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# Natural infection of white grubs (Coleoptera: Scarabaeidae) with entomopathogenic nematodes in the KwaZulu-Natal province of South Africa

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**Abstract**

White grubs are root feeding larvae of beetles (Coleoptera: Scarabaeidae) that are sporadic pests in agriculture and can lead to economic damage. The grubs feed on the roots of plants, while the adult beetle can bore into underground stems, as well as cause defoliation of plants. Sporadic incidence of larvae with symptoms of nematode infections were detected in wattle and sugarcane plantations in the KwaZulu-Natal province of South Africa. The larvae with infection symptoms were isolated, washed, and put on water traps to collect infective juveniles of possible nematode infections. Three species of entomopathogenic nematodes (EPNs) were isolated from the white grub larvae. These included *Steinernema bertusi* isolated from *Maladera* sp. 4., *Oscheius myriophila* from *Maladera* sp. 4 and *Schizonchya affinis,* and *Steinernema fabii* isolated from *Maladera* sp. 4., *Pegylis sommeri,* and *S. affinis*. Of these *S. fabii* was the most common species in the sample (87%). This is the first report of such a high diversity of locally occurring EPNs found naturally associated with white grub species in this region of South Africa.

## Introduction

Entomopathogenic nematodes (EPNs) are microscopic round worms of the order Rhabditida (families Heterorhabditidae & Steinernematidae) that, under laboratory conditions, are known to parasitize nearly all insect orders (Lacey & Georgis 2012). Another family of nematodes, the *Oscheius,* have not always been grouped under EPNs in the past. However, these nematodes have shown similar insect parasitism lifestyles to Steinernematids and the Heterorhabditids (Dilman *et al.* 2012). For example, the recently described *Oscheius chongmingensis* Tumian (= *Heterorhabditidoides*), *Oscheius rugaoensis* Zhang, Liu, Tan, Wang, Qiao, Yedid, Dai, Qiu, Yan, Tan, Su, Lai & Gao (= *Heterorhabditidoides*) and *Oscheius carolinensis* Ye, Torrez-Marragan, Cardoza show potential as EPNs (Ye *et al.* 2010; Torres-Barragan *et al.* 2011; Liu *et al.* 2012). Newly described species from South Africa, *Oscheius safricana* Serepa-Dlamini & Gray and *Oscheius basothovii* Lephoto & Gray, have also been shown to share similar attributes with *Steinernema* and *Heterorhabditis* (Dillman *et al.* 2012; Lephoto *et al.* 2016; Serepa-Dlamini & Gray 2018). In terms of classification as EPNs, the evidence shows that some *Oscheius* species fit the description of true EPNs, although an official classification as such is still lacking.

EPNs are present in a variety of soil habitats (Kaya *et al.* 1993) where they infect soil-dwelling and litter insects. Little is known about their natural insect hosts, as they are mostly baited from the soil using larvae of the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), and mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). For example, in a review done by Peters (1996), information was lacking on natural hosts for seven out of the 18 recognized *Steinernema* species and three out of the six recognized *Heterorhabditis* species. To date, information regarding natural hosts of EPNs remains scant.

EPNs have previously been naturally isolated from white grubs (Coleoptera: Scarabaeidae). Chandel *et al.* (2018) provided a list of up to 11 different species of EPNs that have been naturally isolated from white grubs. White grubs (Coleoptera: Scarabaeidae) are the root feeding larvae of scarab beetles (Jackson & Klein 2006). They are sporadic pests in agriculture and important insect pests in sugarcane and wattle plantations (Echeverri-Molina & Govender 2016). High infestations of white grubs cause economic damage to farmers by feeding on plant roots, while adult beetles bore into underground stems, as well as defoliate plants (Raodeo & Deshpande 1987; Jackson & Klein 2006).

During a white grub sampling and collection trip in the KwaZulu-Natal province of South Africa, white grubs with symptoms of nematode infection were recovered from the collected samples. This finding provided a rare opportunity to isolate and identify the nematode

**Table 1.** Entomopathogenic nematodes (*Steinernema* and *Oscheius* spp.) isolated from white grub (Coleoptera: Scarabaeidae) species from the KwaZulu-Natal province in South Africa, with isolate number, location, host plant of the white grub species, and month of collection

| Isolate | Nematode ID | Grub species | GPS coordinates of collection site | Nearest city | Host plant | Month collected | GenBank ref. |
|---|---|---|---|---|---|---|---|
| SCH10 | *Steinernema fabii* | *Schizonchya affinis* | 29°26'04.9"S 30°38'30.6"E | Watburg | Sugarcane | June 2018 | MW618681 |
| AK02 | *S. fabii* | *S. affinis* | 29°19'22.7"S 30°48'52.9"E | Mbalenhle | Sugarcane | June 2018 | MW618682 |
| AK03 | *S. fabii* | *Pegylis sommeri* | 29°12'52.8"S 30°37'55.5"E | Dalton | Sugarcane | June 2018 | MW618683 |
| AK04 | *S. fabii* | *P. sommeri* | 29°12'40.4"S 30°38'09.0"E | Dalton | Wattle | June 2018 | MW618684 |
| AK05 | *S. fabii* | *S. affinis* | 29°29'30.4"S 30°18'35.9"E | Hilton | Wattle | June 2018 | MW618685 |
| AK06 | *S. fabii* | *S. affinis* | 29°28'33.3"S 30°18'42.2"E | Hilton | Wattle | June 2018 | MW618686 |
| AK07 | *S. fabii* | *S. affinis* | 29°28'33.3"S 30°18'42.2"E | Hilton | Wattle | June 2018 | MW618687 |
| AK08 | *S. fabii* | *S. affinis* | 29°12'52.8"S 30°37'55.5"E | Dalton | Sugarcane | June 2018 | MW618688 |
| AK09 | *S. fabii* | *S. affinis* | 29°12'52.8"S 30°37'55.5"E | Dalton | Sugarcane | May 2019 | MW618689 |
| AK10 | *S. fabii* | *S. affinis* | 29°12'52.8"S 30°37'55.5"E | Dalton | Sugarcane | May 2019 | MW618690 |
| AK11 | *S. fabii* | *S. affinis* | 29°12'52.8"S 30°37'55.5"E | Dalton | Sugarcane | June 2018 | MW618691 |
| AK12 | *S. fabii* | *S. affinis* | 29°12'35.0"S 31°28'06.0"E | Dolphin coast | Sugarcane | June 2018 | MW618672 |
| AK13 | *S. fabii* | *Maladera* sp. 4. | 29°29'30.4"S 30°18'35.9"E | Hilton | Wattle | Dec 2018 | MW618693 |
| AK14 | *S. fabii* | *Maladera* sp. 4. | 29°12'52.8"S 30°37'55.5"E | Dalton | Sugarcane | Dec 2018 | MW618694 |
| AK15 | *S. fabii* | *S. affinis* | 29°25'51.6"S 30°39'21.6"E | Wartburg | Sugarcane | Mar 2020 | MW618695 |
| AK16 | *S. fabii* | *S. affinis* | 29°14'50.7"S 30°39'55.7"E | Wartburg | Sugarcane | Mar 2020 | MW618696 |
| AK17 | *S. fabii* | *S. affinis* | 29°28'47.4"S 30°39'36.3"E | Wartburg | Sugarcane | Mar 2020 | MW618697 |
| AK18 | *S. fabii* | *S. affinis* | 29°28'47.4"S 30°39'36.3"E | Wartburg | Sugarcane | Dec 2018 | MW618698 |
| AK19 | *S. fabii* | *S. affinis* | 29°28'47.4"S 30°39'36.3"E | Wartburg | Sugarcane | Dec 2018 | MW618699 |
| AK20 | *S. fabii* | *P. sommeri* | 29°28'47.4"S 30°39'36.3"E | Wartburg | Sugarcane | Mar 2019 | MW618700 |
| AK21 | *S. fabii* | *P. sommeri* | 29°28'47.4"S 30°39'36.3"E | Wartburg | Sugarcane | Mar 2019 | MW618701 |
| AK22 | *S. fabii* | *P. sommeri* | 29°28'47.4"S 30°39'36.3"E | Wartburg | Sugarcane | Mar 2019 | MW618702 |
| AK23 | *S. fabii* | *S. affinis* | 29°12'35.0"S 31°28'06.0"E | Dolphin coast | Sugarcane | Mar 2019 | MW618703 |
| AK24 | *S. fabii* | *Maladera* sp. 4. | 29°12'35.0"S 31°28'06.0"E | Dolphin coast | Sugarcane | Mar 2019 | MW618704 |
| AK25 | *S. fabii* | *P. sommeri* | 27°07'20.0"S 30°57'04.5"E | Piet Retief | Wattle | Mar 2019 | MW618705 |
| AK26 | *S. fabii* | *P. sommeri* | 27°07'20.0"S 30°57'04.5"E | Piet Retief | Wattle | Mar 2019 | MW618706 |
| AK27 | *Steinernema bertusi* | *Maladera* sp. 4. | 27°07'20.0"S 30°57'04.5"E | Piet Retief | Wattle | May 2019 | MW618707 |
| AK28 | *Oscheius myriophila* | *S. affinis* | 29°29'30.4"S 30°18'35.9"E | Hilton | Sugarcane | Mar 2019 | MW618708 |
| AK29 | *O. myriophila* | *Maladera* sp. 4. | 29°28'33.3"S 30°18'42.2"E | Hilton | Wattle | Dec 2019 | MW618709 |
| AK30 | *O. myriophila* | *Maladera* sp. 4. | 29°12'52.8"S 30°37'55.5"E | Dalton | Wattle | Dec 2019 | MW618710 |

species naturally infecting the white grubs as well as test for their pathogenicity. Nematodes isolated from natural infections have the relative advantage of being naturally adapted to the host and environmental conditions (Abate *et al.* 2017). This would be particularly advantageous for hosts such as white grubs, which have been shown to have developed various resistance mechanisms to EPN infection (Grewal *et al.* 2004). The aim of this study was to identify the EPN species that were isolated from white grubs and to match them to their white grub natural hosts.

## Materials and methods

### *White grub collection and EPN isolation*

White grubs with symptoms of EPN infection were collected from the soil over four seasons, from different geographical regions and two host plants, namely sugarcane and wattle in the KwaZulu-Natal province of South Africa (Table 1). The dug up white grubs were isolated in a 30 ml plastic vial with loose moist soil. The vials were placed in cooler boxes and transported to the insectary at the Forestry and Agricultural Biotechnology Institute (FABI) Biocontrol Centre of the University of Pretoria. White grubs were washed with distilled water, identified to species level, and those showing symptoms of possible nematode infection were kept separately for incubation. Each insect was placed in a small tissue culture Petri dish lined with a moist filter paper. Incubation was done at 25°C and 100% relative humidity. Once the infective juveniles (IJs) started to emerge, the cadavers were transferred to modified White's traps for harvesting (White 1927). Harvested IJs were stored in distilled water at 12°C in horizontally placed culture flasks. The IJs obtained from the White traps were inoculated in *G. mellonella* larvae and reared

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to obtain adult nematodes, which were later used for DNA extraction and identification.

### Identification of EPNs

From each isolate, DNA from a single young female nematode (Table 1) was extracted using the protocols outlined in Nguyen (2007) for single nematode extraction. The lysis buffer used for DNA extraction consisted of 50 mM $MgCl_2$, 10 mM of dithiothreitol (DTT), 4.5% Tween-20, 0.1% gelatine, and 1 µl of proteinase K at 60 µg $m^{-1}$. A first-generation female was placed in a 30 µl drop of the lysis buffer pipetted on the upper side of a 0.5 ml micro centrifuge tube. The nematode was cut into a few pieces, using a sterile insulin needle, and the contents were immediately placed on ice and transferred to -80°C for 20 min. For total lysis of the cells and digestion of the proteins, the tubes were incubated at 65°C for 1 h and at 95°C for 10 min in a thermocycler (GeneAmp 2720) (Thermo Fisher Scientific, Johannnesburg, South Africa). The tube was cooled on ice and centrifuged at 11,600 g at 10°C for 2 min, and 5 µl were pipetted from the supernatant and used in the polymerase chain reaction (PCR) amplification.

PCR amplification of the ITS region was conducted by following the protocol described in Nguyen (2007). The ITS region of the ribosomal DNA was amplified in a 25 µl reaction. The ITS region was amplified using the PCR primers TW81 [5'-GTTTCCGTAGGTGAACCTGC-3'] and AB28 [5'-ATATGCTTAAGTTCAGCGGGT-3']. PCR amplifications were carried out in tubes containing 5 µl nematode lysate, together with 0.5 µM of each primer and 12.5 µl KAPA2G™ Robust Hotstart ReadyMix (KAPA Biosystems, Milnerton, South Africa). The final reaction volume was 25 µl. The cycling conditions were as follows: denaturation at 94°C for 20 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec, with all conditions being repeated for 35 cycles. A 2-min incubation period at 72°C followed the last cycle to complete any partially synthesised strands.

The PCR product was then run on 1% agarose gel in a 1 × TBE buffer and visualised using Gel Red- Sigma- Aldrich Inc. (St. Louis, USA). Post-PCR purification was done using the NucleoFast Purification System -Macherey Nagel (Düren, Germany). Sequencing was performed with the BigDye Terminator V1.3 sequencing kit - Applied Biosystems (Thermo fischer Scientific, Johannesburg, South Africa, followed by electrophoresis on the 3730 × 1 DNA Analyser -Applied Biosystems (Thermo fischer Scientific, Johannesburg, South Africa) at the DNA Sequencing facility, University of Pretoria.

Sequences were assembled, analysed, and edited using the CLC Main Workbench ver. 8.1 (QIAGEN, Aarhus, Denmark). The obtained sequences were compared with sequences in the NCBI GenBank using the nucleotide Basic Local Alignment Search Tool (BLASTn) to determine species identification. Sequences were then aligned using ClustalX 2.1 (Thompson *et al.* 1997), while phylogenetic analyses of sequence data were done using the Maximum Parsimony (MP) method in MEGA5 (Tamura *et al.* 2011). Support for tree branches was evaluated statistically by means of a bootstrap analysis based on 1000 re-samplings of the dataset. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1, in which the initial trees were obtained by means of the random addition of sequences (10 replicates).

## Results and discussion

From the total of 30 infected white grubs from which single nematodes were isolated and sequenced, 26 (87 %) of the nematodes were identified as *Steinernema fabii* Abate, Malan, Tiedt, Wingfield, Slippers & Hurley. Three (10 %) were identified as *Oscheius myriophila* Poinar 1986, and one (3 %) as *Steinernema bertusi* Katumanyane, Malan, Tiedt & Hurley (Table 1). The white grubs found to be infected with EPNs were collected across different seasons in 2018, 2019, and 2020 (Table 1). From *Maladera* sp. 4., the EPNs *S. fabii, S. bertusi,* and *O. myriophila* were isolated. From *Pegylis sommeri*, only *S. fabii* was isolated, while both *S. fabii* and *O. myriophila* were isolated from *S. affinis*. In wattle plantations all three EPN species were found in association with the three grub species, while in sugarcane all white grub species were associated with *S. fabii,* and only one white grub species, *S. affinis*, with *O. myriophila* (Table 1).

Information on the natural hosts of EPNs and their interaction with naturally occurring nematode and insect populations is not readily available, although most recorded naturally occurring nematode infections are from sampling of pest insects, in which case insects occur in high densities (Peters 1996). In our study we isolated three EPN species from naturally infected white grubs. The nematodes were isolated from the white grubs *P. sommeri*, *S. affinis*, and *Maladera* sp. 4. These white grubs are pests of different crops including sugarcane and wattle (Echeverri-Molina & Govender 2016) in the KwaZulu-Natal province. The sugarcane plantations are found in proximity to the wattle plantations, and therefore, there is an overlap of white grub species between these crops (Sivparsad *et al.* 2018).

The EPNs isolated in this study included *S. fabii*, *O. myriophila*, and *S. bertusi. Steinernema fabii,* by far the most dominant EPN isolated from the white grubs, belongs to the *Cameroonense*-clade. The original isolation and subsequent description of *S. fabii* was by trapping with *G. mellonella* larvae from the soil in an *Acacia mearnsii* plantation in the Mpumalanga province of South Africa (Abate *et al.* 2016). *Steinernema bertusi* also belongs to the *Cameroonense*-clade and to date has been isolated twice; from an *Acacia mearnsii* plantation in Tito, Mpumalanga, and from an area with natural vegetation in Port Edward, KwaZulu-Natal, South Africa (Steyn *et al.* 2017; Abate *et al.* 2018; Katumanyane *et al.* 2020). Both isolations were done through baiting with *G. mellonella* larvae. The *Cameroonense*-clade contains EPN species that have only been reported from the African continent. The species in this clade have their origins in the Americas (Spiridonov & Subbotin 2016).

*Oscheius myriophila* has been isolated from various hosts to date (Demirbag 2018; Ye *et al.* 2018; Del Rocio Castro-Ortega *et al.* 2020). The original isolation and description were from the garden millipede, *Oxidis gracilis* Koch (Polydesmida) in California, USA (Poinar 1986). *Oscheius* spp. are divided into two groups that include the Dolichura and Insectivora groups (Liu *et al.* 2012; Ye *et al.* 2010). Species under the Insectivora group are characterized by leptoderan bursa, crochet needle-shaped spicules and normal rectum, whereas the Dolichura group has a peloderan bursa, probe head spicule tips, and expandable rectum (Sudhaus 1976).

In our study, *O. myriophila* grouped close to other *Oscheius* spp. and to the South African isolated new species of *Oscheius safricana* and *Oscheius basothovii* in the Insectivora group. The insect-associated members of the genus *Oscheius* are associated with the insectivorous symbiotic bacteria of *Serratia* (Enterobacterales: Yersiniaceae) (Dillman *et al.* 2012).

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In terms of the pathogenicity of the isolated nematodes on third instar larvae of white grubs, Katumanyane *et al.* (2023) showed that *Steinernema fabii* provided 63 % mortality of *Maladera* sp. 4 in soil bioassays. However, *S. fabii* did not cause any mortality to third instar larvae of *S. affinis* and *P. sommeri* in soil bioassays, while it only killed less than 5% of those tested in Petri dish bioassays. However, *S. fabii* was able to grow in the haemolymph of the white grubs *P. sommeri*, *S. affinis*, and *Maladera* sp. 4. Thus, *S. fabii* has a high ability to reproduce in the tested grubs but a low ability to infect third instar larvae. It is possible that *S. fabii* is able to infect other developmental stages more effectively since these tests were run using only the third instar larvae of white grubs, which is also known to be the most resistant. *Steinernema bertusi* and *O. myriophila* were only tested against *S. affinis* due to limited availability of other white grub species during the season. *Steinernema bertusi* showed a moderate percentage mortality towards third instar larvae of *S. affinis*, while *O. myriophila* resulted in a 40 % mortality of *S. affinis*. However, it was also noted that these two nematodes were unable to keep the cadavers clean, and they were constantly contaminated with mites.

Due to the consistent isolation of *S. fabii* from white grubs over seasons, the relationship between *S. fabii* and white grubs might be a relatively balanced nematode-host association in contrast to being an epizootic. *Steinernema bertusi* and *O. myriophila* were only isolated from white grubs on a few occasions. This could possibly be related to the sampling, and further collections would be needed to confirm the prevalence of the three EPN species, and possibly other EPN species, on naturally infected white grub species.

Our study shows that various white grub species naturally host EPN species, thus demonstrating the potential for EPNs to be used as biological control agents for white grub pest species in forest plantations, sugarcane, and other crops. *Steinernema fabii* was by far the dominant EPN isolated from white grubs and would be a good candidate to further investigate as a potential biocontrol agent. This nematode reproduces quickly in the haemolymph of white grubs but has a low infection potential on its own (Katumanyane et al. 2003). We hypothesize that *S. fabii* is an opportunistic EPN in the field and attacks more susceptible grubs, possibly those undergoing moulting. The low virulence of *S. fabii* to most of the white grub species could be a result of the more resistant targeted growth stage of the host. If this is the case, *S. fabii* can be used as a biological control agent during the seasons when white grubs are moulting.

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**Competing interest.** The authors have no competing interests or conflict of interest to declare.

**Ethical standard.** Not applicable.

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