survival rate were higher both after incubation in cryoprotectant solutions and after cryopreservation (F=258.72, df=30, P=0.00001). There were no significant differences in the two glycerol concentrations before freezing treatment. After cryopreservation, nematodes that had been incubated in glycerol 15%, showed a survival rate of 46%, while when 18% glycerol was used as a cryoprotectant, the nematode activity after thawing increased (56.5%) (Fig. 3.10).

The results of the survival of *H. bacteriophora* IJs which were incubated 48 h in 13% and 15% glycerol before cryopreservation are showed in Figure 3.11. Significant differences were detected both after incubation in two cryoprotectant solutions and after cryopreservation (F=227.71, df=30, P=0.00001). The lowest survival rates were recorded with glycerol 15%. After 48 h incubation, almost the half of nematodes had died and the survival rate decreased further after cryopreservation treatment (44.5%). A good result was obtained using 13% glycerol as cryoprotectant. The survival of IJs after freezing was 71.2%.

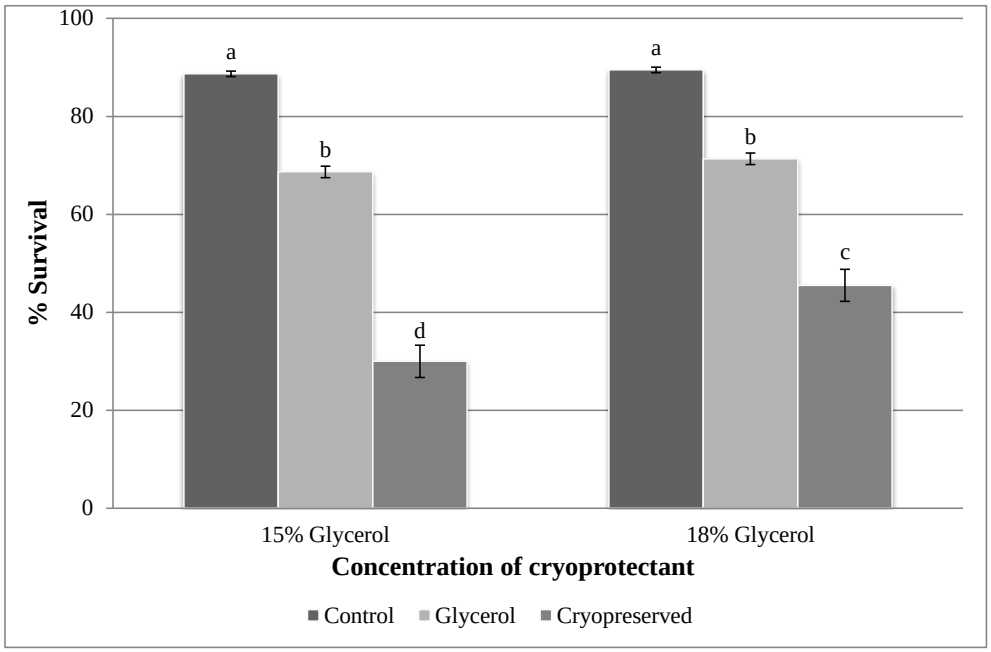

Fig. 3.9 – Mean percentage *Steinernema affine* (ItS-FO1) survival (95% Confidence interval) after incubation for 48 hours in glycerol 15% and 18% (one-way ANOVA and Tukey HDS test).

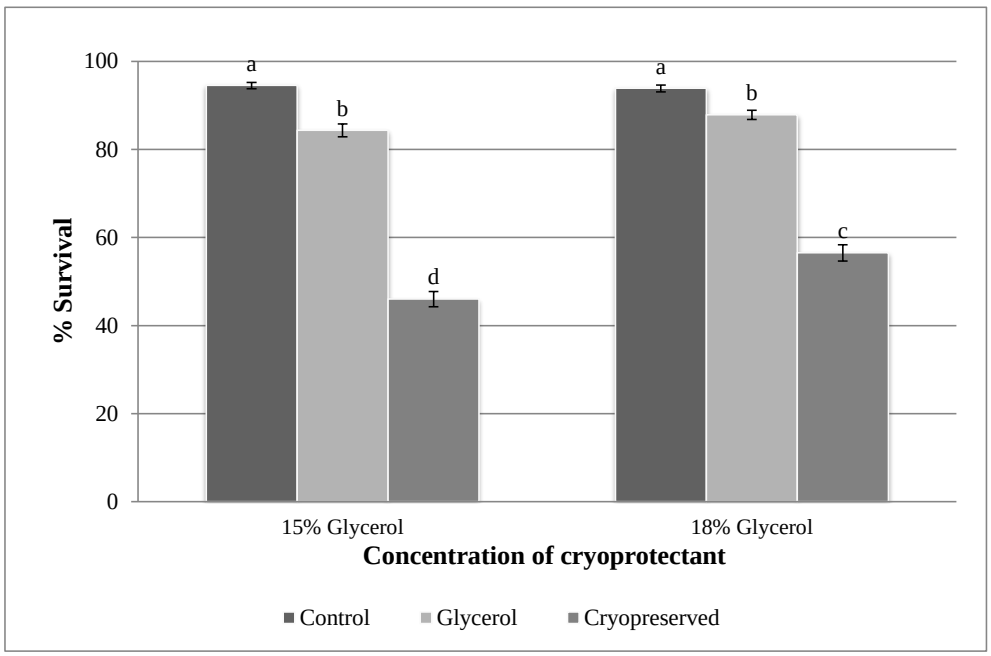

Fig. 3.10 – Mean percentage *Steinernema feltiae* (ItS-OT15) survival (95% Confidence interval) after incubation for 48 hours in glycerol 15% and 18% (one-way ANOVA and Tukey HDS test).

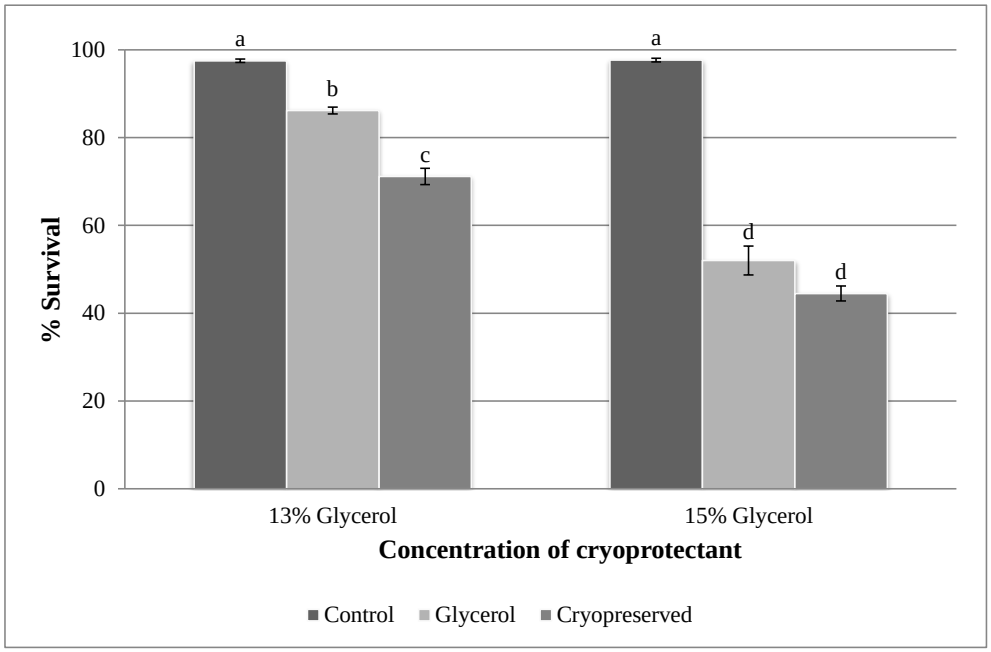

Fig. 3.11 – Mean percentage *Heterorhabditis bacteriophora* (ItH-LU1) survival (95% Confidence interval) after incubation for 48 hours in glycerol 13% and 15% (one-way ANOVA and Tukey HDS test).

The results of the survival of IJs of *S. apuliae* which were incubated in 18% glycerol with and without cryopreservation were analysed and significant differences in the survival rate were found between different periods of incubation (F=164.18; df=30; P=0.00001) (Fig. 3.12). After 24 h in cryoprotectant, the survival rate was similar to the control in water, but a decrease in this rate was observed when samples were incubated for 48 h. After 1 day of storage at -140 °C the nematode survival percentage was reduced (25.0% and 14.8% for nematodes incubated in glycerol 18% for 24 h and 48 h respectively).

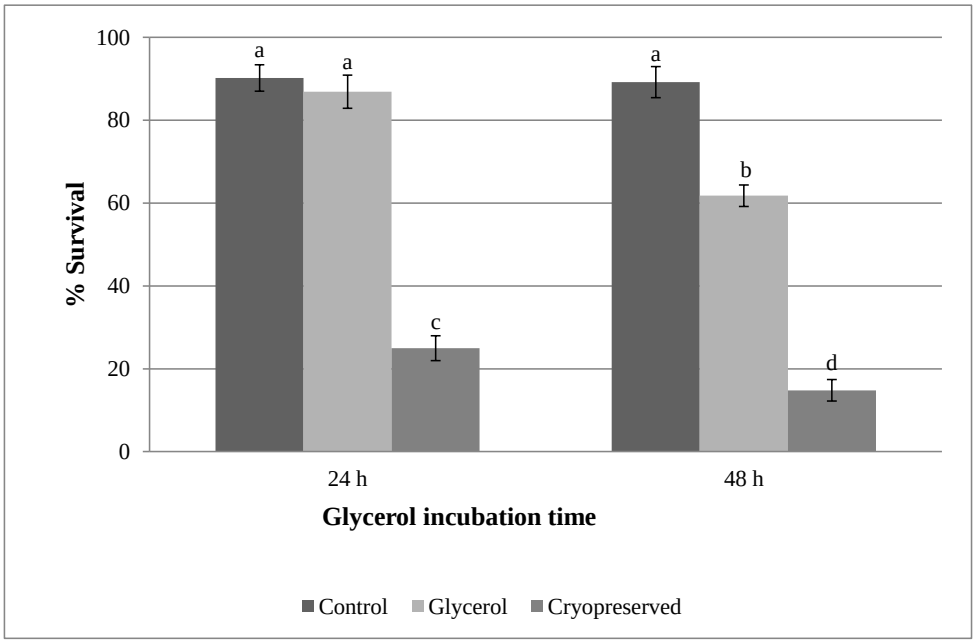

Fig. 3.12 – Mean percentage *Steinernema apuliae* (ItS-CS3) survival (95% Confidence interval) after incubation for 24 h and 48 h in glycerol 18% (one-way ANOVA and Tukey HDS test).

3.3.2.2 Virulence assays

The surviving IJs retained their original virulence against *G. mellonella* larvae. In all three EPNs species tested, only minor differences in virulence were highlighted between nematodes subjected to cryopreservation and those from the controls (kept at 12 °C). IJs of all three nematode species emerged from all cadavers within 10-12 days after host death. In Table 3.2 were shown the results of these assays.

Table 3.2 – Results of Virulence assays of *Steinernema affine, Steinernema feltiae* and *Heterorhabditis bacteriophora.*

EPNs species	Treatment	Mortality (%)	LT_{50} (days)	LT_{90} (days)	Emergence from cadavers	Time of emergence
S. affine	Control	100%	1.9	2.8	100%	10 days
	Cryopreserved	100%	1.7	2.7	100%	12 days
S. feltiae	Control	100%	1.9	2.8	93%	10 days
	Cryopreserved	100%	2.4	2.9	93%	10 days
H. bacteriophora	Control	100%	1.9	2.8	80%	10 days
	Cryopreserved	100%	2.2	2.9	80%	10 days

3.4 DISCUSSION

In the current study, a cryopreservation protocol for *S. carpocapsae* was developed. An optimum survival rate of 91% was obtained using the following protocol: (i) storage of IJs in distilled water for 15 days at 12 °C after the emergence from *G. mellonella* larvae, (ii) 48 h incubation in 18% glycerol at 24 °C, (iii) 10 min in 70% methanol at 0 °C, (iv) several minutes in liquid nitrogen and finally (v) storage in mechanical freezer at -140 °C.

Sayre & Hwang (1975) suggested that certain conditions affect the survival of nematodes, such as the age and vigour of nematodes before freezing, the length of time nematodes are in contact with the cryoprotectants, and concentration of the cryoprotectant. As regard the age of nematodes, many authors (i.e. Nugent *et al*., 1996) suggested using freshly IJs, but no one specified how many days IJs have been stored before cryopreservation. Cosi *et al*. (2008) used for cryopreservation experiments IJs stored for maximum two weeks at 10 °C, while in others works, EPNs were stored at 13 °C up to 45 days before experimentation (Bai *et al*, 2004) or for longer periods, but not exceeding 3 months (Popiel & Vasquez, 1991).

In the first experiment of this study an optimum time for cryopreserve EPNs after the emergence of IJs from *G. mellonella* larvae was determined. The results indicate that the survival rate and the reproduction rate of IJs after cryopreservation is highly dependent on the length of the days of storage at 12 °C. Nematode survival increases with increasing storage time up to an optimum level at 15 days before it decreases again.

Probably differences between nematodes stored 1 day and 15 days depend on the accumulation of trehalose. This important disaccharide is a stress protectant in biological systems as it interacts with and directly protects lipid membranes and proteins from the damage caused by environmental stresses such as desiccation and freezing (Behm, 1997). In vitrification process, trehalose prevents fusion of adjacent membranes and trap tissues in the high-viscosity, low molecular mobility medium formed by the carbohydrate glass which limits a variety of deterioration process (Franks, 1985).

Qiu & Bedding (1999) demonstrated that when IJs of *S. carpocapsae* were incubated aerobically in tap water at temperatures ranging from 2 to 14 °C, increased their trehalose levels. Jagdale & Grewal (2003) observed a positive correlation between cold acclimation induced trehalose accumulation and enhanced freezing tolerance of *S. carpocapsae*. May be after 15 days of storage, as described to Nugent *et al*. (1996), IJs began to be old with reduced food reserves and their post-cryopreservation survival was poor.

In the second experiment the results indicate that the survival rate of IJs after storage in mechanical freezer depends on the length of the incubation period in cryoprotectant. Nematode survival increased with increasing incubation time. No differences concentration of glycerol were tested in this study, because prior studies on *S. carpocapsae* cryopreservation demonstrated that acceptable survival can be obtained with the use of 18% glycerol respect others concentrations (Bai *et al.*, 2004; Cosi *et al.*, 2008; Curran *et al.*, 1992). It was shown by Popiel & Vasquez (1991) that, although incubation of nematodes in glycerol alone was sufficient, their survival rate was enhanced by a 10 minute methanol wash, because it reduced the cooling rate required

for vitrification. In the current study, filter paper strips were used as they had been proven to enhance post-cryopreservation survival rates, compared with those achieved by freezing nematodes as a liquid suspension (Nugent *et al*., 1996).

In the third experiment the effect of long term storage at -140 °C on nematode survival is currently under study, in order to assess if the 75% survival percentage observed after 30 days storage not significantly change during longer storage periods. The current study supports findings by Popiel & Vasquez (1991). Their work is the only currently present that evaluates the long term maintenance, observing that survival rate of *S. carpocapsae* thawed after 24 h was slightly highest. In subsequent thawings this percentage decreased, remaining constant.

Results from Brown & Gaugler (1998) showed that the infectivity and pathogenicity of IJs are reduced by prolonged exposure to cryopreservation. Jagdale & Grewal (2003) observed that trehalose helped to preserve the original virulence of nematodes; a result, when EPNs were either heat- or freeze-stressed, their original virulence against *G. mellonella* larvae was largely preserved. The decrease in survival and infectivity of IJs cryopreserved is entirely due to the freezing, storage and thawing procedure. Concurring to Van der Beek *et al*. (1996), there is no significant difference in infectivity after storage at ultra-low temperature.

Starting with the protocol developed for *S. carpocapsae*, some experiments were performed in order to cryopreserve also other EPN species. Prior studies on cryopreservation demonstrated that acceptable survival of all species and isolates of EPNs can be obtained with the use of 15% glycerol, and Curran *et al*. (1992) obtained a mean survival rate of 69% for isolates of *Steinernema* and 68% for isolates of *Heterorhabditis.* Results of different experiments in the present study showed that 15%

glycerol was not the better concentration for all EPNs, for example *H. bacteriophora* increase survival rate (71.2%) if incubated in 13% glycerol, while *S. feltiae* and *S. affine* had a highest survival rate if incubated in 18% glycerol.

According to Curran *et al*. (1992), larger nematode species such as *S. glaseri* (Steiner, 1929) Wouts, Mrácek, Gerdin & Bedding, 1982, show lower survival rates after thawing and results from this study have confirmed their observations, with an overall lower survival rate (25%) for *S. apuliae*, in comparison with *S. carpocapsae*. The cause of the difference in survival post-cryopreservation is due to the difference in size between the two nematode species; *S. apuliae* is a larger nematode, with a IJs body length of 1064±66 μm (Triggiani *et al*., 2004), compared to *S. carpocapsae* ItS-CAO1 strain, which has a body length of 559.0 ± 23.8μm (Torrini *et al*., 2014).

In all EPNs species tested, surviving IJs retained their original virulence against *G. mellonella* larvae. Only minor differences in virulence between nematodes subjected to cryopreservation and those from the controls (kept at 12 °C) were detected.

Long-term cryopreservation of EPNs offers many benefits over the normal laboratory maintenance of nematode cultures. It obviates labour, requirements for space and the maintenance of controlled environmental conditions, all of which are costly. The cryopreservation protocols developed in the present study can be applied to preserve different EPN species to genetic variation and to minimise the risk of contamination. Wang & Grewal (2002) showed the maintenance of stress tolerance and other fitness traits by the cryopreserved nematodes. They also suggested that keeping the strains in the laboratory for only few generations could lead to laboratory selection.

Protocols developed during this study can serve to cryopreserve each new species or EPNs isolate which have been found or will be find during surveys in Italy.

Chapter 4

BIOASSAYS BEFORE AND AFTER CRYOPRESERVATION

4.1 INTRODUCTION

For an EPN isolate to be used successfully in biological control programmes, it must have good pathogenicity to insects and be easy to propagate on a large scale. Although the commercial and scientific interests in EPNs are increased, no standard universal assays for evaluation of nematode quality or potency exist. The need to evaluate nematode insecticidal activity in the laboratory has resulted in the development of variety of assays that measure nematode infectivity by recording host mortality.

Information on the pathogenicity of *Steinernema carpocapsae* ItS-CAO1, isolated from the soil was obtained through different bioassays, using *Galleria mellonella* as insect host. The same bioassays were repeated with nematodes of the same strain cryopreserved, in order to evaluate if after thawing the nematode ability to infect insect host can be prejudiced.

Four types of bioassays were carried out to determine the infectivity and stability of the nematodes: Penetration (Caroli *et al*., 1996), Exposure time (Glazer, 1991), Sand column (Griffin & Downes, 1994) and One-on-one (Miller, 1989). The Penetration assay reflects nematode ability to penetrate into the insect; the Exposure time assay was carried out to determine the minimum time required by the EPNs to penetrate and kill

an insect host; the Sand column assay measures nematode ability to locate and penetrate the target host and the One-on-one assay represents the overall infection process.

4.2 MATERIALS AND METHODS

4.2.1 Nematodes and Insects

The EPN used was *S. carpocapsae* ItS-CAO1 strain, before and after cryopreservation. In the first case EPNs were cultured *in vivo* in the greater wax moth, *G. mellonella* larvae and IJs were recovered using modified White traps and stored in distilled water at 12 °C for three weeks. In the second case, IJs thawed were stored in distilled water one week at 12 °C.

Assays were performed using last instar *G. mellonella* larvae, with an average weight of 0.2-0.4 g. Larvae of *Galleria* are used extensively as the hosts of choice for the in *vivo* culture and study of EPNs because they are eminently suitable and susceptible hosts. They are easily reared in the lab and are widely available from many commercial sources (Kaya & Stock, 1997; Ehlers, 2001).

All nematode suspensions and larvae were kept at 20 °C for 48 h prior to testing. Adjustment nematode concentrations were effectuated using the formula (1) as described in section 3.2.2.

4.2.2 Bioassays

The first three assays, indicated above, were performed in 12-well plates (COSTAR®, Corning, New York) with filter paper (Whatman No.1) at the bottom of each one of wells. Sand column assay were performed using plastic containers (40 mm height x 45 mm diameter) contained moist sterile sand (autoclaved). In all bioassays, the incubation periods were carried out in the dark at 24 °C.

4.2.2.1 Penetration assay

A nematode suspension of 4000 IJs ml^{-1} was prepared and with a micropipette a 0.5 µl aliquot was transferred to each one of twelve wells followed by a single last instar *G. mellonella* larva. Larval mortality was recorded daily. After an additional 48 h in 6 cm Petri dishes with moist filter paper, cadavers of larvae were dissected. Using a dissecting microscope the number of nematodes established in the host was counted. The penetration rate was expressed as the percentage of the initial IJs inoculum that had invaded the insect host.

4.2.2.2 Exposure time assay

Every single *G. mellonella* larva was exposed to nematodes (400 IJs in 0.5 µl /well) for periods of 20, 40, 60, 120 and 180 minutes (twelve replicates per treatment). At the end of each exposure period, insects were rinsed in steril water to remove any nematodes from the surface and transferred to 6 cm Petri dishes with moist filter paper. The mortality was recorded 48 h after initial nematodes exposure.

4.2.2.3 One-on-one assay

This assay was conducted using two 12-well plates. In every well was placed only one IJ in 50 µl and one *G. mellonella* larva. Twelve wells with 0.5 µl of deionized water were used as control. The lids were replaced and the plates were sealed with parafilm to prevent desiccation. Mortality within 24, 48 and 72 h was recorded.

4.2.2.4 Sand column assay

Fifteen plastic containers (40 mm height x 45 mm diameter) were used for this assay. One last instar *G. mellonella* larva was placed in the bottom and then the container was filled with moist sterile sand. A suspension of 100 IJs in 100 µl was added to the top of each container. After 24 h, larvae were removed from the sand, rinsed in tap water and incubated in 6 cm Petri dishes with moist filter paper for a further 3 days. Larval mortality and penetration rate was recorded.

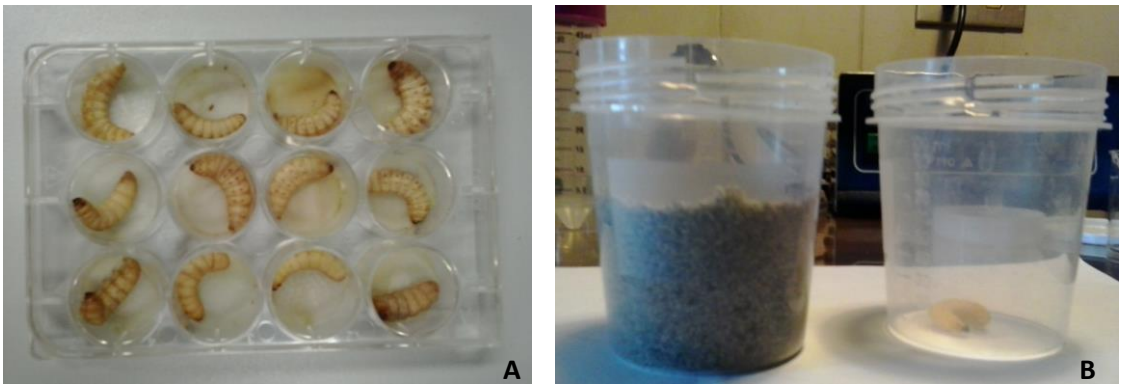

Fig. 4.1 – A: Multiwell plate (12 wells) containing last-instar larvae of *Galleria mellonella* used for Penetration assay, Exposure time assay and One-on-one assay. B: Plastic containers with one last instar *G. mellonella* larvae and sterile sand used for Sand column assay.

4.2.3 Data analysis

Penetration rate data from Penetration assay and Sand column assay were analysed by using a one-way ANOVA. The differences were compared using Tukey HDS test (CoStat statistical software package - http://www.cohort.com/costat.html). Percentage data were arcsine-transformed prior to analysis; non-transformed means are presented in tables and figures.

In One-on-one assay ET_{50} values (the exposure time of nematodes to insects that are required to achieve 50% insect mortality) were calculated using probit analysis (SPSS 15.0). Regression analysis was used in Exposure time assay and One-on-one assay to examining the relationship between the exposure time and insect-host mortality.

4.3 RESULTS

4.3.1 Penetration assay

S. carpocapsae ItS-CAO1 achieved 100% mortality of *G. mellonella* larvae after 48 h of exposition both the control and nematodes cryopreserved. Penetration rate (P) was calculated using the following formula (Glazer & Lewis, 2000):

$$(Nx100)/T=P \quad (2)$$

Where:

- *N*=average number of nematodes counted in each cadaver,

- *T*=original average number of nematodes in the well (in this case *T*=200)

- *P*=percentage penetration.

Significant differences were observed between mean penetration rate of the control and cryopreserved nematodes (F=37.35, df=22, P=0.00001). The highest ability of IJs to penetrate in the host was recorded for nematodes which were not cryopreserved and they were faster to kill insect hosts (Tab. 4.1). The sex ratio of nematodes recovered from cadavers of *Galleria* larvae not differed significantly between before and after freezing treatment. Females were slightly predominant both for the control and for nematodes cryopreserved.

Table 4.1 – Insect mortality, time to death, penetration rate and sex ratio in Penetration assay with *Steinernema carpocapsae* ItS-CAO1 before (Control) and after cryopreservation (Cryopreserved). Average of twelve replicates. Means (±SD).

Nematodes	Insect mortality (%)	Days to die	P (total) *	P (males)	P (females)	Sex ratio (F/M)
Control	100%	0.25±0.5 (1-2)	57.6±9.5 **a** (40.0-73.0)	23.6±6.3 (7.5-31.0)	34.0±5.7 (24.5-47.0)	59.5/40.5 ±8.2
Cryopreserved	100%	1.75±0.4 (1-2)	33.8±9.5 **b** (25.0-54.5)	14.1±5.0 (7.5-23.0)	19.7±5.0 (13.0-19.5)	58.9/41.1 ±5.8

* Means with different letters are significantly different (ANOVA, P<0.05).

4.3.2 Exposure time assay

Results at 48 h are presented in figures 4.2 and 4.3 below. The overall trend showed a gradual increase in mortality with increasing exposure time. No significant differences were found between control and nematodes cryopreserved. Only after 20 min of exposition, in the second case there was one cadaver more than the control. In both nematodes, complete mortality was achieved just when *G. mellonella* larvae were exposed to 400 IJs for 60 min and the ET_{50} was < 20 min.

After 24 h from the exposition (data not show) no *G. mellonella* larvae were died for cryopreserved nematodes, while for the control, percentage mortality of insect hosts was variable (from 0% for 20 min to 83.3% for 180 min).

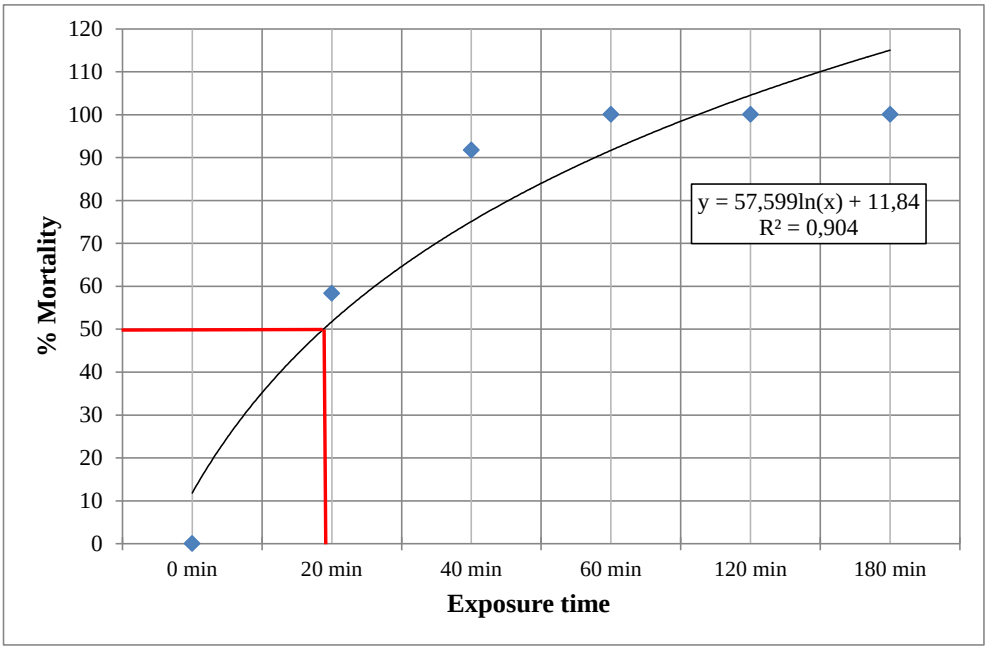

Fig. 4.2 – Percentage insect mortality following exposure of *Galleria mellonella* larvae to 400 *Steinernema carpocapsae* IJs (control)/insect, for different time (min) periods. Regression equation is shown.

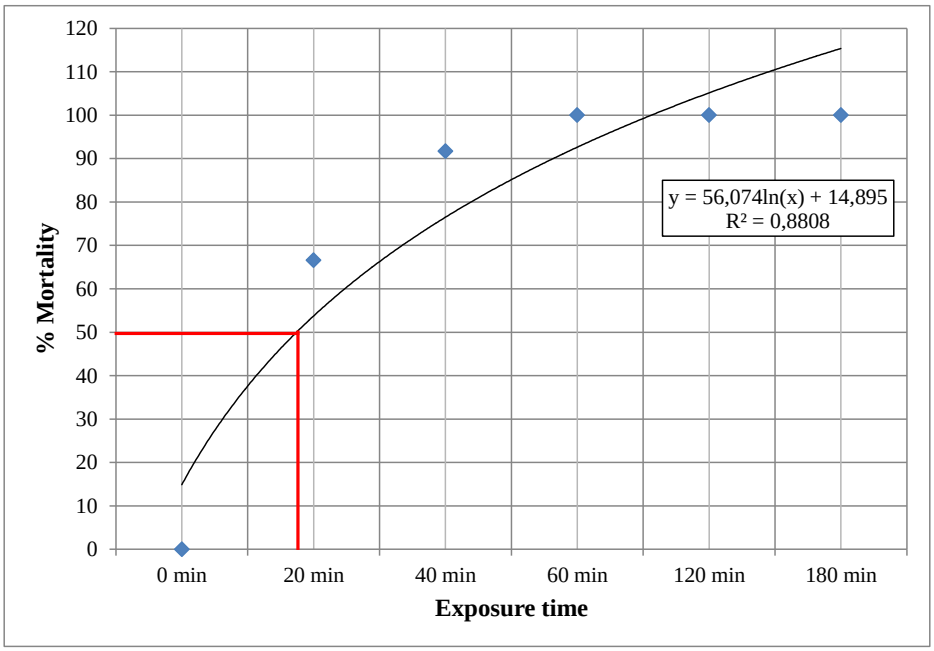

Fig. 4.3 – Percentage insect mortality following exposure of *Galleria mellonella* larvae to 400 *Steinernema carpocapsae* IJs (cryopreserved)/insect, for different time (min) periods. Regression equation is shown.

4.3.3 One-on-one assay

In the one-on-one assay, nematode virulence was measured by insect mortality over 72 h. Both in the experiment with nematodes cryopreserved and in that with nematodes as control, one IJ was capable of killing the insect host. After 24 h no *Galleria* larvae were died, while after a period of 48 h and 72 h mortality was highest for nematodes cryopreserved respect to the control (Figs. 4.4 and 4.5). Mortality at 72 h was significantly correlated with mortality at 48 h both for the control and for nematode cryopreserved. All mortality was assumed to be caused by nematode infection because no mortality was observed in the control.

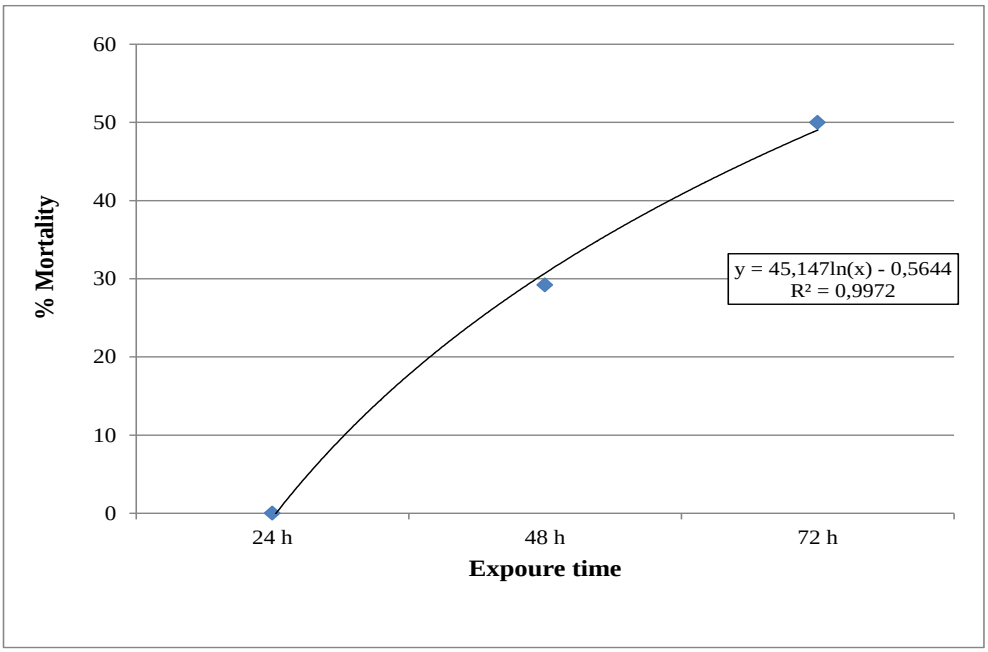

Fig. 4.4 – Percentage insect mortality following exposure of *Galleria mellonella* larvae to one *Steinernema carpocapsae* IJ (control)/insect, for different time (hours) periods. Regression equation is shown.

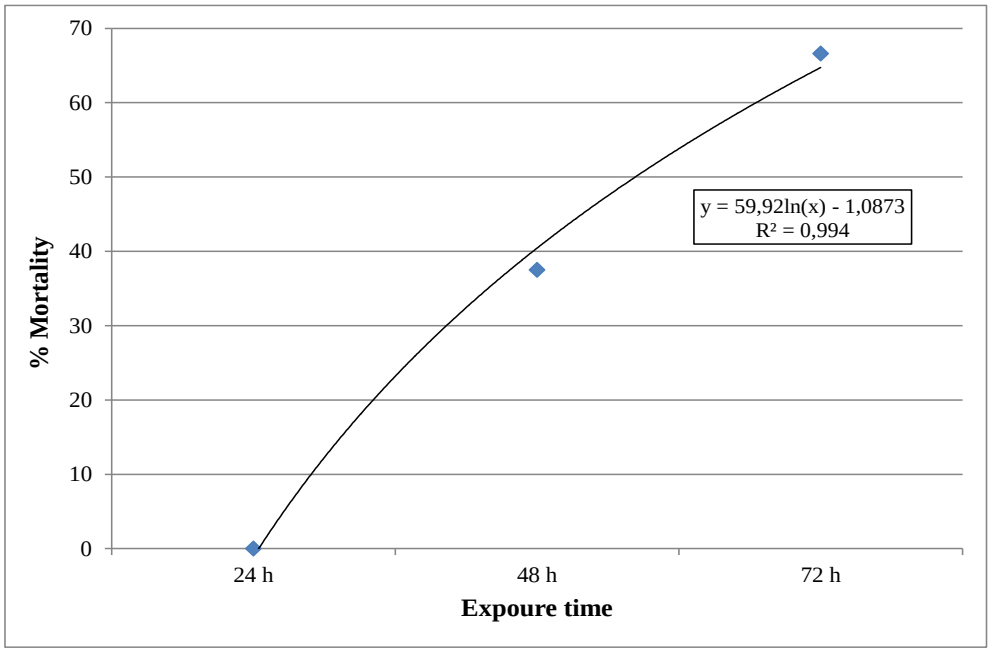

Fig. 4.5 – Percentage insect mortality following exposure of *Galleria mellonella* larvae to one *Steinernema carpocapsae* IJ (cryopreserved)/insect, for different time (hours) periods. Regression equation is shown.

4.3.4 Sand column assay

Steinernema carpocapsae ItS-CAO1 achieved 100% mortality of *G. mellonella* larvae after 72 h of exposition for nematodes cryopreserved, while for the control mortality was 93.3%. Penetration rate (P) was calculated with the formula (2) used for the Penetration assay (as described in section 4.3.1).

Significant differences were observed between mean penetration rates of the control and cryopreserved nematodes (F=9.78, df=28, P=0.004). The highest ability of IJs to penetrate in the host was recorded for nematodes cryopreserved and they were faster to kill insect hosts (Tab. 4.2). The sex ratio of nematodes recovered from cadavers of *Galleria* larvae differed between before and after freezing treatment. In the first case, male were slightly predominant, in the second case nematodes cryopreserved showed a sex ratio of nearly 50%.

Table 4.2 – Insect mortality, time to death, penetration rate and sex ratio in Sand column assay with *Steinernema carpocapsae* ItS-CAO1 before (Control) and after cryopreservation (Cryopreserved). Average of twelve replicates. Means (±SD).

Nematodes	Insect mortality (%)	Days to die	P (total)*	P (males)	P (females)	Sex ratio (F/M)
Control	93%	2.28±0.5 (2-3)	42.9±8.0 **a** (31.0-63.0)	23.3±6.8 (15.0-42.0)	19.6±4.9 (14.0-31.0)	46.0/54.0 ±8.7
Cryopreserved	100%	2.06±0.3 (2-3)	59.4±10.3 **b** (48.0-80.0)	29.1±5.7 (22.0-39.0)	30.3±6.1 (22.0-44.0)	51.0/49.0 ±5.8

* Means with different letters are significantly different (ANOVA, P<0.05).

4.4 DISCUSSION

Screening of EPN species is an important step before initiating mass production or field evaluation. Laboratory studies are essential for maximising the chances of success in field experiments (Bedding, 1990). Mannion & Jansson (1992) recommended the use of more than one type of bioassay for evaluation of nematode biological control potential. For this reason, in the present study, the virulence of *S. carpocapsae* ItS-CAO1 was evaluated using four bioassays, according to different steps in the infection process.

The Exposure time assay indicated indirectly how quickly insects were infected by nematodes. This assay tested *S. carpocapsae* ItS-CAO1 IJs cryopreserved and not-cryopreserved (control) against *G. mellonella* larvae. In both nematodes, mortality increased with longer exposure time but already in the firsts 20 min, IJs were able to penetrate inside the body host. Complete mortality was achieved just when *G. mellonella* larvae were exposed to 400 IJs for 60 min. These findings suggest that this strain is very quick in penetrating the host, because it needs only 20 min of exposure to enter the *G. mellonella* and causing a percentage of mortality above 50%, respect *S. carpocapsae* All strain (Glazer & Lewis, 2000) needs about 65 min to achieve 50% insect mortality.

Steinernema carpocapsae ItS-CAO1 is not only very quick during the penetration step, but the results of the One-on-one assay suggested that it is also very virulent against *Galleria* larvae, as a single nematode was able to kill them. Single nematode of both thesis (cryopreserved and control) has needed more time to kill the insect host than bioassays with 100-400 IJs/host, because high penetration rates increase

the infection rates, which in turn increase toxins produced by nematodes and their symbiotic bacteria (Burman, 1982) and so they kill the insect host more rapidly by septicaemia. After 72 hours insect mortality caused both nematodes cryopreserved and control (66.6% and 50.0% respectively) was in agreement with results reported by Glazer & Lewis (2000). When an EPN is highly virulent, the use of probit analysis to estimate the lethal level (LD_{50}) is not useful, sins a single steinernematid IJ was capable of killing a host (Huber & Hughes, 1984). For this reason the Dose-response assay (Morris *et al*., 1990) was not carried out.

Hominick & Reid (1990) proposed the use of invasion efficiency as a direct measure of nematode infectivity. These authors assumed that the nematode with the greatest efficacy against a target insect would have the highest invasion efficiency. Fan & Hominick (1991) reported that the invasion efficiency index is more sensitive than host mortality to determine nematode activity. In the present study, Penetration assay and Sand column assay were conducted to evaluate *S. carpocapsae* ability to penetrate into the host. In the first assay nematodes and insects were kept in close contact, in the second one, natural environmental conditions were simulated to measure nematode ability to locate and penetrate the target host. Insect mortality was very high in Penetration (100% in both cases) and Sand column assay (100% for nematodes cryopreserved and 93% for the control). In the first assay, penetration rate was higher for the control, while in the second assay the highest ability of IJs to penetrate in the host was recorded for IJs cryopreserved.

Assays conducted in multi-well plates exclude host behavioural and ecological barriers to infectivity and limit the influence of foraging strategies. The direct contact assay thus measured both (i) the virulence of the parasite due to the presence of

symbiont and (ii) invasiveness of the nematode, without the interference of any possible host resistance. Furthermore Lewis *et al.* (1992) reported that *S. carpocapsae* demonstrates a behaviour of 'ambushing' adopting a 'sit and wait' strategy in order to make contact with insects. This strategy implies that IJs have a lower mobility in soil, where it prefers to stay near the surface, closed to its application place (Campbell & Gaugler, 1993). Results of Sand column assay showed that *S. carpocapsae* ItS-CAO1 was able to find and infect *Galleria* larvae in the sand soil even if it is classified as 'not actively host searcher'.

Sex ratio was evaluated in Penetration and Sand column assay. In the first one, results were in agreement with the *S. carpocapsae* sex ratio reported by Bednarek *et al.* (1986) for a Polish strain (F/M = 57/43) and by Gaugler *et al.* (1990) for Fondation strain (F/M = 56/44). The sex ratio of nematodes recovered from cadavers of *Galleria* larvae not differed significantly between before and after freezing treatment. Females were slightly predominant both for the control and for nematodes cryopreserved. On the contrary, in Sand column assay the sex ratio of nematodes differed between before and after cyopreservation but differed also from Penetration assay, because male were predominant (control) or nearly 50% (nematodes cryopreserved).

Grewal *et al*. (1993) proposed that male steinernematids are the colonizing sex; that they disperse, locate and establish in distant hosts before females. In addition, they reported that in *S. carpocapsae* the highest percentage of nematodes that invaded hosts located 10 cm away from the point of nematode application were male, while the sex ratio in hosts in close contact was female-biased. Findings of the present study confirmed the presence of higher proportion of males in hosts that were invaded following migration in the sand assy.

Chapter 5

CONCLUSION

The first objective of this study was to use light microscopy, morphometrics, morphology and molecular analysis, SEM, and drawings to describe a new species of *Oscheius*, a new strain of *Steinernema carpocapsae* (ItS-CAO1) and other EPNs isolated from soil samples, collected during different surveys conducted in the three years of study.

The results of virulence assay have showed that *S. carpocapsae* was very quick to kill the insect host. For this reason the attention was focused on this nematode.

The second aim was to develop simplified protocols for the long-term storage of infective juveniles (IJs) of EPN species. Different cryopreservation experiments were carried out to obtain an optimum survival of *S. carpocapsae*. An optimum time for cryopreserve IJs after their emergence from *Galleria mellonella* larvae was determined. The influence of exposure of nematodes to different cryoprotectants for different time periods was assessed, and the effect of different periods of preservation at ultra low temperatures on nematode survival was evaluated.

The better cryopreservation survival rate (91%) was obtained when IJs were stored in distilled water for 15 days at 12 °C after the emergence from *G. mellonella* larvae. After this period, IJs were incubated 48 h in 18% glycerol at 24 °C and washed for 10 min in 70% methanol at 0 °C, before to be plunged in liquid nitrogen for several minutes and finally stored in mechanical freezer at -140 °C.

Thawing of nematodes was completed as rapidly as possible (within 10 seconds), because it is a critical step. Nematodes revive was observed within four hours of thawing, with videos and photos. A morphological comparison between living and died nematodes after cryopreservation was carried out to evaluate freezing damages.

Virulence of nematodes following cryopreservation was not lost and there was no difference in virulence between IJs kept at -140 °C for one or 30 days. They successfully completed their life cycles in *G. mellonella* larvae. Reproductive potential after cryopreservation was evaluated. Nematodes were reproduced in all larvae inoculated and in the treatment with IJs stored 15 days at 12 °C before cryopreservation differences in the number of progeny were not observed respect to IJs not cryopreserved.

Starting with the protocol developed for *S. carpocapsae*, some experiments were performed in order to cryopreserve also other EPNs. All species tested were successfully cryopreserved although with different survival rates: *Heterorhabditis bacteriophora* (71.2%), *S. feltiae* (56.5%), *S. affine* (45.5%) and *S. apuliae* (25.0%) and they were able to infect *G. mellonella* larvae after cryopreservation and complete their life cycles.

The third and last objective of this study was to obtain information on the pathogenicity of *S. carpocapsae* ItS-CAO1 before and after cryopreservation, in order to evaluate if after thawing the nematode ability to infect insect host can be prejudiced. Four bioassays were carried out to determine the infectivity and stability of the nematodes against *G. mellonella* larvae.

Results of One-on-one assay indicated that a single IJ was capable of killing a host; Exposure time assay showed that nematodes were very quick to penetrate inside the body of *Galleria* larvae (ET_{50}<20 min). Sand column assay has revealed nematode ability to locate and penetrate the target host located under 4 cm of sand soil, although *S. carpocapsae* was classified as ambusher species. Also Penetration assay reflected nematode ability to penetrate into the insect host, but under laboratory conditions.

Although it is necessary to repeat these assays with other insects and compare the results with field assays, this study indicates the possibility of using this strain for the biological control of insects such as the *G. mellonella* and especially it shows that the cryopreservation process does not alter the virulence of EPNs, rather the long term preservation offers many benefits over the normal laboratory maintenance of nematode cultures. It obviates labour, requirements for space and the maintenance of controlled environmental conditions, it preserves genetic variation of nematode species, and minimises the risk of contamination.

APPENDIX A

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Oscheius onirici sp. n. (Nematoda: Rhabditidae): a new entomopathogenic nematode from an Italian cave

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Abstract

Oscheius onirici **sp. n.** (Nematoda: Rhabditidae) was isolated from a karst cave soil of Central Italy. Molecular and morphological analyses were performed. Total DNA was extracted from individual nematodes and the mitochondrial COI, the ITS containing region, the D2-D3 expansion domains of the 28S rRNA gene and the 18S rRNA gene were amplified and sequenced. BLAST search at NCBI by using all molecular markers revealed that this taxon is similar to *Oscheius* species. Phylogenetic trees of ITS, 28S and 18S rDNA revealed that *O. onirici* **sp. n.** belongs to *Dolichura*-group. *Oscheius onirici* **sp. n.** is characterized by small body size and stoma rhabditoid type. Female reproductive system is amphidelphic. Males are rare with peloderan bursa, spicules slender and small, nine pairs of papillae of different lengths, arranged in a 1+1+1/3+3 pattern. Entomopathogenicity bioassay revealed that this nematode is capable of infecting larvae of *Galleria mellonella* and *Tenebrio molitor*.

Key words: 18S, D2-D3, Description, *Dolichura*-group, entomopathogenicity bioassay, ITS containing region, mitochondrial COI, taxonomy

Introduction

The subterranean environment hosts unique biological communities of remarkable diversity that, however, are still understudied in large parts of Europe (e.g. Gibert & Culver 2009) and species are continuing to be described in well-known taxa (e.g. Mazza *et al*. 2013).

The search for entomopathogenic nematodes (EPNs) has resulted in numerous surveys all over the world, in order to acquire new resources for biological control of insect pests. EPNs are ubiquitous (Hominick 2002), but some habitats, such as the cave soils, are understudied in comparison to the classic ones, at least in Italy (Tarasco *et al.* 2014).

The genus *Oscheius* Andrássy, 1976 comprises two main groups, *Dolichura* and *Insectivorus* (Sudhaus & Hooper 1994) and until now EPNs were found only in the *Insectivorus*-group (Pervez *et al*. 2013).

During a survey for subterranean entomofauna in a karst cave in Central Italy, soil samples were collected on October 2013. Only one rhabditid nematode was isolated by using the *Galleria* trap method. Morphological and molecular evidence indicated this is a new *Oscheius* species belonging to the *Dolichura*-group. Presently, seven species are recognized in this group, namely: *Oscheius bengalensis* Timm 1956, *O. dolichura* Schneider 1866, *O. dolichuroides* Anderson & Sudhaus 1985, *O. guentheri* Sudhaus & Hooper 1994, *O. pseudodolichura* Körner 1954, *O. sechellensis* Potts 1910 and *O. tipulae* Lam & Webster 1971. *Oscheius pherosophi* Smart & Nguyen 1994 has most of the characteristics of this group, but it is unique in this genus for the presence of fused spicules. The

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new taxon, *O. onirici* **sp. n.**, is herein described and its phylogenetic relationships with other *Oscheius* species are discussed.

Material and methods

Isolation of EPNs from soil

Soil samples were collected during a survey for subterranean fauna in October 2013 inside a karst cave in Central Italy (see below for further details). EPNs were isolated by the *Galleria* bait method (Bedding & Akhurst 1975). Soil samples (about 2 dm^3) were placed in two plastic containers and one steel mesh pocket containing three last-instar *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae was placed in each container and kept at room temperature (20 ± 3°C) in the Nematology laboratory of C.R.A. – Agrobiology and Pedology Research Centre, Florence (Italy). Cadavers with nematodes were placed on White trap (Kaya & Stock 1997). Juveniles emerging from the *Galleria* larvae were collected and kept in a laboratory culture in 50 ml plastic tubes. Part of them was sent to the Institute of Sustainable Plant Protection (IPSP)-CNR, Bari (Italy) for molecular characterization.

Morphological observations. Nematodes were heat-killed in warm water at 60°C, fixed in 4% formalin for 48 h, processed to glycerin by a modification of a glycerin-ethanol series of Seinhorst's (1959) rapid method and permanently mounted on microscope glass slides.

Specimens were examined and measured with a Leitz ORTHOPLAN light microscope at up to 1000×magnification and photographed using CANON Power Shot S50. Illustrations were made using a drawing tube attached to the microscope.

Scanning electron microscopy. Nematodes were rinsed in distilled water and transferred by pipette to 0.5 ml tube containing 3% glutaraldehyde and 1.8% sucrose solution in 0.1 M sodium cacodylate buffer, pH 7.2, for 16 h at 4°C. Samples were washed three times with the same buffer, post-fixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, for 16 h at 4°C in the dark, washed in three more changes of the same buffer and then dehydrated through a graded ethanol series, all on ice. Specimens were mounted on SEM stubs, coated with gold using a JEOL JFC-1300 and then examined with a JEOL NeoScope JCM-5000 (Scanning Electron Microscope - SEM), operating at 10 Kv, at CRA-ABP of Florence.

Molecular analyses. DNA extraction, PCR amplification and sequencing. Specimens for molecular analysis were kept in Ringer's solution. Genomic DNA was extracted from fifteen individual nematodes as described by De Luca *et al.* (2004). The crude DNA isolated from each individual nematode was directly amplified. The portion of the mitochondrial cytochrome oxidase c subunit 1 (*mt*COI) gene was amplified with this primer set: COI-F1 (5'-CCTACTATGATTGGTGGTTTTGGTAATTG-3') and COI-R2 (5'-GTAGCAGCAGTAAAATAAGCACG-3') (Kanzaki & Futai 2002); the ITS1-5.8S-ITS2 regions were amplified using the forward primer TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and the reverse primer AB28 (5'-ATATGCTTAAGTTCAGCGGGT -3') (Joyce *et al.* 1994); the D2A-D3B expansion segments of 28S rRNA gene using the primers D2A (5'-ACAAGTACCGTGGGGAAAGTTG-3') and the D3B (5'-TCGGAAGGAACCAGCTACTA-3') and the 18S rDNA was amplified using the 18SnF (5'-TGGATAACTGTGGTAATTCTAGAGC-3') and 18SnR (5'-TTACGACTTTTGCCCGGTTC-3') (Zeng *et al.* 2007). PCR cycling conditions used for amplification were: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 50s, annealing at 55°C for 50s and extension at 72°C for 1 min and a final step at 72°C for 7 min. The size of the amplification products was determined by comparison with the molecular weight marker ladder 100 (Fermentas, St. Leon-Rot, Germany) following electrophoresis of 10 µl on a 1% agarose gel.

PCR products of the partial mitochondrial COI, the ITS containing region and the 18S rRNA gene from three individual nematodes for each marker were purified for sequencing using the protocol listed by manufacturer (High Pure PCR elution kit, Roche, Germany). Purified DNA fragments were cloned and sequenced in both directions. PCR products of the D2-D3 expansion domains of the 28S rRNA gene were directly sequenced. A BLAST (Basic Local Alignment Search Tool) search at NCBI (National Center for Biotechnology Information) was performed in order to confirm their nematode origins and species (Altschul *et al.* 1997). The newly obtained sequences for ITS containing region, the D2-D3 domains of the 28S rRNA gene and the partial 18S gene were aligned using ClustalW (Larkin *et al.* 2007) with default parameters with the corresponding published gene sequences of

Oscheius and *Rhabditis*. Sequence alignments were manually edited using BioEdit. Outgroup taxa for each dataset were chosen according to the results of previously published data. Phylogenetic trees, obtained for both ITS and 18S datasets, were constructed with Neighbour-Joining (NJ), Minimum Evolution (ME), Maximum Likelihood (ML) and Maximum Parsimony (MP) methods using MEGA version 6 software (Tamura *et al.* 2013). No significant conflict in branching order and support level among methods is observed, only ML tree is shown for each marker. ML analysis under a general time reversible and a gamma-shaped distribution (GTR + G) model for ITS and 18S datasets was performed. The phylograms were bootstrapped 1,000 times to assess the degree of support for the phylogenetic branching indicated by the optimal tree for each method. The newly obtained sequences were submitted to GenBank with the following accession numbers LN613268-LN613269 for *mt*COI; LN613265-LN613267 for the ITS; LN613263-LN613264 for the D2-D3 expansion domains of the 28S rRNA gene LN613261-LN613262 for the 18S rRNA gene.

RFLP analysis. Ten µl of each product, containing the ITS region, from two individual nematodes from *O. onirici* **sp. n.** isolate were digested with the following restriction enzymes: *Alu* I (Roche), *Ava* II (Roche), *Bam* HI (Roche), *Dde* I (Roche), *Hinf* I (Roche), and *Rsa* I (Roche) (5 U of enzyme for each digestion) at 37°C overnight. The digested DNA fragments were loaded onto 2.5% agarose gel and visualized by red staining gel according to the manufacturer.

Nematode entomopathogenicity. Nematode entomopathogenicity of *O. onirici* **sp. n.** was evaluated in comparison to *Steinernema carpocapsae* (Weiser 1955) Wouts, Mráček, Gerdin & Bedding 1982, using two host insects, in pathogenicity bioassays. A Petri dish (3.5 cm diam.) with two layers of filter paper (Whatman No. 1) containing one last instar larva of *G. mellonella* or *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) was used as experimental unit. A distilled water suspension of 250 µl containing about 400 Infective Juveniles (IJs) was inoculated into each Petri dish. Only distilled water was used as control. Petri dishes were stored at 24°C in darkness. Mortality of larvae was evaluated every 24 h for a week. Fifteen larvae/host insect were used for each bioassay and the experiments were repeated three times. Half of the dead larvae were dissected to evaluate the presence and development of nematodes inside and half were transferred to White traps to determine nematode emergence.

In virulence tests, mortality data were corrected for natural mortality in controls using Abbott's formula (Abbott 1925). Lethal time to 50% mortality (LT_{50}) was estimated.

Description

Oscheius onirici sp. n.

(Figs. 1–3, Table 1)

***Female*:** Body almost straight to slightly bent on ventral side when relaxed by gentle heat. Cuticle finely annulated. Lateral fields with basically three longitudinal ridges, delimited by four lines. In few specimens, an additional short running line could be seen in the middle part of body (Fig. 2H). Six lips, separate, continuous with body contour; six bristle-like labial sensilla, one on each lip. Amphidial apertures elliptical (Fig. 3E), located on lateral lips. Stoma rhabditoid type, *ca* four times longer than diam. Cheilostom not sclerotized, gymnostom culticularized, stegostom (pharyngeal collar) conspicuous, enveloping *ca* 50% of stoma length. Glottoid apparatus well developed, isomorphic. Pharyngeal corpus cylindrical, devoid of valvate median bulb, ending in a round basal bulb. Nerve ring localized at *ca* 64–67% of pharyngeal length. Excretory pore conspicuous, ventrally located at level ranging 73–100% of pharyngeal length. Hemizonid not clearly observed. Reproductive system amphidelphic. Ovaries reflexed anteriorly. Vulva in form of a transverse slit. Vagina *ca* 16% of corresponding body diameter. Rectum long, ranging 2.4–2.8 anal body diam. long, sometimes expanded proximally. Spermatheca filled with sperm. Tail conical, gradually tapering to a fine point, *ca* 4 anal body diam. long. Phasmids pore-like, located behind anus at 14 to 25 % of tail length.

***Male*:** Rare (only twelve, were found). Body straight to open C when heat-killed. General morphology similar to female except for smaller size and reproductive system. Testis single, anterior end reflexed ventrally. Spicules paired, of equal length, separate, with a small, rounded manubrium; lamina expanded in its proximal part, with a more or less evident velum and ending in a rounded tip. Gubernaculum slender and slightly curved, *ca* 46% of

spicule length. Bursa peloderan, not open; nine pairs of papillae of different lengths, arranged in a 1+1+1/3+3 pattern. Pairs 4–6 and 7–9 more closely spaced than pairs 1–3. Pairs 5 and 8 curved dorsally and not reaching rim of bursa. Tail *ca* 1.1 anal body diam. long. In three males, the tail protruded slightly outside the bursa (Fig. 1I)

***Dauer juveniles*:** Body straight when heat-killed. Stoma and pharynx morphology similar to adult. Lateral fields with two longitudinal ridges. Nerve ring localized at *ca* 67% of pharyngeal length. Excretory pore located at *ca* 83% of pharyngeal length. Tail elongate, conical tapering to a hyaline part 11.5 ± 1.6 (9.5–16) µm long.

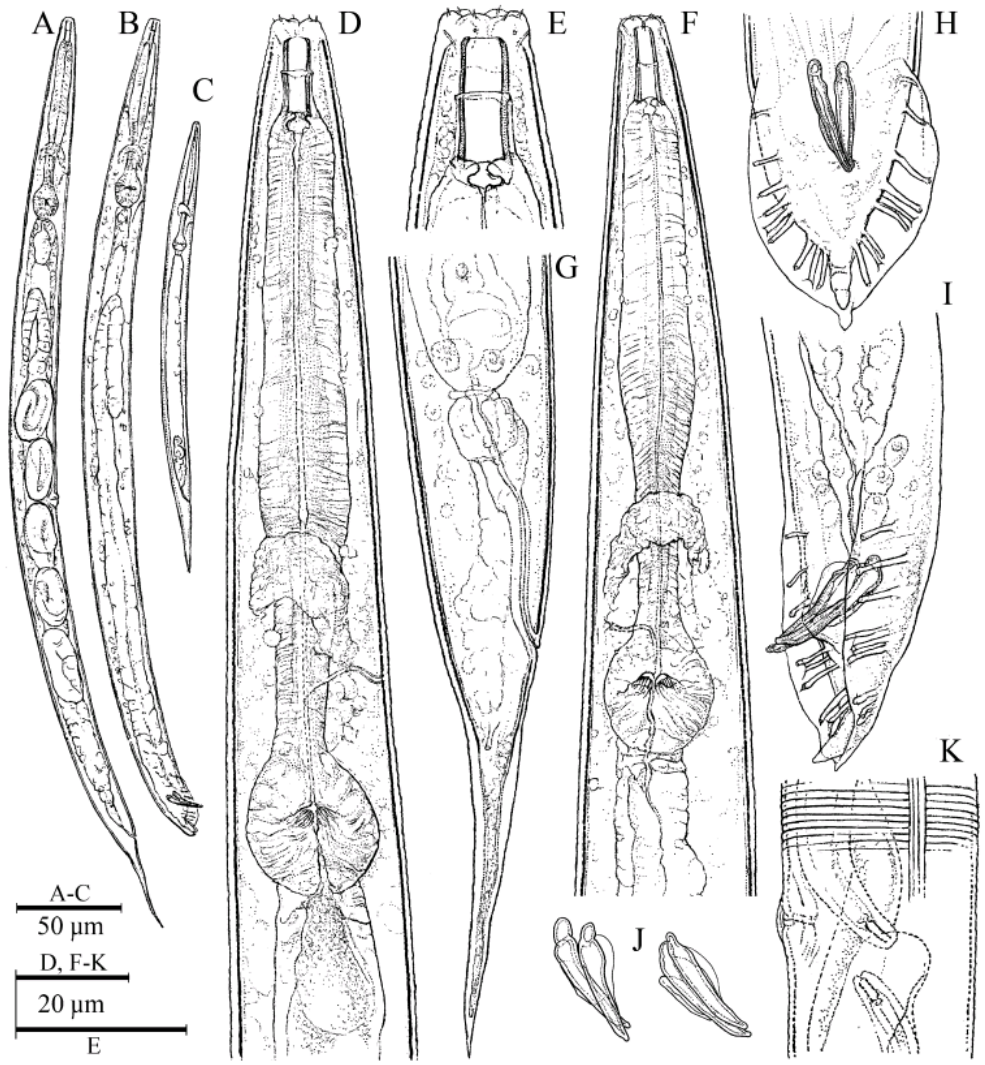

FIGURE 1. *Oscheius onirici* **sp. n.** A–C: Entire body of female (A), male (B) and dauer juvenile (C); D: Female pharyngeal region; E: Stoma; F: Male pharyngeal region; G: Female tail region; H, I: Male tail in ventral and lateral view, respectively; J: Morphology of male spicule and gubernaculum; K: Female vulval region.

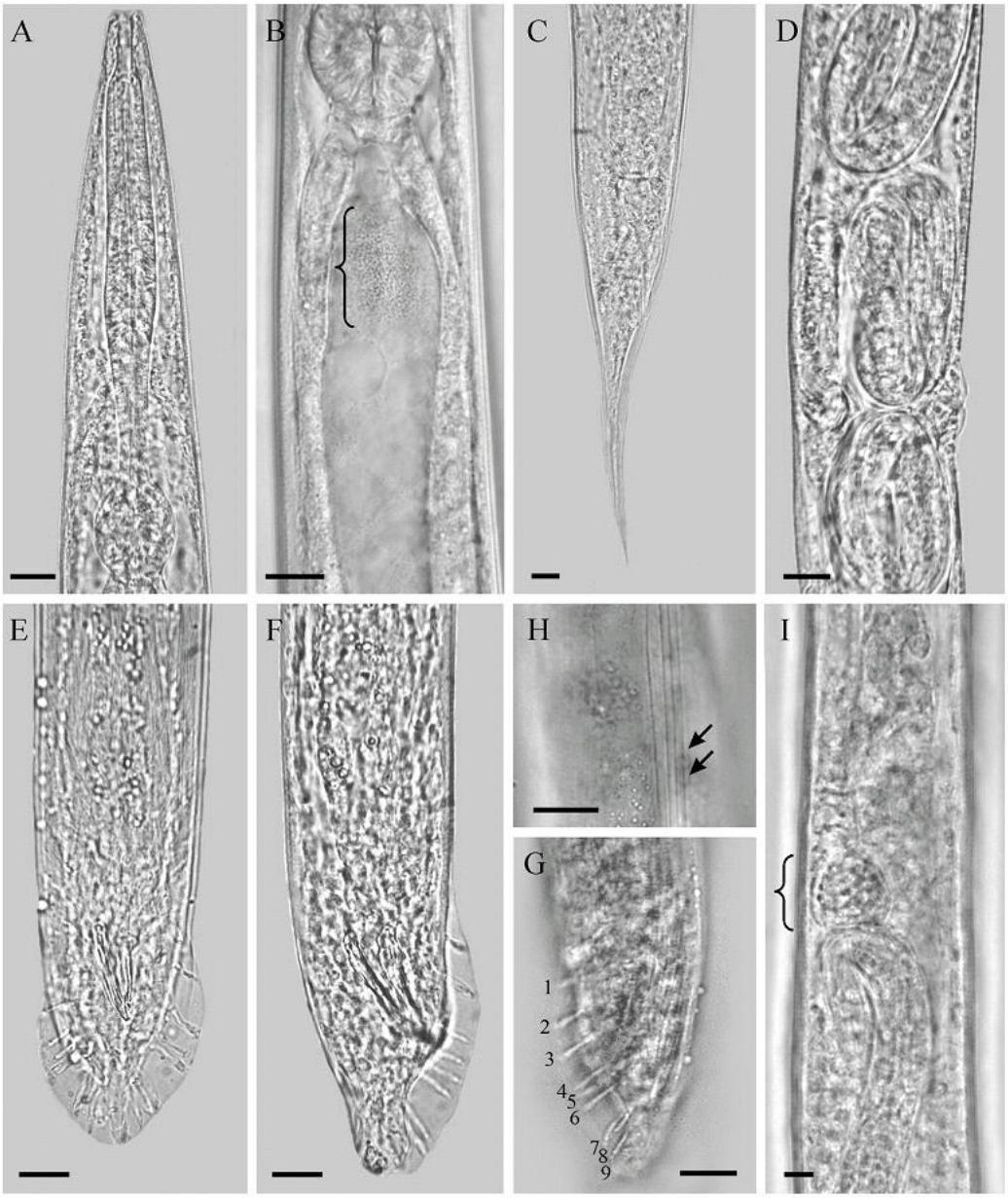

FIGURE 2. Light micrographs of *Oscheius onirici* **sp. n.** A: Female pharyngeal region; B: Female anterior part of intestine, showing bacteria (bracket); C: Female tail; D: Vulval region, with embryonated eggs in the uterus; E: Ventral view of male tail; F: Lateral view of male tail; G: Male tail showing bursal rays arrangement; H: Female lateral field at midbody; arrowed is a discontinuous fifth line, occasionally visible in some of the specimens; I: Female anterior genital tract showing the spermatheca (bracket). (Scale bars = 10 μm).

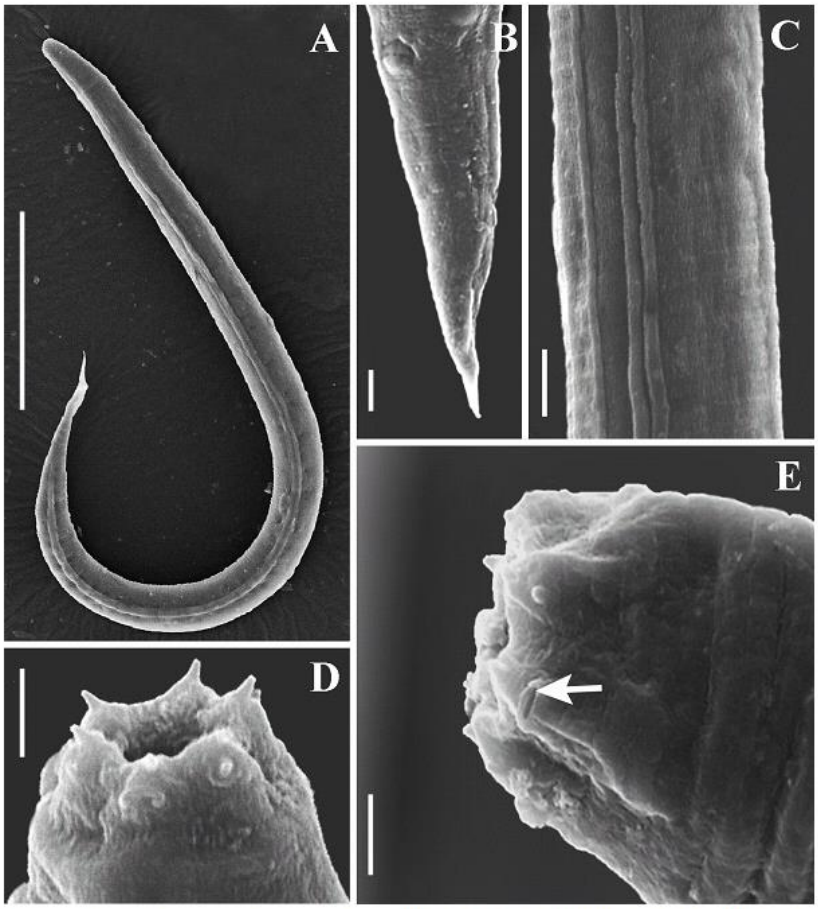

FIGURE 3. Scanning electron micrographs of *Oscheius onirici* **sp. n.** (A, B, C: Juvenile; D, E: Female): A: Entire body; B: Tail; C: Lateral field at mid-body; D, E: Lip region, showing lips with their labial sensilla (D) and amphidial aperture (E, arrowed). (Scale bars: A = 50 µm; B, C = 5 µm; D, E = 2 µm) .

Type host and locality. Natural host unknown, since material was recovered by soil samples inside the karst cave "Grotta del Lago" in the Monti Pisani area: 10°28'24.8"E, 43°44'17.7"N, municipality of San Giuliano Terme, Pisa Province, Tuscany Region, Central Italy (Mazza *et al*. 2014).

Type material. Holotype male, ten paratype females and ten dauer juveniles are mounted on glass slides and deposited at the laboratory of Nematology of the CRA-ABP of Florence (Italy). Two glass slides with two paratype males, two females and five dauer juveniles were deposited in the laboratory of Department of Soil, Plant and Food Sciences, Section of Entomology and Zoology, University of Bari "A. Moro", Bari (Italy) and in the nematode collection at the Institute for Sustainable Plant Protection (IPSP), CNR, Bari, Italy. Additional paratypes were distributed to the United States Department of Agriculture Nematode Collection, Beltsville, MD, University of California Riverside Nematode Collection and WaNeCo, Plant Protection Service, The Netherlands, Wageningen (one glass slide with two males and two females for each Collection).

Etymology. The name of the species refers to the dream [from greek ὄνειρον, -ου, τὸ (oneiron)] because one of the authors had a premonitory dream on the discovery of this new species with the indication on the precise locality of the collection.

Nematode entomopathogenicity. *Oscheius onirici* **sp. n.** infected 46% of *Galleria* larvae and 58% of *Tenebrio* larvae, while *S. carpocapsae* killed 100% of these insects in one week (LT_{50}: 6.8 (*Galleria*) and 5.2

(*Tenebrio*) days for *O. onirici,* **sp. n.** and 1.4 days for *S. carpocapsae*). IJs of both nematodes emerged from all cadavers within 72 h after host death, and from all the dissected dead larvae nematodes of different developmental stages were recovered. *Galleria* and *Tenebrio* larvae death was sufficiently rapid with a 33% of insect larvae death within 120 hours.

TABLE 1. Morphometrics of *Oscheius onirici* **sp. n.** All measurements are in µm: mean ± s.d. (range).

Character	Male		Female	Infective juveniles
	Holotype	Paratypes	Paratypes	Paratypes
n	–	10	20	20
L	648	510±35.2 (443–553)	671±65.5 (584–801)	334±19.6 (304–370)
a	17.5	16.1±1.2 (14.6–18.2)	19±2.0 (15.8–22.5)	18.8±2.6 (14.9-23.9)
b	4.3	4.1±0.3 (3.8–4.6)	4.9±0.5 (4.4–6.0)	3.6±0.1 (3.4-3.8)
c	26	24.2±3.1 (21.2–30.4)	9.7±0.9 (8.6–11.8)	6.9±0.3 (6.3-7.6)
c'	1.3	1.2±0.2 (0.9–1.5)	4.3±0.4 (3.5–5.0)	32.6±2.7 (27.9-36.7)
V	–	–	51.0±1.3 (47.3–52.6)	–
Max. body diam.	37	32±1.8 (29–35)	36±5.3 (30–51)	18±2.2 (16–22)
Pharynx	150	125±5.4 (117–134)	137±6.2 (126–146)	94±4.2 (86–103)
Stoma length	14	13±0.9 (12–15)	14±1.1 (12–16)	12±1.1 (10–13)
Stoma diam.	4	3±0.5 (3–5)	4±0.4 (3–4)	2±0.2 (2–3)
Basal bulb length	21	20±1.2 (18–22)	23±1.3 (21–25)	13±1.1 (11–15)
Basal bulb diam.	18	17±1.8 (15–21)	19±1.2 (17–21)	10±0.6 (9–11)
Excretory pore	118	93±6.7 (85–104)	108±13.2 (92–146)	78±5.0 (64–88)
Diam. at excretory pore	29	26±1.4 (24–29)	28±4.3 (22–39)	15±1.1 (13–18)
Nerve ring	85	82±6.2 (75–95)	90±5.0 (80–98)	63±3.2 (58–68)
Tail length	25	21±2.9 (18–25)	69±5.5 (63–81)	49±3.3 (41–53)
Anal body diam.	19	19±2.3 (16–22)	16±2.2 (13–23)	10±1.0 (9–12)
Testis reflex	63	61±12.4 (46–78)	–	–
Spicule length	23	22±1.2 (20–24)	–	–
Spicule width	5	4±0.8 (4–6)	–	–
Gubernaculum length	9	10±1.4 (9–13)	–	–
SW	1.2	1.2±0.2 (1.0–1.5)	–	–
GS%	39.1	47.7±6.7 (35.1–56.9)	–	–
D%	78.7	74.6±6.7 (63.5–85.5)	78.9±9.6 (63.4–101.7)	83.5±5.6 (74.5–92.2)
E%	–	–	–	161.6±15.8 (135.8–200.2)

Diagnosis and relationships. *Oscheius onirici* **sp. n.** is characterized by a small size (mean body length of the female is 671 µm, of the male 510 µm and of the dauer juvenile 334 µm), cuticle finely annulated, stoma rhabditoid type, *ca* three times longer than its diameter and a lateral field marked by three ridges (four longitudinal lines) in adults. Female have an amphidelphic reproductive system, a long rectum (*ca* 2.4 anal body diam. long), a spermatheca with sperm, and a conical tail, gradually tapering to a finely pointed terminus (*ca* 4 anal body diam. long). Males are characterized by a short, rounded tail, peloderan bursa with nine pairs of papillae of different lengths, arranged in a 1+1+1/3+3 pattern, spicules small and slender.

The peloderan bursa, expansile rectum and the not crochet-needle-shaped spicules place *O. onirici* **sp. n.** in the *Dolichura*-group of *Oscheius* (Sudhaus & Hooper 1994, Sudhaus & Fitch 2001). Species comparisons in the *Dolichura*-group are presented in Table 2.

TABLE 2. Comparison of species in the *Dolichura*-group of *Oscheius*. All measurements are in µm: mean ± s.d. (range). Important characters are indicated in bold.

	O. bengalensis	*O. dolicura*	*O. dolicuroides*	*O. guentheri*	*O. onirici* sp. n.	*O. pseudodolicura*	*O. sechellensis*	*O. tipulae*
Type locality	Bengal	–	Kenia	Vietnam	Italy	–	Seychelles	Germany
Type host/habitat	Algae (*Siphonocladus*)	–	Scarabaeidae larva	Rice plants (*Oryza sativa*)	Soil cave	–	Moss	*Tipula paludosa* larvae
Lateral lines	4	2	4	4	4	4	–	4
Female								
L	1160–1643	686–1110	1355 ± 129 (167–1580)	863 (733–1071)	671 ± 65.5 (584–801)	915–1216	680	603 (505–691)
a	17.0–23.4	14–19.8	27 ± 2.7 (22–31)	18.3 (15.8–20.7)	19 ± 2.0 (15.8–22.5)	15.4–19.9	–	18.4 (16.3–19.9)
b	5.5–7.5	5.3–8.6	5.4 ± 0.5 (4.6–6.2)	6.1 (5.6–6.6)	4.9 ± 0.5 (4.4–6.0)	6.6–8.0	–	4.3 (3.9–4.9)
c	12.4–17.1	7.7–11.2	9.4 ± 0.7 (8.2–11.2)	6.1 (5.4–7.0)	9.7 ± 0.9 (8.6–11.8)	7.4–8.3	–	7.0 (6.2–8.5)
c'	–	–	7.0 ± 0.7 (5.9–8.2)	7.7 (6.9–9.6)	4.3 ± 0.4 (3.5–5.0)	–	–	5.2 (4.2–6.4)
V	48–52	48.7–55	54 ± 1.7 (51–57)	53 (50–56)	51.0 ± 1.3 (47.3–52.6)	45.5–49.1	50.6*	48.6 (46.4–51.7)
Max. body diam.	57–96	54–74	–	47 (38–61)	36 ± 5.3 (30–51)	46–79	–	33 (29–42)
Pharynx	197–234	130–197	255 ± 10.5 (231–273)	143 (126–162)	137 ± 6.2 (126–146)	–	128	140 (129–151)
Excretory pore	–	–	207 ± 25 (178–225)	–	108 ± 13.2 (92–146)	–	–	105 (84–120)
Nerve ring	–	–	–	–	90 ± 5.0 (80–98)	–	–	100 (81–109)
Tail length	83–117	73–116	144 ± 13.5 (121–172)	145 (116–172)	69 ± 5.5 (63–81)	–	120	83 (70–95)

.....continued on the next page

TABLE 2. (Continued)

	O. bengalensis	*O. dolicura*	*O. dolicuroides*	*O. guentheri*	*O. onirici* sp. n.	*O. pseudodolicura*	*O. sechellensis*	*O. tipulae*
Male								
L	845–1375	528–886	1118 ± 129 (1045–1286)	745 (655–799)	510 ± 35.2 (443–553)	764	496	676
a	19.4–22.2	16.5–19.1	30 ± 2.3 (27–35)	19.7 (18.4–20.4)	16.1 ± 1.2 (14.6–18.2)	20.1	–	17.2
b	4.8–6.6	4.5–5.8	4.9 ± 0.3 (4.5–5.7)	5.5 (4.9–5.8)	4.1 ± 0.3 (3.8–4.6)	6.4	–	5.4
c	26.8–48.1	19.5–28.9	43 ± 6 (32–56)	19.5 (18.4–20.8)	24.2 ± 3.1 (21.2–30.4)	25.5	–	24
c'	–	–	1.3 ± 0.2 (1.0–1.7)	–	1.2 ± 0.2 (0.9–1.5)	–	–	–
Max. body diam.	28–63	27–51	37 ± 3.3 (30–41)	38 (36–39)	32 ± 1.8 (29–35)	38	–	39
Pharynx	163–210	108–197	228 ± 6.6 (216–241)	136 (134–140)	125 ± 5.4 (117–134)	–	–	126
Tail length	24–34	24–32	66 ± 3.8 (60–74)	38 (36–40)	21 ± 2.9 (18–25)	–	–	28
Bursa arrangement	1+1+1/3+3	–	1 + 1 +1/3 +3	1+ 1+1/1+2+ 1+2+r or 1+ 1+1/1+2+ 2+1+r	1 + 1 +1/3 + 3	1 +1 + 1/3 +3	1 + 1 + 1/3 + 3**	1 + 1 + 1/3 +3
Spicule length	26–39	32–35	46 ± 2.0 (41–48)	19 (17–21)	22 ± 1.2 (20–24)	27	–	25
Gubernaculum length	14–18.5	19–27	26 ± 2.3 (22–29)	–	10 ± 1.4 (9–13)	–	–	–
Reference	Sudhaus (1974)	Völk (1950)	Anderson & Sudhaus (1984)	Sudhaus & Hooper (1994)	present study	Körner (1954)	Potts (1910)	Sudhaus (1993)

* Calculated

** After original drawing (Potts, 1910) and reported by Sudhaus & Hooper (1994)

Oscheius onirici **sp. n.** is most closely related to *O. tipulae* (Sudhaus 1993), for similar body size, but can be separated by shorter body length (510 *vs* 676) and shorter spicules in males and shorter tail length in both adult stages. Females also differ in the value of ratio c (9.7 *vs* 7.0 in *O. tipulae*). Body length of the adult of the new species is similar to *O. sechellensis*, but it is clearly different for the presence of four lateral lines *vs* two.

Oscheius onirici **sp. n.** can be distinguished from *O. pseudodolichura* (Körner 1954) by smaller size of both adult stages. They also differ in the value of ratio b (female: 4.4–6.0 *vs* 6.6–8.0; male: 3.8–4.6 *vs* 6.4) and females differ in the value of ratio c (8.6–11.8 *vs* 7.4–8.3). *Oscheius onirici* **sp. n.** differs from *O. bengalensis* (Sudhaus 1974), *O. dolichuroides* by smaller size of both adult stages and also for shorter pharynx, shorter male spicules and gubernaculum and shorter female tail (see Table 2). Based on measurements by Völk (1950), *O. onirici* **sp. n.** differs from *O. dolichura* (Schneider 1866) in having a shorter pharynx in males (126–146 *vs* 130–197) shorter tail in both adult stages, shorter male spicules and gubernaculum and four *vs* two lines in lateral fields .

Finally, *O. onirici* **sp. n.** can be separated from *O. guentheri* by the shorter adults body size, by the shorter tail and by bursa arrangement, though in *O. guentheri* is uncertain. Adults of *O. onirici* **sp. n.** also differ in the value of ratio c (female: 8.6–11.8 *vs* 5.4–7.0; male: 21.2–30.4 *vs* 18.4–20.8).

Since different standard were used to assess classification of EPNs, according to Dillman *et al.* (2012) *O. onirici* **sp. n.** is an entomopathogenic nematode, because of its ability to reproduce inside the body of the insects and because *Galleria* and *Tenebrio* larvae death was sufficiently rapid that it can be distinguished from phoretic, necromenic, and other parasitic associations. This new species is the first EPN of *Dolichura*-group.

Molecular analysis

PCR-RFLP analysis. Amplification of the ITS region in *O. onirici* **sp. n.** from Italy produced a fragment of 881 bp in length for all specimens. PCR-ITS-RFLP patterns for *O. onirici* **sp. n.** are given in Figure 4. Restriction of the PCR products by each of six enzymes, *Alu* I, *Ava* II, *Bam* HI, *Dde* I, *Hinf* I and *Rsa* I, produced species-specific profiles for this species. Sequence analysis of *O. onirici* **sp. n.** ITS revealed the presence of two *Bcl* I restriction sites as reported for isolates PS2068 (AJ297891), PS335 (AJ297892), JU179 (AJ291722) and JU77 (AJ297893) belonging to sp. 2 mating group, by Felix *et al.* (2001).

Sequence analysis. The amplification of the *mt*COI from *O. onirici* **sp. n.** produced a fragment of 642 bp in length. No intraspecific variation was determined between specimens belonging to Italian *O. onirici* **sp. n.** isolate. The nucleotide sequences of *mt*COI of *O. onirici* **sp. n.** obtained in this study showed 93% similarity with the corresponding COI sequence of *Parapristionchus giblindavisi* (Kanzaki *et al.* 2012). No corresponding COI sequences of other *Oscheius* spp. are present in the database.

The sequences of the entire ITS of *O. onirici* **sp. n.** were determined for two specimens. No intraspecific differences were found in the sequences of these specimens. The ITS sequences of *O. onirici* **sp. n.** were compared with all sequences of *Oscheius* spp. present in the database. The result revealed that *O. onirici* **sp. n.** is identical to the corresponding sequences of *Oscheius* isolates PS 335 (AJ29789), PS 2068 (AJ297892), JU 179 (AJ297893) and JU77 (AJ291722), 67-P20 (KJ938578) and 69-P20 (KJ938580) (Table 3). All sequences showing identical sequences to *O. onirici* **sp. n.** were also characterized by the presence of two *Bcl* I restriction sites confirming that *O. onirici* **sp. n.** corresponds to sp. 2 mating group as reported by Felix *et al.* (2001).

TABLE 3. Percentage of ITS similarity with the closest *Oscheius* sequences present in the database.

	Oscheius PS 335	*Oscheius* PS 2068	*Oscheius* JU 179	*Oscheius* JU 77
O. onirici **sp. n.**	100	99	99	99

continued.

	Oscheius 67–P20	*Oscheius* 69–P20	*O. tipulae*
O. onirici **sp. n.**	99	99	82

The D2-D3 expansion domains of the 28S rRNA gene was amplified and sequenced in *O. onirici* **sp. n.** The direct sequencing of two specimens produced a fragment of 624 bp in length and no intraspecific variability was determined between specimens of the Italian population. The pairwise comparisons of the D2-D3 expansion

domains of *O. onirici* **sp. n.** are reported in Table 4. The sequencing of the partial 18S rRNA gene produced a sequence of 1588 bp. The partial 18S sequence was 99% identical to *Oscheius* spp. isolate PS 335 (HQ130503), 1586/1588 identities and 1 gap, 99% similar to *O. tipulae*, 1564/1583 identities and 2 gap, 97% similar to *O. dolichura* isolate PS330, 1517/1588 identities, 94% similar to *O. guentheri* and 93% to *O. dolichuroides*.

TABLE 4. Percentage of D2–D3 similarity with the closest *Oscheius* sequences present in the database.

	O. tipulae	*O. guentheri*	*O. dolichura*	*O. dolichuroides*	*O. myriophilus*
O. onirici **sp. n.**	98	86	84	84	84

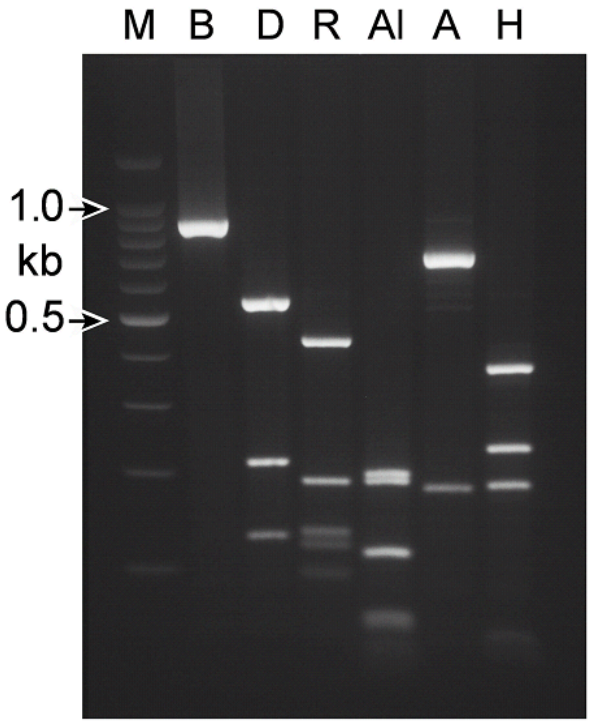

FIGURE 4. Restriction fragments of amplified ITS containing region of *Oscheius onirici* **sp. n.** M:100 bp ladder, B: *Bam*HI, D: *Dde*I, R: *Rsa*I, AI: *Alu*I, A: *Ava*II, H: *Hinf*I.

Phylogenetic relationships. Phylogenetic trees generated by Neighbour-Joining (NJ), Maximum Likelihood (ML), Minimum Evolution (ME) and Maximum Parsimony (MP) showed no significant conflict in branching order and support level, thus only ML tree is shown for each marker.

ITS is a highly variable DNA fragment. As the ITS sequences of *O. onirici* **sp. n.** matched well with few closely related *Oscheius* species, only these sequences were included in the phylogenetic analysis. The phylogenetic tree based on the ITS sequences of closely related *Oscheius* species present in the GenBank sequence database (http://www.ncbi.nlm.nih.gov/) is reported in Fig. 5. *Heterorhabditis bacteriophora* Poinar 1976 and *Steinernema carpocapsae* were used as outgroups. The phylogenetic tree revealed that *O. onirici* **sp. n.** grouped with *Oscheius* sp. isolates PS 335, JU 179, PS 2068 and JU 77, 67-P20 and 69-P20 with 100% bootstrap support confirming that all these isolates are closely related to *O. onirici* **sp. n.** Furthermore, the analysis shows that *O. onirici* **sp. n.** is a sister species of *O. tipulae* and *Oscheius* isolate JU 75. For the last isolate, JU 75, Felix *et al.* (2001) also demonstrated that it was a biological species closely related to *O. tipulae* and to the other isolates showing identical sequences with *O. onirici* **sp. n.**

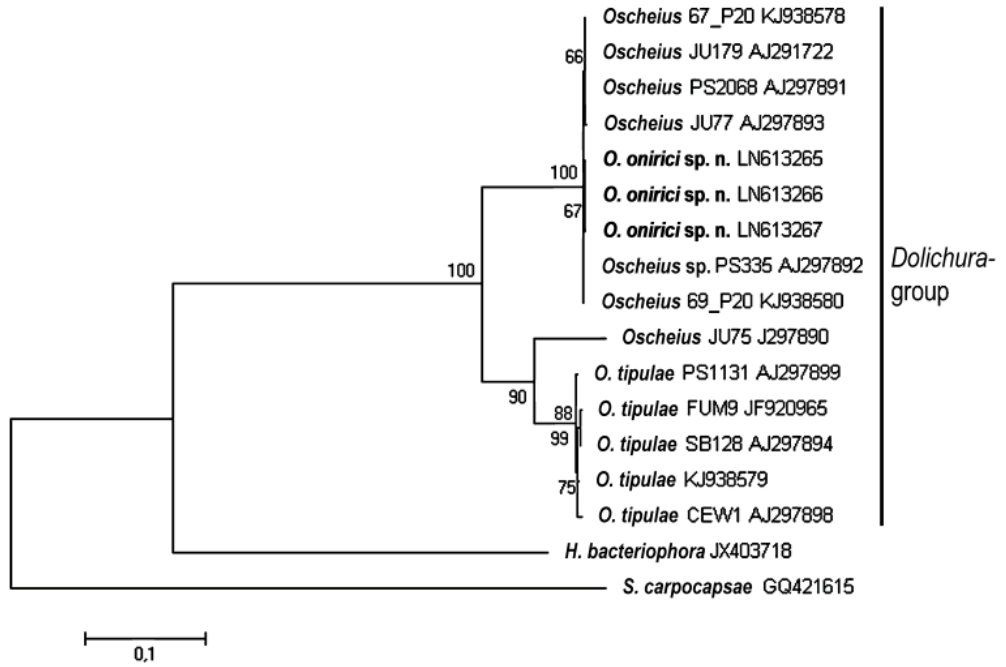

FIGURE 5. Phylogenetic trees of ITS containing region of *Oscheius onirici* **sp. n.** and the closest species. Sequences were analysed using Maximum Likelihood method. Numbers at nodes indicate bootstrap values.

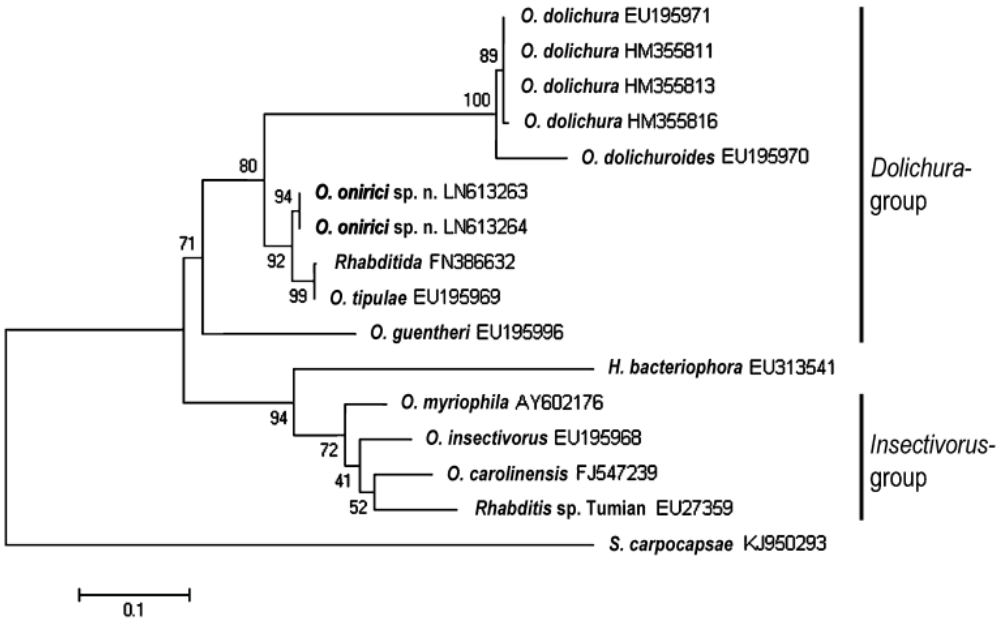

FIGURE 6. Phylogenetic trees of partial D2-D3 expansion domains of 28S rRNA gene of *Oscheius onirici* **sp. n.** and the closest species. Sequences were analysed using Maximum Likelihood method. Numbers at nodes indicate bootstrap values

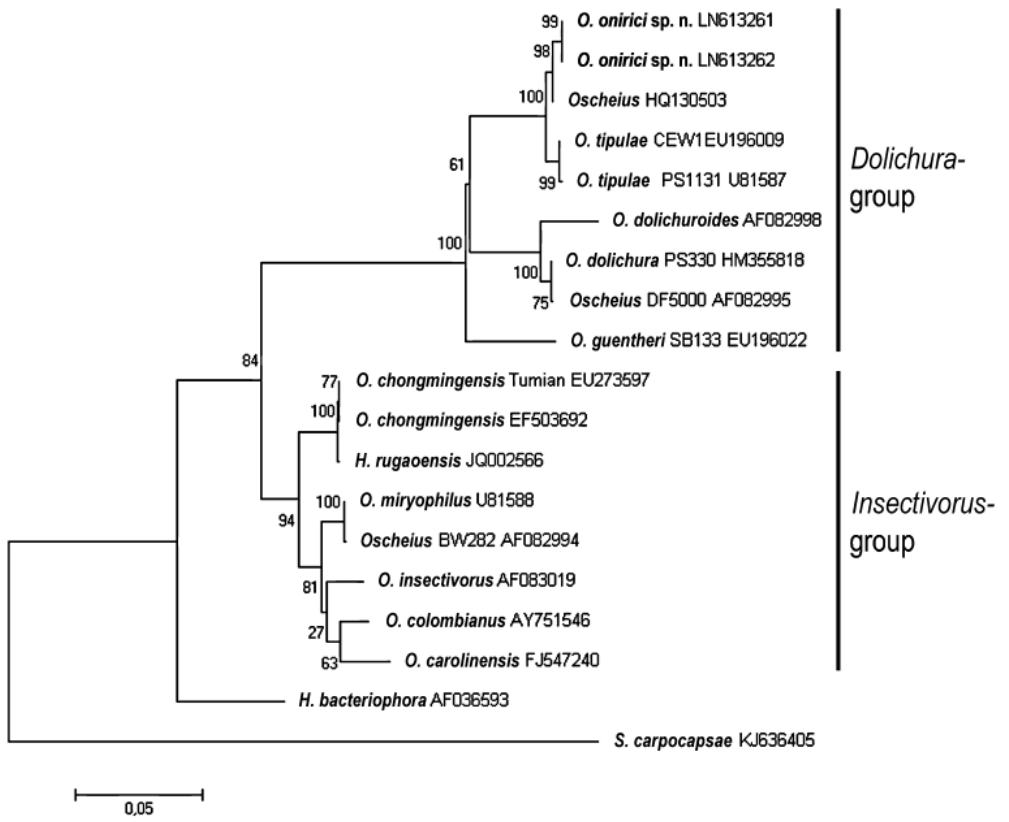

FIGURE 7. Phylogenetic trees of partial 18S rRNA gene of *Oscheius onirici* **sp. n.** and the closest species. Sequences were analysed using Maximum Likelihood method. Numbers at nodes indicate bootstrap values.

The phylogenetic tree based on D2-D3 sequences of all *Oscheius* spp. and *Rhabditis* sp. present in the GenBank sequence database (http://www.ncbi.nlm.nih.gov/) is reported in Fig. 6. *H. bacteriophora* and *S. carpocapsae* were used as outgroups. The results revealed that *O. onirici* **sp. n.** is a sister species of *O. tipulae* and Rhabditida environmental sample (FN386632). Furthermore, *O. onirici* **sp. n.** clustered with *O. guentheri*, *O. dolichura* and *O. dolichuroides* forming the *Dolichura*-group. The outgroup *H. bacteriophora* (EU313541) was embedded in the *Insectivorus*-group suggesting that the D2–D3 sequences are not sufficient to resolve high level relationships.

Figure 7 shows a phylogenetic tree based on the nearly full-length 18S rDNA in which all species of *Oscheius* formed two well defined groups with 88% support, clearly separated from all other genera. Among the *Oscheius* species included in the phylogenetic analysis, two groups were well defined: the *Insectivorus*-group and *Dolichura*-group, each with 100% support as reported by Ye *et al.* (2010). The *Insectivorus*-group includes *O. carolinensis* Ye *et al.* 2010, *O. insectivorus* Körner 1954, *O. colombianus* Stock *et al.* 2005, *O. myriophilus* Sudhaus & Schulte 1989, *O. chongmingensis* Zhang *et al.* 2008, *Heterorhabditidoides rugaoensi* Zhang *et al.* 2012 and one undescribed species (AF082994). The *Dolichura*-group includes *O. onirici* **sp. n.**, *O. tipulae*, *O. dolichura*, *O. dolichuroides*, *O. guentheri* and two undescribed *Oscheius* sp. (HQ130503, AF082995). *Oscheius onirici* **sp. n.** clustered together with *O. tipulae* and undescribed *Oscheius* sp. (HQ130503) with 100% bootstrap support confirming it as sister species of *O. tipulae*. This finding is consistent with the morphological data obtained in this study and with those reported by Sudhaus & Hooper (1994). The clustering of *H. rugaoensis* with *Rhabditis*-

Tumian and *O. chongmingensis*, with 100% support, suggests a re-examination of this species in order to establish if it belongs to *Oscheius* genus.

Overall, the phylogenetic trees examined here confirm that the *Insectivorus*-group and *Dolichura*-group formed monophyletic groups with high support. The results obtained herein demonstrated that all markers allocated *Oscheius* species to their corresponding groups and in particular *O. onirici* **sp. n.** clustered within *Dolichura*-group.

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APPENDIX B

REDIA, XCVII, 2014: 89-94

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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF A *STEINERNEMA CARPOCAPSAE* (NEMATODA STEINERNEMATIDAE) STRAIN ISOLATED IN VENETO REGION (ITALY)

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Torrini G., Landi S., Benvenuti C., De Luca F., Fanelli E., Troccoli A., Tarasco E., Bazzoffi P., Roversi P.F. – Morphological and molecular characterization of a *Steinernema carpocapsae* (Nematoda Steinernematidae) strain isolated in Veneto region (Italy).

A new strain of *Steinernema carpocapsae* (Weiser, 1955) was isolated from soil collected in a lagoon plain in Veneto region (North-East Italy). This new strain was named ItS-CAO1. Molecular and morphological analyses were performed. The ITS region and the 18S rRNA gene were amplified and sequenced. The ITS products were then digested with six restriction enzymes in order to unequivocally identify this species. Nematode virulence was tested against last instar of *Galleria mellonella* (L.) using different laboratory assays. Insect mortality of this new strain is very high in penetration (100%) and sand column assay (93.3%) and the percentage of penetrating infective juveniles was 57.6 and 42.9, respectively. Larval mortality in one-on-one quality assay was 50% and in exposure time assay it was 50% at 19 minutes. With the results of infectivity assays we can evaluate the possibility to use this new strain in biological control programs.

KEY WORDS: entomopathogenic nematode, ITS region, PCR-RFLP, infectivity assay, biological control

INTRODUCTION

Entomopathogenic nematodes (EPN) in the genera *Heterorhabditis* and *Steinernema* are capable of controlling a wide variety of economically important insect pest worldwide (KAYA *et al.*, 2006). In the entomopathogenic nematode infection process, infective juveniles (IJs) penetrate into the insect haemolymph and release the symbiotic bacteria (*Xenorhabdus* spp. for steinernematids and *Photorhabdus* spp. for heterorhabditids) that kill the insect host, usually in short time.

EPN have been used successfully as biological control agents to suppress insect populations (SHAPIRO-ILAN *et al.*, 2002). At present, mainly non-native strains have been used in nematode applications, but these strains may not be well adapted to environmental conditions, whereas native species are adapted to local climatic conditions and have therefore more likely to survive after application.

During a survey in Veneto region, a new steinernematid strain of *Steinernema carpocapsae* were found in soil samples collected. The objectives of this study were (i) to isolate and identify EPN, (ii) to characterize at molecular level the Italian isolate, (iii) determine their survival and infectivity to *Galleria mellonella* larvae using different types of laboratory bioassays. This information will serve as basic knowledge to develop new biological control agents from native insect parasitic nematodes.

MATERIALS AND METHODS

ISOLATION OF EPN FROM SOIL

A survey was conducted on September 2013 in a site located at farm Vallevecchia of Veneto Agricoltura (45°37'51.21"N-12°58'10.29"E), in Caorle (Venezia) in lagoon plain at 1 m a.s.l. The mean annual air temperature of the site is 13.5°C with average maximum temperatures in July-August (28.4°C) and average minimum temperatures in January (-0.8°C). The mean annual precipitation is 806 mm concentrated in October (109 mm). The soil texture was classified as silt-loam with 19.5% (standard error 0.38) of sand, 63.4% (0.19) of silt and 17.1% (0.23) of clay. Until 2008, a rotation based on maize and sorghum was applied. Three different set-aside regimes were established in 2008. The total area of three plots was 1.5 ha (0.5 ha per plot) with ecotonal zone on one side for each. The fields of the three set-aside managements were covered by mowing on May, on July or none, respectively.

For each location, in each plot, 6 scores were randomly sampled and then mixed to form one composite sample of approximately 2 Kg each. The soil was transported in sterile polythene bags to the laboratory and 5-6 *Galleria mellonella* L. (Lepidoptera, Pyralidae) larvae were inserted in a long-handled tea infuser in the middle of each sample (TARASCO & TRIGGIANI, 1997). Then the samples were incubated at 25°C for 7 days in the laboratory of Namatology of the C.R.A. – Research Centre for Agrobiology and Pedology, Florence (Italy). Then died

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Galleria larvae were placed individually in modified White traps (KAYA & STOCK, 1997) and juveniles emerged from the cadavers were collected from water solution and established in a laboratory culture in 50 ml plastic tubes. Some of them were transported to the Institute of Sustainable Plant Protection (IPSP)-CNR, Bari (Italy) for molecular characterization, and some infective juveniles were used to fresh *G. mellonella* larvae to produce nematodes (IJs and males) used for identification.

MORPHOLOGICAL IDENTIFICATION

Nematodes were heat-killed in warm water at 60°C and fixed in triethanol-amine-formalin (TAF) (COURTNEY *et al.*, 1955), processed to glycerin by a modification of a glycerin-ethanol series of Seinhorst's (1959) rapid method and then permanently mounted in anhydrous glycerin on microscope slides.

Specimens were examined and measured with a Leitz ORTHOPLAN light microscope at up to 1000 magnification. IJs and males were identified using morphological and morphometric characters based on NGUYEN & SMART (1996) and NGUYEN & HUNT (2007).

MOLECULAR ANALYSIS

Specimens of *S. carpocapsae* for molecular analysis were collected from *Galleria* larvae and directly processed. Genomic DNA was extracted from fifteen individual nematodes as described by DE LUCA *et al.* (2004). The crude DNA isolated from each individual nematode was directly amplified by using the forward primer TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and the reverse primer AB28 (5'-ATATGCTTAAGTTCAGCGGGT -3') (JOYCE *et al.*, 1994) for the ITS region, and by using the 18SnF (5'-TGGATAACTGTGGTAATTCTAGAGC-3') and 18SnR (5'-TTACGACTTTTGCCCGGTTC-3') for the 18S rRNA gene. PCR cycling conditions used for amplification were: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 50s, annealing at 55°C for 50s and extension at 72°C for 1 min and a final step at 72°C for 7 min. The size of the amplification products was determined by comparison with the molecular weight marker ladder 100 (Fermentas, St. Leon-Rot, Germany) following electrophoresis of 10 l on a 1% agarose gel.

PCR products of the ITS containing region and the 18S rRNA gene from three individual nematodes were purified for sequencing using the protocol listed by manufacturer (High Pure PCR elution kit, Roche, Germany). Purified DNA fragments were cloned and sequenced in both directions. A BLAST (Basic Local Alignment Search Tool) search at NCBI (National Center for Biotechnology Information) was performed using to confirm their nematode origins and species (ALTSCHUL *et al.*, 1997). The newly obtained sequences for the ITS were aligned using ClustalW (LARKIN *et al.*, 2007) with default parameters with corresponding published gene sequences of *Steinernema*. Sequence alignments were manually edited using BioEdit in order to improve the default parameters of the multialignment. Outgroup taxa for each dataset were chosen according to the results of previously published data. Phylogenetic trees, obtained for both datasets, were performed with Neighbour-Joining (NJ), Minimum Evolution (ME) and Maximum Parsimony (MP) methods using MEGA version 6 software (TAMURA *et al.*, 2013). The phylograms were bootstrapped 1,000 times to assess the degree of support for the phylogenetic branching indicated by the optimal tree for each method.

The newly obtained sequences were submitted to GenBank with the following accession numbers LN624756-LN624757 for the 18S rRNA gene, LN624758-LN624759 for the ITS region .

For Restriction Fragment Length Polymorphism (RFLP) analysis, ten µl of each product, containing the ITS region, from two individual nematodes of *S. carpocapsae* isolate were digested with the following restriction enzymes: *Alu* I (Roche), *Ava* II, *Bam* HI, *Dde* I (Roche), *Hinf* I (Roche), and *Rsa* I (Roche) (5 U of enzyme for each digestion) at 37°C overnight. The digested DNA fragments were loaded onto 2.5% agarose gel and visualized by gel red staining gel. All gel images were stored digitally.

INFECTIVITY ASSAYS

The pathogenicity of the new strain was assessed using a method modified from that proposed by GLAZER & LEWIS (2000). Penetration, exposure time, one on one and sand column assays were performed using last instar *G. mellonella* larvae, with an average weight of 0.2-0.4 g. IJs were stored at 12°C for 3 weeks before use. The first three assay, indicated above, were performed in 12-well plates (COSTAR®, Corning, New York) with filter paper (Whatman No. 1) at the bottom of each one of wells. Sand column assay were performed using plastic containers (40 mm height x 45 mm diameter) contained moist sterile sand (autoclaved). In all bioassays, the incubation periods were carried out in the dark at 24°C.

Penetration assay

A nematode suspension of 4000 IJs ml^{-1} was prepared and with a micropipette a 0.5 µl aliquot was transferred to each one of twelve wells followed by a single last instar *G. mellonella* larva. Larval mortality was recorded daily. After an additional 48h in 6-cm Petri dishes with moist filter paper, cadavers of larvae were dissected. Using a stereomicroscope the number of nematodes established in the host was counted. The penetration rate was expressed as the percentage of the initial IJ inoculum that had invaded the insect host (CAROLI *et al*, 1996).

Exposure time assay

Every single *G. mellonella* larva was exposed to nematodes (400 IJs in 0.5 µl /well) for periods of 20, 40, 60, 120 and 180 minutes (twelve replicates per treatment). At the end of each exposure period, insects were rinsed and transferred to 6-cm Petri dishes with moist filter paper. The mortality was recorded 48 h after initial nematodes exposure and the ET_{50} values (the exposure time of nematodes to insects that are required to achieve 50% insect mortality) was calculated using probit analysis (SPSS 15.0).

One-on-one assay

This assay was conducted using two 12-well plates. In every well was placed only one IJ in 50 µl and one *G. mellonella* larva (MILLER, 1989). Twelve wells with 0.5 µl of deionized water were used as control. The mortality was recorded after 72 h of incubation.

Sand column assay

Fifteen plastic containers (40 mm height x 45 mm diameter) were used for this assay. One last instar *G. mellonella* larva was placed in the bottom and then the container was filled with moist sterile sand. A suspension of 100 IJs in 100 µl was added to the top of each column.

After 24 h, the larvae were removed from the sand and incubated in 6-cm Petri dishes with moist filter paper for a further 3 days. Larval mortality and penetration rate was recorded.

RESULTS

The ItS-CAO1 strain was found in set-aside no mowing regime.

MORPHOLOGICAL IDENTIFICATION

Morphometric data of first generation males and infective juveniles are in table 1.

Males: Body C-shaped when heat-relaxed. Cuticle smooth. Stoma shallow. Pharynx with cylindrical procorpus, slightly enlarged metacarpus, followed by an isthmus and a basal bulb with valve. Nerve ring surrounding isthmus. Excretory pore anterior to nerve ring. Reproductive system monorchic, testis reflexed. Spicules paired, symmetrical, slightly curved. Gubernaculum length *ca* 65% of spicule, boat-shaped in lateral view. Tail short, tip with a mucron and *ca* 70% of anal body diam. long. Bursa absent.

Female: Variable in size (n=10, L: 6493.7 ± 971). Body in form of spiral on heat-relaxation. Anterior region similar to male. Reproductive system amphidelphic, reflexed. Vulva in form of transverse slit. Tail short, conical.

Infective juvenile: Body slender, enclosed in a sheath. Pharynx long and narrow, often degenerate, with valvate basal bulb. Nerve ring localized at 68% of pharyngeal length. Tail elongate and conical.

MOLECULAR ANALYSIS

PCR-RFLP and sequencing analyses

Amplification of the ITS region in *S. carpocapsae* from Italy produced a fragment of approximately 0.8 kb in length for all specimens. A restriction profile of the ITS region from the Italian isolate of *S. carpocapsae* is shown in Fig. I. The restriction patterns with *Alu* I, *Dde* I, *Hinf* I and *Rsa* I enzymes resulted the same of those reported by REID *et al.*, 1997 and SUSURLUK & TOPRAK, 2006. *Alu* I enzyme yielded two polymorphic bands that were not present in the restriction profiles of other *S. carpocapsae* isolates identifying specifically the Italian isolate of *S. carpocapsae* (HOMINICK *et al.*, 1997; NGUYEN, 2003).

The sequences of the entire ITS of *S. carpocapsae* were determined for 2 specimens for the Italian population. No intraspecific differences were found in the sequences of these specimens. The ITS sequences of *S. carpocapsae* from Italy showed an intra-specific variability with the

Table 1 – Morphometrics of Infective juveniles and first generation males of *Steinernema carpocapsae* (ItS-CAO1). All measurements are in µm: mean ± s.d. (range).

Character[a]	Male	Infective Juveniles
n	20	20
L	1608.96 ± 143.3 (1339.5-1845.1)	559.0 ± 23.8 (505.8-597.0)
a	13.8 ± 1.1 (11.3-15.5)	21.3 ± 2.0 (18.7-27.1)
b	11.4 ± 1.0 (9.1-13.9)	4.7 ± 0.4 (4.2-5.5)
c	49.9 ± 4.4 (42.1-59.0)	10.7 ± 0.4 (10.2-11.5)
W	117.2 ± 12.5 (102.3-156.5)	26.4 ± 2.6 (20.1-30.4)
ES	141.9 ± 5.8 (130.9-151.1)	118.5 ± 9.0 (99.7-139.0)
EP	68.0 ± 5.5 (60.2-78.2)	36.2 ± 3.4 (28.2-40.3)
NR	101.0 ± 6.2 (93.3-114.4)	81.3 ± 5.5 (70.0-90.0)
T	32.3 ± 2.0 (29.5-36.1)	52.5 ± 2.7 (48.8-57.0)
ABD	47.1 ± 3.0 (41.8-54.2)	13.4 ± 0.5 (12.7-14.2)
Testis reflex	525.9 ± 59.3 (396.0-624.0)	–
SL	69.4 ± 4.3 (54.8-75.2)	–
Spicule width	13.8 ± 1.3 (11.4-15.1)	–
GL	45.4 ± 1.8 (42.1-48.2)	–
SW	1.5 ± 0.1 (1.3-1.6)	–
GS%	65.7 ± 5.3 (58.3-79.6)	–
D%	48.0 ± 4.2 (41.7-55.1)	30.7 ± 3.7 (23.9-40.1)
E%	211.6 ± 24.1 (166.8-265.1)	69.1 ± 6.4 (53.6-79.0)

[a] L = body length, W = max. body diam, ES = pharynx, EP = excretory pore, NR = nerve ring, T = tail length,
ABD = anal body diam., SL = spicule length, GL = gubernaculum length.
Ratios: a = L/W, b = L/ES, c = L/T, SW = SL/ABD, GS% = GL/SL x 100, D% = EP/ES x 100, E% = EP/T x 100

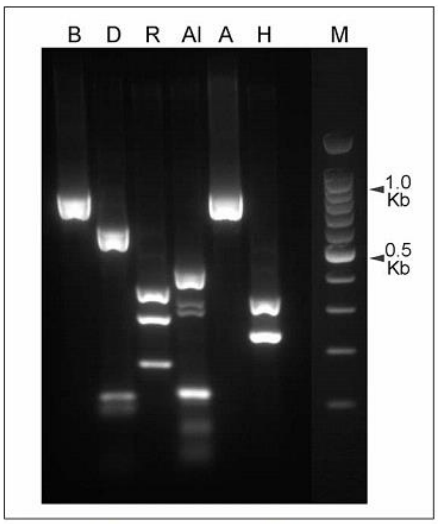

Fig. I - Restriction fragments of amplified ITS containing region of *Steinernema carpocapsae*. Al: *Alu*I, A: *Ava* II, B: *Bam* HI, D: *Dde*I, H: *Hinf* I, R: *Rsa* I and M:100 bp ladder.

corresponding sequences of *S. carpocapsae* present in the database ranging from 0 to 2% (5-16 bp different).

The sequencing of the 18S rRNA gene produced a sequence of 1633 bp. The partial 18S sequence was 99% identical to *S. carpocapsae* present in the database, 1627/1630 identities and 0 gap and 99% similar to *S. monticolum* (FJ040423), 1585/1589 identities and 1 gap.

For phylogenetic analysis of the ITS region, the corresponding sequences of *S. carpocapsae, S. feltiae, S. surketense, S. nepalese, S. tami, S. siamkayai, S. minutum and Steinernema* sp. from the database were aligned along with the ITS sequences obtained in this study. Phylogenetic trees generated by Neighbour-Joining, Maximum Parsimony and Minimum Evolution methods showed no significant conflict in branching order and support level, so only ML tree is shown (Fig. II). ML tree supported five clades within *Steinernema*. The Italian isolate of *S. carpocapsae* formed a well supported clade (90% support) with all sequences of *S. carpocapsae*, despite the high sequence variability as reported by SPIRIDONOV *et al.* (2004). The *S. carpocapsae* cluster resulted closely related to *S. surketense, S. nepalese* and *Steinernema* sp. (clade II). Clade III included only one taxon, *S. scapterisci*. Clade IV grouped *S. tami, S. siamkayai* and *S. minutum*. Clade V contained only one taxon, *S. feltiae* from the database, located at the basal position of the tree. Most clades were supported by high bootstrap values giving strong support to these associations.

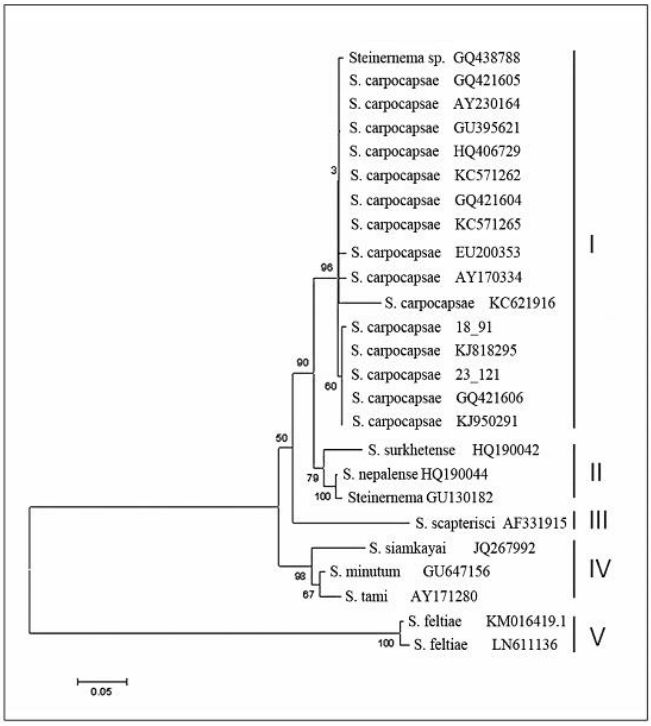

Fig. II - Phylogenetic trees of ITS containing region of *Steinernema carpocapsae* and the closest species. Sequences were analysed using Maximum Likelihood method. Numbers at nodes indicate bootstrap values.

Table 2 – Results of different infectivity assays with *Steinernema carpocapsae* strains.

Bioassay strain	Penetration	Sand column		One-on-one		Exposure time	
	ItS-CAO1	ItS-CAO1	Uk[c]	ItS-CAO1	All[c]	ItS-CAO1	All[c]
Insect mortality (%)	100	93.3	42	50 (33.3-66.6)	50 (45-85)		
Days to die	1.25 ± 0.5 (1-2)	2.3 ± 0.5 (2-3)					
P[a]	57.6 ± 9.5 (40-70)	42.9 ± 8.0 (31-63)	1.5				
ET_{50} (min)[b]						19	65 (50-70)

[a] P: penetration rate (percentage of initial nematodes that invades an insect host)
[b] ET_{50}: exposure time in minutes that is required by nematode to acheive 50% insect mortality
[c] Data from GLAZER & LEWIS (2000)

INFECTIVITY ASSAY

ItS-CAO1 strain caused high levels of mortality of the insect tested, achieving the 100% after 48 hours from the nematodes exposure (Table 2). The ItS-CAO1 penetration rate is higher compared to that reported by CAIROLI *et al.* (1996) at the same conditions. In the exposure time ET_{50} was lower than *S. carpocapsae* All strain. In the one-on-one quality bioassay, ItS-CAO1 virulence was the same to All strain (50%). In sand column bioassays, ItS-CAO1 showed insect mortality (93.3%) and penetration rate greater than UK strain.

DISCUSSION

S. carpocapsae was previously isolated from soil sample in Veneto region (EHLERS *et al.*, 1991) but it was not morphologically or molecularly characterized.

Molecular identification of *Steinernema* species is a prerequisite for proper classification, biodiversity study and their potential use in biological control programs.

DNA sequence and phylogenetic analyses of entomopathogenic nematodes provide additional criteria for identifying and delimiting species within *Steinernema*. Sequence analyses of the ITS and the 18S rRNA gene well supported the identification of *S. carpocapsae* isolate from Italy. In particular, sequence analyses of the ITS containing region has allowed to assess the heterogeneity among isolates of *S. carpocapsae*, and among *Steinernema* species, even for those that are closely related (HOMONICK *et al.*, 1997). This result was also confirmed by RFLP profiles that were in accordance with the patterns reported previously for *S. carpocapsae* (Fig. I) (SPIRIDONOV *et al.*, 2004; SUSURLUK & TOPRAK, 2006). The alignment of the optimised ITS sequences from different species of *Steinernema* spp. revealed multiple insertion and deletion events (indels) among taxa of varying size (1-20 bp) and the longest indels were localized in the ITS1 (data not shown). Furthermore, the multiple alignment constructed revealed several conserved sequence motifs characteristic for each *Steinernema* species.

The phylogenetic analyses with ML, NJ and MP methods yielded congruent phylogenetic trees. Notably, one highly supported group was evident in both analyses, consisting of all *S. carpocapsae* sequences (Fig. II) closely related to *S. surkhetense, S. nepalense*, *Steinernema* sp. and *S. scapterisci*.

In conclusion, the ITS sequences allowed clear separation of *Steinernema* species in spite of the high intra-specific variability. The alignment revealed small species-specific DNA sequences suitable for the construction of potentially useful species-specific primers or for a more promising approach for DNA barcoding of entomopathogenic nematodes.

Screening of EPN species is an important step before initiating mass production or field evaluation. Laboratory studies are essential for maximising the chances of success in field experiments (BEDDING, 1990). The bioassays used in this study examined nematode activity in the infection process. Insect mortality with ItS-CAO1 strain is very high in penetration and sand column assay (100% and 93.3% respectively). Nevertheless, the penetration ability is an important characteristic for EPNs. High penetration rates would increase the infection rates, which in turn increase toxins produced by developing nematodes and their symbiotic bacteria (BURMAN, 1982) and so will enhance septicaemia and kill the insect host more rapidly. In ItS-CAO1 strain Infective juveniles' penetration was 57.6% in penetration assay and 42.9% in sand column assay.

The exposure time assay indicated indirectly how quickly insects were infected by the nematodes (ET_{50} 19 min). In one-on-one assay mortality at 72h was 50%. With the results of infectivity assays we can evaluate the possibility to use this new strain in biological control programs.

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