The diversity and distribution of entomopathogenic nematodes in the United Kingdom and the first confirmed UK record of *Steinernema carpocapsae*

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Running head: EPNs found in the U.K.

Abstract

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae are lethal parasites of insects that have been commericalised as biological control agents to provide protection against several pestiferous species in agriculture and forestry. EPNs have been isolated from across the world but the current distribution and diversity of EPNs found in the U.K. is poorly understood. To remedy this we collected 518 soil samples from a diverse range of habitats across the U.K. and baited them with *Galleria mellonella* to isolate EPNs. Dead *G. mellonella* were placed in White traps and emergent EPNs underwent DNA barcoding analyses. From the 518 collected soil samples, 3.5% were positive for EPNs. No species of *Heterorhabditis* were found, but we found seven isolates of *Steinernema glaseri,* one isolate of *S. feltiae*, eight isolates of *S. affine* and two isolates of *S. carpocapsae*. This was the first confirmed record of *S. carpocapsae* in the U.K.

Keywords

Steinernema carpocapsae, S. glaseri, S. feltiae, S. affine, Heterorhabditis, Lundy Island, entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* are lethal pathogens of hundreds of insect species (Laumond, 1979; Georgis et al., 2006; Grewal et al., 1994). They have been formulated into biological control agents for use against insect pests in agriculture and forestry (Campos-Herrera, 2015) including *Diaprepes abbreviates, Otiorhynchus sulcatus*, *Thrips* spp., *Delia radicum*, *Phyllopertha horticola*, *Cydia pomonella* and several other Dipteran and Lepidopteran larvae (Georgis et al., 2006). Nematodes are applied to soil where they are attracted to cues exuded from insects including host derived odorants, carbon dioxide and faeces (Dillman et al., 2012; Hallem et al., 2011, Grewal et al., 1993). They then penetrate through the mouth, spiracles and anus (Dowds & Peters, 2002) where they then release their symbiotic bacteria (*Xenorhabdus* for *Steinernema* and *Photorhabdus* for *Heterorhabditis*), which kills insects in 24-48 hours (Forst et al., 1997). The bacteria produce a selection of toxins, haemolysins and lipopolysaccharides (LPS) that are responsible for killing the insect host (Chaston et al., 2011), and the nematodes feed on the proliferating bacteria. When the food source is depleted they arrest development at dauer stage and go in search of more potential insect hosts in the soil. Their lethality, effectiveness in the field and the mass production technology (Shapiro-Ilan & Gaugler, 2002) make them excellent alternatives to chemical control (Campos-Herrera, 2015).

EPNs have been isolated worldwide with more than 95 species of *Steinernema* and 16 species of *Heterorhabditis* described so far (Hunt & Nguyen, 2016). There have been many surveys over the past few decades looking at EPN diversity and distribution including recent surveys of Thailand (Thanwisai et al., 2012) and New Zealand (Ali & Wharton 2017). Over the last 30 years there have been several surveys of EPNs in Scotland, England, Wales and Northern Ireland (Boag et al., 1992; Blackshaw, 1988; Hominick & Briscoe, 1990a,b; Gwynn & Richardson, 1996), though no recent surveys have been conducted. These surveys have produced mixed results in terms of success of isolating EPNs. For example, Boag et al.

(1992) sampled Scotland in 40 x 40 km grids and achieved a return rate of approximately 2.2% from their sampling regime finding only *Steinernema feltiae* and a nematode they suspected was *S. carpocapsae*. However, Homininck & Briscoe (1990a,b) had a higher success rate with 48% of samples being positive for finding EPNs such as *S. bibionis*, an unidentified *Steinernema* sp. and an unidentified *Heterorhabditis* sp. There were several factors that may contribute to this difference in success rate, one of which may be the modifications to the standard method of isolating EPNs, the *Galleria mellonella* trap method (Glazer & Lewis 2000). For example, some studies baited soil samples with *G. mellonella* only once (Griffin et al., 1991) but others tried two times (consecutively) and at two different temperatures (Homininck & Briscoe, 1990a,b). Also some studies used *Tenebrio molitor* instead of *G. mellonella* (Boag et al., 1992), which may affect the numbers and species of EPNs isolated. As there have been mixed results discovering what EPN species are present in the U.K., and the last survey was over 20 years ago (Gwynn & Richardson, 1996), we decided to carry out a survey of the biodiversity and distribution of the U.K. EPN fauna by collecting soil samples and baiting them with *G. mellonella*, followed by using White traps to grow any potential EPNs. Identification of species used standard genotyping procedures.

Materials and Methods

Collection of soil samples from across the U.K.

In total 518 soil samples were collected across the U.K. (Fig 1A; Supplementary Fig 1). The collection sites were chosen to get a broad distribution across the U.K. including a wide range of habitats, land use and soil types (e.g. deciduous forest, coastal, salt marsh, urban environments, improved grassland and livestock farmland) as described by Joint Nature Conservation Committee (JNCC) Handbook for Phase 1 habitat survey (JNCC, 2010)

and using the UKSO Soils map viewer (NERC, 2017). Between target land uses and habitats, a random drive method was employed, as described by Hominick & Briscoe (1990a,b), to cover as much area as possible. Each soil sample (300-500 g) was removed using a hand-trowel to a depth of 10-15 cm. At the time of each collection, GPS coordinates and a photograph were taken, and land use, soil type and any other distinguishing habitat information were recorded.

In addition to mainland U.K. fine scale sampling of the island of Lundy was conducted (n = 46 soil samples), which had never been previously sampled for EPNs. Lundy Island is a small, flat topped, granite island situated 18 km from the coast of North Devon with the Atlantic Ocean on its west side and the Bristol Channel on its east. It is a small island extending to 5 km long and 1 km wide with an area of 345 ha (JNCC 2017). A patchwork of different habitats covers the top of the island including open heathland, rough and improved grassland, patches of moorland with acidic bogs and pools and rare waved heathland to the north of the island (JNCC, 2017). Samples were taken based on a grid system covering the whole of the island and were collected between September 2014 and July 2015.

Isolation of EPNs using baiting with *G. mellonella* as bait

Once soil samples were transported back to the laboratory they were placed into non-airtight plastic boxes (10 x 8 x 5 cm) and each box was baited with six late instar *G. mellonella* and maintained at room temperature (15-20°C) (Glazer & Lewis, 2000). Every 48 hours any dead or moribund *G. mellonella* were removed, rinsed with distilled water and placed into individual White traps (White, 1927). This process was repeated every 2-3 days

for 22 days until all *G. mellonella* were removed from the box. A second bait was performed using the same temperature regime with the same soil samples after 22 days.

Molecular identification of EPN species

Any nematodes that were found en-masse in the surrounding water of the White trap after 28 days were identified using molecular analysis. Between 30-50 dauer juveniles were placed in a 1.5 ml Eppendorf tube and their DNA extracted using a Qiagen DNeasy Blood and Tissue DNA extraction kit following the manufacturer's recommended protocol. Nematode DNA was then used for PCR amplification of the 18S rRNA gene for species identification (Blaxter et al., 1998) using the primers SS18U (5'-AAAGATTAAGCCATGCATG-3') and SS26R (5'-CATTCTTGGCAAATGCTTTCG-3'). The PCR cycling conditions were as follows: 94 °C for 5 mins, 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min and then 72 °C for 7 mins. To aid differentiation between *Steinernema* species, PCR of the internal transcribed spacer regions ITS-1, 5.8 and ITS-2 using primers TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') was performed (Spiridonov et al., 2004). Successful amplification of both genes was then checked using gel electrophoresis and PCR products purified using a Thermo Scientific GeneJET PCR Purification Kit. Amplicons were sequenced in both directions by GATC (Constance, Germany). Sequences were manually checked and edited and used to identify to species level using a BLASTN search in NCBI database (Altschul 1990).

Results

Identification of EPNs from soil samples from around the U.K.

From 518 soil samples collected across the length and breadth of the U.K. (Fig 1A; Supplementary Fig 1), 18 (3.5%) were positive for EPNs (Fig 1B; Supplementary Fig 1). The 18 individual sites had four different species of EPN from the genus *Steinernema* including eight isolates of *S. affine*, seven isolates of *S. glaseri*, one of *S. feltiae* and two of *S. carpocapsae* (Fig 1B; Table 1). The sampling sites that contained EPNs were predominantly from rural, sparsely populated settings, the only exception was one isolate of *S. feltiae*, which was found in an urban car park. No *Heterorhabditis* spp. were isolated from the soil samples despite numerous samples being taken from habitats where *Heterorhabditis* spp. had previously been detected in the UK such as sandy coastal areas (Hominínck et al., 1995).

The majority of the EPNs were found in the south west of England in the counties of Devon and Cornwall (Fig 1B; Table 1). In total ten out of the 18 samples were found there with representatives of three *Steinernema* species (*S. glaseri, S. carpocapsae* and *S. affine*). However, no *S. feltiae* were found. The habitat types where EPNs were found in this area were diverse including moorland, grassland and farmland, variously consisting of loam and peat soils. The other eight EPN isolates were sporadically found across the U.K., but with a tendency for positive samples to be more common in the northern parts of the U.K. *Steinernema affine* was found in Northern Ireland, Scotland and northern England. *Steinernema glaseri* was isolated near Whitby and Scarborough as well as near Oxford. The only isolate of *S. feltiae* was found in northern England near Alnmouth from loam soil collected near a car park. Interestingly, there have been no confirmed records of *S. carpocapsae* from the U.K. before, but we isolated two samples, both from the south west of England. The island of Lundy also harboured EPNs as we found one rich loam sample from

farmland had a single isolate of *S. glaseri*.

Identification of other nematode species

Only 18 (3.5%) of the soil samples had EPNs present, however another 155 (29.9%) of all the soil samples had other nematode species from a variety of trophic groups present. The genera identified were: *Acrobeloides* (104 isolates), *Aphelenchoides* (3), *Aphelenchus* (8), *Cervidellus* (1), *Choriorhabditis* (2), *Chiloplacus* (6), *Mesorhabditis* (1), *Oscheius* (2), *Panagrolaimus* (2), *Pelodera* (2), *Phasmarhabditis* (1), *Pristionchus* (15) and *Rhabditis* (8). In some cases two nematode species were found in one sample, including a soil sample that contained *S. feltiae* and *Oscheius* sp. but they were separated based on general morphology before molecular identification. As these other nematode genera were not being surveyed and are often found on dead *G. mellonella* from such surveys (Mráček, 1980) they were not studied further.

Discussion

The purpose of this survey was to understand the diversity and distribution of EPNs in the U.K. and to investigate whether the EPN landscape had changed since the last survey 21 years ago (Gwynne & Richardson, 1996). Previous surveys of the U.K. isolated *S. bibionis, S. affine, S. feltiae* and *S. kraussei* and a *Heterorhabditis* spp. (Homininck & Briscoe, 1990a,b; Griffin et al., 1991; Blackshaw, 1988; Homininck et al., 1995; Chandler et al., 1997; Gwynne

& Richardson 1996). We found *S. affine*, *S. feltiae*, *S. glaseri* and *S. carpocapsae*, with the latter species having no previous confirmed records in the U.K.

From 518 soil samples 3.5% had nematodes present - similar to the survey results of Boag et al. (1992) who found 2.2% of soil samples were positive for EPNs, but is lower than Gwynne & Richardson (1996) who found 11% of samples had EPNs present. Other surveys have recorded much higher success at finding EPNs in the U.K. Homininck & Briscoe (1990a) recovered EPNs from 48.6% of soil samples and Homininck et al. (1995) found 38.2% of soil samples with EPNs. Boag et al. (1992) suggested the high return rate might be due to second baiting of soil samples with *G. mellonella*. We also performed a second *G. mellonella* bait on all 518 soil samples, however the recovery rate did not increase. Other studies suggest that baiting soil samples at different temperatures is an important consideration as some species are more active at different temperatures e.g. *S. kraussei* is able to tolerate colder temperatures (Mráček et al., 2005). Two of the previous U.K. based surveys baited at different temperatures, which increased EPN isolation (Homininck & Briscoe 1990a,b; Gwynne & Richardson, 1996). However, in this study we carried out baiting at room temperature which fluctuated from 15 to 20°C. Boag et al. (1992) baited their soil samples with *T. molitor* as well as *G. mellonella* as the latter may not be the most suitable host for all EPN species (Spiridonov & Moens, 1999). However, they did not isolate more species or achieve a higher recovery rate.

EPN identification has previously been based solely on morphology and crossbreeding of isolates with known EPNs strains (Poinar 1979; Stock, 2002). However, the validity of crossbreeding as a means of species identification was called into question following the discovery of hermaphroditic Steinernematids by Griffin et al. (2001). Genetic identification methods have become more common over time and this study is the first to employ direct sequencing of the 18SrRNA and ITS1 genes with samples from a U.K. wide

survey. Hominick et al. (1990a), Chandler et al., (1997) and Gwynne & Richardson (1996) all used Restriction Fragment Length Polymorphism (RFLP) to identify EPN species. However, there are problems with this technique (Linacre & Tobe, 2009) and it does not provide sufficient resolution to understand variation within species (Powers et al., 1997; Szalanski et al., 2000). Similarly, there are also issues with morphological identification of nematodes too as there is a lack of diagnostic traits in members of *Steinernema* and *Heterorhabditis* which can be problematic even for trained experts (Stock, 2002) therefore we believe we have used a potentially more accurate method to determine what species were present.

One of the most interesting species isolated was *S. carpocapsae* as it has not previously been recorded in the U.K. In the U.K. the use of EPNs as biocontrol agents centres on four nematode species: *S. feltiae*, *S. kraussei*, *H. bacteriophora* and *S. carpocapsae*. However, *S. carpocapsae* is considered a non-native EPN species in the U.K. and its use is strictly controlled by the Wildlife and Countryside Act (1981). It is still possible to use *S. carpocapsae* to control pests such as *Sciaridae*, or *Otiorhynchus* spp. (Kim et al., 2003) however, its release is only allowed by holders of a government-issued licence (FERA-DEFRA, 2017). As well as our study there have been some previous references to possible findings of *S. carpocapsae* in the U.K. (Georgis & Hague, 1981; Boag et al., 1992) but none of these have been confirmed by molecular methods (Torr et al., 2007b). *S. carpocapsae* is described as having a cosmopolitan distribution (Poinar, 1979; Hominick et al., 1996; Gaugler, 2002) and has been recorded in a wide range of geographic locations including the USA, Argentina, Australia and Mexico (Peters, 1996). These geographic regions comprise a vast range of ambient temperatures and habitats. However, it has not frequently been found in the temperate areas of Europe (Kary et al., 2009; Mráček et al 2005, Sturhan, 1999). Both isolates of *S. carpocapsae* were found in rural locations (wooded layby and rural farm) and

222 therefore it is unlikely that they originated from EPNs released from control usages. This
223 confirmed presence of *S. carpocapsae* on mainland UK has ramifications for the controlled
224 use of this EPN.

225 EPNs exhibit habitat preferences, for example *S. kraussei* is found more frequently in
226 woodlands (Mráček et al., 2005; Ali & Wharton 2017), and *S. glaseri* is associated with
227 looser soil textures (Koppenhofer & Fuzy, 2006). It has previously been considered a
228 nematode of temperate climates (Gaugler, 2002) although Al-Own (2013), using the same
229 molecular methods as this survey, identified 16 isolates in the South West of the U.K.
230 mainland. *Steinernema affine* is associated with grassland habitats (Torr et al., 2007a) and is
231 often found with *S. feltiae* (Sturhan, 1999). *Steinernema feltiae* is found globally and in a
232 wide range of habitats such as pastures, roadsides and any areas where human disturbance is
233 minimal (Hominínck et al., 1996). Our strains of *S. affine* were found in soil from a selection
234 of habitats including moorland, a hedgerow and grassland and our single isolate of *S. feltiae*
235 was found in an urban car park.

236 We also focused on the island of Lundy, which has never been previously sampled for
237 EPNs. Lundy Island is a biologically and geologically diverse habitat and has been the
238 subject of much scientific interest. This small island displays three unique endemic species
239 including the Lundy cabbage (*Coincya wrightii*), the bronze Lundy cabbage flea beetle
240 (*Psylliodes luridipennis*) and the Lundy cabbage weevil (*Ceutorhynchus contracus spp.*
241 *pallipes*). From 46 soil samples we found *S. glaseri* is present on Lundy. How or when this
242 species was introduced to the island is unknown but further work is needed to investigate the
243 interactions of this species with the unique and diverse flora and fauna that have evolved on
244 Lundy, particularly the indigenous insect species.

As well as EPNs there were numerous other nematodes that were also isolated using the *G. mellonella* bait technique. These included members of the *Oscheius* genus, which are commonly found in soil and live on decaying matter and some are thought to be entomopathogenic (Ye et al., 2010). *Pristionchus* nematodes were also found which are bacterivorous and transported by beetles (Herrmann et al., 2006). However, the most abundant nematodes found were those of the genus *Acrobeloides*. These nematodes are bacterial-feeding nematodes found in agricultural soil (Bird & Ryder, 1993) and are routinely found in soil surveys (Campos-Herrera et al., 2016). There are no reports of these nematodes killing insects so they must have been isolated because they reproduced on dead *G. mellonella*. It should be noted that we had difficulties positively identifying these nematodes using just analysis of the 18SrRNA gene (Blaxter et al., 1998) and when first examined the majority of isolates were identified as *Cervidellus vexilliger*. This is due to the complex evolutionary relationships of members of the Cephalobidae that are difficult to identify at the morphological and molecular level (Smythe & Nadler, 2006) and several genes should be used to separate these genera. Other nematodes isolated included a member of the gastropod parasitic genus *Phasmarhabditis* (designated species VP2016a); whilst not a parasite of insects (Wilson et al., 1994) these nematodes readily eat bacteria and hence would be found on rotting *G. mellonella*. We failed to isolate any *Heterorhabditis* sp. even though we sampled from sand dunes, beach hinterlands and sandy soils where they can be found (Rolston et al., 2005). However, this is not unusual as it seems that *Heterorhabditis* spp. are infrequently recovered from these types of surveys (Sturhan & Liskova, 1999) with only two out of six U.K. based sampling surveys finding *Heterorhabditis* (Homininck & Briscoe 1990a; Homininck et al., 1995).

In conclusion, we have carried out a U.K. based soil survey looking for indigenous EPNs. From 518 soil samples we managed to find four species of *Steinernema*, which is a

similar number of species present in all six surveys conducted over 29 years in the U.K. (Hominick & Briscoe, 1990a,b; Griffin et al., 1991, Blackshaw, 1988; Hominick et al., 1995; Chandler et al., 1997; Gwynne & Richardson, 1996) but we have found, for the first time, *S. carpocapsae* in U.K. soils.

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Figure legends

Fig 1: Location of soil samples collected throughout the U.K (A) and those that were positive for EPNs including *S. affine* (white circle), *S. feltiae* (grey circle), *S. carpocapsae* (black circle) and *S. glaseri* (black star) (B). Each dot represents a location where at least one soil sample was taken. Scale bar represents 100 miles.

Supplementary Figure legends

Suppl. Fig 1: Interactive map of exact location of soil samples collected throughout the U.K. which can be found at: https://www.google.com/maps/d/viewer?mid=1P70uNnG_HR5nAj_H_0J1pCTx_sk&ll=54.34787995568756%2C-5.651589203125013&z=7

Each yellow dot represents location where at least one soil sample was taken and the exact location of soil samples where EPNs were recorded included: *S. affine* (red star), *S. feltiae* (purple cross), *S. carpocapsae* (green square) and *S. glaseri* (blue diamond).

Table legends

Table 1: Date of collection, collection location, land use and soil type of samples collected throughout the U.K which had EPNs present.

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