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

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12 Running head: EPNs found in the U.K.

13 **Abstract**

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

25

26 **Keywords**

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

29

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

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

55 (1992) sampled Scotland in 40 x 40 km grids and achieved a return rate of approximately
56 2.2% from their sampling regime finding only Steinernema feltiae and a nematode they
57 suspected was S. carpocapsae. However, Hominick & Briscoe (1990a,b) had a higher
58 success rate with 48% of samples being positive for finding EPNs such as S. bibionis, an
59 unidentified Steinernema sp. and an unidentified Heterorhabditis sp. There were several
60 factors that may contribute to this difference in success rate, one of which may be the
61 modifications to the standard method of isolating EPNs, the Galleria mellonella trap method
62 (Glazer & Lewis 2000). For example, some studies baited soil samples with G. mellonella
63 only once (Griffin et al., 1991) but others tried two times (consecutively) and at two different
64 temperatures (Hominick & Briscoe, 1990a,b). Also some studies used Tenebrio molitor
65 instead of G. mellonella (Boag et al., 1992), which may affect the numbers and species of
66 EPNs isolated. As there have been mixed results discovering what EPN species are present in
67 the U.K., and the last survey was over 20 years ago (Gwynn & Richardson, 1996), we
68 decided to carry out a survey of the biodiversity and distribution of the U.K. EPN fauna by
69 collecting soil samples and baiting them with G. mellonella, followed by using White traps to
70 grow any potential EPNs. Identification of species used standard genotyping procedures.

71

72 **Materials and Methods**

73 **Collection of soil samples from across the U.K.**

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

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

85 In addition to mainland U.K. fine scale sampling of the island of Lundy was
86 conducted (n = 46 soil samples), which had never been previously sampled for EPNs. Lundy
87 Island is a small, flat topped, granite island situated 18 km from the coast of North Devon
88 with the Atlantic Ocean on its west side and the Bristol Channel on its east. It is a small
89 island extending to 5 km long and 1 km wide with an area of 345 ha (JNCC 2017). A
90 patchwork of different habitats covers the top of the island including open heathland, rough
91 and improved grassland, patches of moorland with acidic bogs and pools and rare waved
92 heathland to the north of the island (JNCC, 2017). Samples were taken based on a grid
93 system covering the whole of the island and were collected between September 2014 and July
94 2015.

95

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

97

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

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

105

106 **Molecular identification of EPN species**

107

108 Any nematodes that were found en-masse in the surrounding water of the White trap
109 after 28 days were identified using molecular analysis. Between 30-50 dauer juveniles were
110 placed in a 1.5 ml Eppendorf tube and their DNA extracted using a Qiagen DNeasy Blood and
111 Tissue DNA extraction kit following the manufacturer's recommended protocol. Nematode
112 DNA was then used for PCR amplification of the 18S rRNA gene for species identification
113 (Blaxter et al., 1998) using the primers SS18U (5'-AAAGATTAAGCCATGCATG-3') and
114 SS26R (5'-CATTCTTGGCAAATGCTTTCG-3'). The PCR cycling conditions were as
115 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
116 and then 72 °C for 7 mins. To aid differentiation between Steinernema species, PCR of the
117 internal transcribed spacer regions ITS-1, 5.8 and ITS-2 using primers TW81 (5'-
118 GTTTCCGTAGGTGAACCTGC-3') and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3')
119 was performed (Spiridonov et al., 2004). Successful amplification of both genes was then
120 checked using gel electrophoresis and PCR products purified using a Thermo Scientific
121 GeneJET PCR Purification Kit. Amplicons were sequenced in both directions by GATC
122 (Constance, Germany). Sequences were manually checked and edited and used to identify to
123 species level using a BLASTN search in NCBI database (Altschul 1990).

124

125 **Results**

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

127

128 From 518 soil samples collected across the length and breadth of the U.K. (Fig 1A;
129 Supplementary Fig 1), 18 (3.5%) were positive for EPNs (Fig 1B; Supplementary Fig 1). The
130 18 individual sites had four different species of EPN from the genus Steinernema including
131 eight isolates of S. affine, seven isolates of S. glaseri, one of S. feltiae and two of S.
132 carpocapsae (Fig 1B; Table 1). The sampling sites that contained EPNs were predominantly
133 from rural, sparsely populated settings, the only exception was one isolate of S. feltiae, which
134 was found in an urban car park. No Heterorhabditis spp. were isolated from the soil samples
135 despite numerous samples being taken from habitats where Heterorhabditis spp. had
136 previously been detected in the UK such as sandy coastal areas (Hominick et al., 1995).

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

150 farmland had a single isolate of S. glaseri.

151

152 **Identification of other nematode species**

153

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

164

165 **Discussion**

166

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

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

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

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

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

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

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

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

270 similar number of species present in all six surveys conducted over 29 years in the U.K.
271 (Hominick & Briscoe, 1990a,b; Griffin et al., 1991, Blackshaw, 1988; Hominick et al.,
272 1995; Chandler et al., 1997; Gwynne & Richardson, 1996) but we have found, for the first
273 time, S. carpocapsae in U.K. soils.

274

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276

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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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