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Development of *Phasmarhabditis hermaphrodita* (and members of the *Phasmarhabditis* genus) as new genetic model nematodes to study the genetic basis of parasitism

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Running head: *P. hermaphrodita* - A model nematode to study parasitism

1 **Abstract**

2

3 The genetic mechanisms of how free-living nematodes evolved into parasites
4 are unknown. Current genetic model nematodes (e.g. *Caenorhabditis elegans*) are not
5 well suited to answer this and mammalian parasites are expensive and logistically
6 difficult to maintain. Here we propose the terrestrial gastropod parasite
7 *Phasmarhabditis hermaphrodita* as a new alternative to study the evolution of
8 parasitism and outline the methodology of how to keep *P. hermaphrodita* in the lab
9 for genetic experiments. We show that *P. hermaphrodita* (and several other
10 *Phasmarhabditis* species) are easy to isolate and identify from slugs and snails from
11 around the U.K. We outline how to make isogenic lines using ‘semi-natural’
12 conditions to reduce in lab evolution and how to optimise growth using NGM agar
13 and naturally isolated bacteria. We show that *P. hermaphrodita* is amenable to
14 forward genetics and that *unc* and *sma* mutants can be generated using formaldehyde
15 mutagenesis. We also detail the procedures needed to carry out genetic crosses.
16 Furthermore, we show natural variation within our *Phasmarhabditis* collection with
17 isolates displaying differences in survival when exposed to high temperatures and pH,
18 which facilitates micro and macro evolutionary studies. In summary, we believe that
19 this genetically amenable parasite that shares many attributes with *C. elegans* as well
20 as being in Clade 5 which contains many animal, plant and arthropod parasites, could
21 be an excellent model to understand the genetic basis of parasitism in the Nematoda.

22

23 **Keywords:** *C. elegans*, *P. pacificus*, genetic model, parasitism, evolution

24

25 **Introduction**

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Nematodes have evolved to parasitise arthropods, plants and mammals (Blaxter et al., 1998) but the genetic mechanisms of how parasitism evolved are unknown. Pre-adaptations that are thought to be key for this evolutionary transition include close associations with arthropod hosts (Blaxter & Koutsovoulos, 2015) and the ability to arrest development which can aid with coping with stressful conditions such as host enzymes (Poulin, 1998; Weischer & Brown, 2000). There are several parasitic species that have been developed as genetic models but are unsuitable to answer this question as they require intermediate and/or definitive mammal hosts (e.g. *Brugia* sp.) to complete their lifecycle, which can be financially and logistically prohibitive and laborious (Lok & Unnasch, 2013). Due to these problems genetic experiments can be difficult. More developed genetic model nematodes are associated with invertebrates. *Caenorhabditis elegans* is thought to have a loose association with slugs and snails (Petersen et al., 2015; Schulenburg & Félix, 2017) and the diplogastrid nematode *Pristionchus pacificus* has a necromenic relationship (coined by Schulte, 1989) with scarab beetles where it infects hosts waits for them to die and reproduces on their cadaver (Morgan et al., 2012). However, both species are not parasitic (Rae & Sommer, 2011; Herrmann et al., 2006); therefore provide little information about the underlying evolution of genetic mechanisms that are used to infect, parasitise and even kill their hosts. They are however formidable at unravelling genes involved with a plethora of biological important and ecologically important traits (The *C. elegans* Research Community, 2005; Sommer, 2015). Both of these species are successful as nematode genetic models as they can be isolated easily, can be kept in culture and can be grown in large amounts (on Nematode Growing Media (NGM) plates fed *Escherichia coli* OP50), they can be mutagenized and can be mated

1 easily (Brenner, 1974; Sommer et al., 2000). Furthermore, as well as full genome
2 sequence (*C. elegans* Sequencing Consortium, 1998; Dieterich et al., 2008) post
3 genomic tools such as reverse genetic techniques; first RNAi (Fire et al., 1998;
4 Cinkornpumin & Hong, 2011) now CRISPR-Cas9 (Lo et al., 2013; Witte et al., 2015)
5 can be carried out in both species to understand gene function as well as transgenic
6 techniques facilitating the analysis of gene expression (Chalfie et al., 1994; Schlager
7 et al., 2009). Similar techniques can be carried out in mammalian parasites e.g.
8 *Brugia malayi*, *Nippostrongylus brasiliensis* and *Ascaris suum* but the efficiency is
9 variable and only a selection of genes can be inhibited (Geldhof et al., 2006; 2007). A
10 promising genetic model to study nematode parasitism would combine the ease of
11 keeping and growing *C. elegans* and *P. pacificus* en masse in the lab with the ability
12 to collect different species and strains easily to facilitate micro and macro
13 evolutionary studies. Also it would be closely related to other parasitic and
14 necromenic species that would allow genomic comparison of the evolution of
15 potential parasitism genes from different parasitic lifestyles. Furthermore, it would be
16 able to be genetically manipulated that would allow an in-depth analysis of gene
17 function.

18 A nematode that theoretically meets all these criteria is the terrestrial
19 gastropod parasite *Phasmarhabditis hermaphrodita* (Fig 1A). *P. hermaphrodita* can
20 complete its life cycle in several ways. First, it can infect and kill several susceptible
21 slug species (e.g. *Deroceras reticulatum*) (Wilson et al., 1993; Rae et al., 2009).
22 Second, it can infect and remain inside larger slug and snail species waiting for the
23 host to die, where it reproduces on the decaying cadaver when the host dies (termed
24 ‘necromeny’) (Rae et al., 2009). Third, it can reproduce on decomposing organic
25 matter such as leaf litter, dead earthworms and slug faeces (Macmillan et al., 2009;

1 Tan and Grewal, 2001a). Therefore, it is not an obligate parasite that requires a host
2 to survive but a bacterivorous nematode that can be grown in the lab without slugs but
3 is still able to retain its pathogenicity towards slugs. Due to its pathogenic potential it
4 has been formulated into a biological control agent (Nemaslug® from BASF-
5 Agricultural Specialities) for farmers and gardeners to control slugs and snails (Rae et
6 al., 2007). Nematodes are applied to soil where they actively seek out slugs and infect
7 and kill them 4-21 days later (Wilson et al., 1993; Tan and Grewal, 2001a). *P.*
8 *hermaphrodita* has been shown to provide protection against slug damage in many
9 agriculturally important crops (Wilson & Rae, 2015). Although, *P. hermaphrodita*
10 has received considerable attention as an agricultural biopesticide it is interesting
11 from a fundamental evolutionary perspective and has been proposed as an excellent
12 candidate as a genetic model to elucidate how parasitism has arisen in free-living
13 species (Rae, 2017; Wilson et al., 2015). It was even a potential candidate as Sydney
14 Brenner's nematode of choice instead of *C. elegans* (Cold Spring Harbour Laboratory
15 Archives, 2017; <http://libgallery.cshl.edu/items/show/75709>). *P. hermaphrodita* is the
16 only nematode from an estimated 1 million (Lambhead, 1993) that has evolved to
17 parasitise and kill gastropods. Also there are over 108 species of nematodes that
18 parasitise molluscs and four out of five clades of the Nematoda have members that
19 parasitise gastropods (Grewal et al., 2003; Blaxter et al., 1998). Therefore, parasitism
20 of gastropods is a very important lifestyle choice amongst nematodes; but the genes
21 involved to infect and survive in these hosts are unknown.

22 The majority of research on *P. hermaphrodita* has focused on optimizing
23 application techniques in the field (see Rae et al., 2007), host range studies (Wilson et
24 al., 1993; Grewal et al., 2003; Rae et al., 2009), taxonomic descriptions and surveys
25 charting abundance and diversity of *Phasmarhabditis* in different countries

1 (Tandingan De Ley et al., 2014, 2016; Wilson et al., 2012; Nermut et al., 2016a,b,c;
2 Ross et al., 2012; 2015). There are few details and no methods about how to keep this
3 nematode under lab conditions like *C. elegans* and whether it could be amenable to
4 genetic manipulation. There is little information about life history traits and how they
5 change with bacterial diet or temperature. Also there have been few experiments
6 looking at natural variation in the genus *Phasmarhabditis* in any ecological traits as
7 all studies so far have focused on using the commercial strain of *P. hermaphrodita*
8 (DMG0001) that has been in culture for over 20 years (Rae et al., 2007).

9 Here we provide information on how to grow, maintain, mutagenize and mate
10 not only *P. hermaphrodita* but also several species of the *Phasmarhabditis* genus
11 under lab conditions. We also provide information on how to isolate, identify and
12 make isogenic lines of *P. hermaphrodita*. Taken together, these results show that
13 many of these species can be easily maintained under lab conditions and could make
14 excellent candidates as genetic models to understand the evolution of parasitism in
15 the Nematoda.

16

17 **Materials and methods**

18

19 **Terrestrial gastropod survey and molecular identification of parasitic nematodes**

20

21 Slugs (*Deroceras panormitanum*, *D. reticulatum* (Fig 1B), *Arion subfuscus*, *A.*
22 *ater*, *A. hortensis*, *Limax flavus*, *L. maximus*, *Lehmannia valentiana*, *Milax*
23 *budapestensis* and *M. sowerbyi*) and snails (*Cepaea nemoralis*, *Cornu aspersum* and
24 *Oxychilus draparnaudi*) were collected from several locations in Liverpool, U.K
25 including: Priory wood near St Michael's station (Grid reference number

1 SJ3673586862) (n = 107), Sefton Park (SJ3787187058) (n = 195) and Otterspool
2 (SJ3707686321) (n = 57). Slugs were also collected from the Cruickshank Botanic
3 gardens at the University of Aberdeen (NJ9376008556) (n = 48) and from Dale,
4 Wales (SM809057) (n = 19). Once collected they were transported back to the lab
5 where they were chopped in half and placed in a 5 cm Petri dish with a few drops of
6 distilled water and stored at room temperature. Over 4 days the slugs were examined
7 for presence of nematodes. Any nematodes that morphologically resembled
8 *Phasmarhabditis*-like nematodes (Fig 1) e.g. over 1 mm hermaphrodites, females or
9 males were individually transferred to modified White traps (White, 1927) (see below
10 for description) to make isogenic lines. After 21 days when the food was exhausted
11 and the nematodes had reached the dauer stage they were present in the surrounding
12 water and were removed and centrifuged at 16,000 rpm to concentrate and their DNA
13 was extracted using a DNA extraction kit (Qiagen). Using PCR three genes were then
14 amplified (ITS1, 18SrRNA and the D2-D3 domain of LSU rDNA) (Blaxter et al.
15 1998; Tandingan De Ley et al., 2014) and sequenced in both directions for species
16 identification. For the ITS1 gene the primers used were N93 (5'-
17 TTGAACCGGGTAAAAGTCG-3') and N94 (5'-TTAGTTTCTTTTCCTCCGCT-3').
18 The 18SrRNA gene was amplified using 18A (5'-AAAGATTAAGCCATGCATG-
19 3') and 26R (5'-CATTCTTGGCAAATGCTTTCG-3'). The D2-D3 LSU rDNA
20 primers were D2 (5'-AGCGGAGGAAAAGAACTAA-3') and D3 (5'-
21 TCGGAAGGAACCAGCTACTA-3'). As well as these three genes *P. hermaphrodita*
22 specific primers were also used which were based on 150-200 bp of the Cytochrome
23 Oxidase I gene created by Read et al. (2006), which consisted of Ph-F-1754 (5'-
24 TGGGTGCCCTGATATAAGAT-3) and Ph-R-1887 (5-
25 CGGATGACCAAGGGTACTTAAT-3). These primers were used to examine if they

1 could provide a rapid and cheap method for identifying *P. hermaphrodita* without
2 DNA sequencing as they have been used previously to determine if *P. hermaphrodita*
3 was present in mites and collembolans (Read et al. 2006). PCR cycling conditions
4 consisted of the following: 3 mins at 95°C followed by 35 cycles of 15 secs at 95°C,
5 30 secs at 55°C, 1.5 in at 72°C and a final step of 8 mins at 72°C. The PCR products
6 were then purified and sequenced in both forward and reverse directions for each
7 gene (ITS1, 18SrRNA and the D2-D3 domain of LSU rDNA). Gene sequences of
8 nematodes were then compared with NCBI database sequences using BLASTN
9 searches using similarly matches of 99%. For PCR using primers designed by Read et
10 al. (2006) the 200 bp product was visualised after gel electrophoresis where a positive
11 band indicates presence of *P. hermaphrodita* and no sequencing was carried out.

12

13 **Semi-natural conditions for growth of *Phasmarhabditis* species to make isogenic** 14 **lines and dauer juveniles**

15

16 Any potential *Phasmarhabditis* like nematodes observed growing on the
17 collected rotting slugs and snails were transferred to modified White traps (White,
18 1927) for maintenance, to make isogenic lines and to quantify the number of dauers
19 that were produced per individual hermaphrodite mother. Modified White traps
20 consisted of a 10 cm Petri dish half filled with tap water. A 5 cm lid of a Petri dish
21 was then placed on top of the water, which had a 4.5 cm diameter piece of pre-
22 moistened Whatman number 1 filter paper inside. To each White trap a 3 mm slice of
23 *L. flavus* was added as a food source. *L. flavus* were collected from LJMU
24 greenhouses and frozen at -80°C for 1 hour to kill any nematodes present. *L. flavus*
25 was used for *Phasmarhabditis* food for several reasons including: 1. It is a common

1 slug species easily collected 2. It is a large slug, which allows many White traps to be
2 made at one time 3. From preliminary experiments it consistently produces large
3 numbers of nematodes. Also it should be stressed that a previous study has shown that
4 feeding on this slug species in White traps produces consistently virulent *P.*
5 *hermaphrodita* (Rae et al., 2010). A single hermaphrodite mother was then
6 transferred via a worm pick to each White trap and then sealed with Parafilm® and
7 stored at 20°C for 21 days. After which dauer stage nematodes were found in the
8 surrounding water. This procedure was repeated for all of the *Phasmarhabditis*
9 collection to make isogenic lines.

10 In a separate experiment the numbers of dauer juveniles produced by one self-
11 fertilising hermaphrodite mother was quantified for two strains of *P. hermaphrodita*
12 (commercial strain DMG0001 and the naturally isolated strain DMG0007) and 1
13 strain of *P. californica* (DMG0017). These strains were chosen to understand if there
14 was a difference between the growth of the commercial strain (*P. hermaphrodita*
15 DMG0001; Hooper et al., 1999) and natural strains of *P. hermaphrodita* as the
16 commercial strain has been in culture for over 20 years and fed solely on the
17 monoxenic diet consisting of the bacterium *Moraxella osloensis* (Wilson et al.,
18 1995a,b). In total the number of dauer juveniles produced by a single hermaphrodite
19 mother of *P. hermaphrodita* DMG0001, DMG0007 or *P. californica* (DMG0017)
20 was quantified by making 5 White traps containing either 0.025 g or 0.25 g of *L.*
21 *flavus* and left for 21 days at 20°C (Fig 1D). The experiment was repeated twice.

22

23 **Brood size of *Phasmarhabditis* species exposed to lab and naturally isolated**
24 **bacteria**

25

1 We investigated whether *Phasmarhabditis* nematodes could grow on several
2 different bacteria including *Escherichia coli* OP50 (the food of *C. elegans* and *P.*
3 *pacificus*, Brenner, 1974; Sommer et al., 2000) and *E. coli* BR (a strain used for
4 cloning in molecular biology and easily available) and two naturally isolated bacterial
5 species found associated with *Pristionchus entomophagus* called *Pseudomonas* sp. 1
6 and *Bacillus* sp. 1 (Rae et al. 2008) (Fig 1C). Bacteria were grown in nutrient broth at
7 30°C overnight. The following morning 100µl of each bacterium was spread onto five
8 NGM (Nematode Growing Media; Hope, 1999) plates (5 cm) and were then
9 incubated at 30°C overnight. An individual dauer stage nematode was transferred to
10 each plate via a worm pick and incubated at 20°C. The numbers of offspring were
11 then recorded per plate after 6 days. The experiment consisted of using *P.*
12 *hermaphrodita* DMG0001 and DMG0007 and *P. californica* DMG0017. This
13 experiment was repeated three times with all four bacteria and with all three
14 nematode isolates.

15 In order to understand the feeding behaviour of *Phasmarhabditis* nematodes
16 in more detail we also recorded the pharyngeal pumping rate whilst they were eating.
17 This has been observed easily in both *C. elegans* and *P. pacificus* (Kroetz et al., 2012)
18 but never for any *Phasmarhabditis* species. The pharyngeal pumping rate of an
19 individual of three *Phasmarhabditis* species (*P. hermaphrodita* DMG0007, *P.*
20 *neopapillosa* DMG0012 and DMG0016; and *P. californica* DMG0017) was counted
21 for 60 secs and repeated 10 times with separate worms.

22

23 **Investigating the effect of temperature on the brood size of *Phasmarhabditis***
24 **species**

25

1 To examine what the optimum temperature for growth of *Phasmarhabditis*
2 nematodes was under laboratory conditions fifteen 5 cm NGM plates were seeded
3 with 100µl of *Pseudomonas* sp. 1 and then incubated at 30°C overnight.
4 *Pseudomonas* sp. 1 was chosen as out of all 4 bacterial species tested
5 *Phasmarhabditis* nematodes produced a large number of offspring, which were easy
6 to see in the bacterial lawn due to its translucent nature. A single L4 hermaphrodite
7 was placed onto each NGM plate and groups of 5 plates were incubated at either
8 10°C, 15°C or 20°C for 6 days. On day 3 and 6 the numbers of offspring were then
9 recorded. This experiment was repeated three times using the same nematode species
10 and strains as above.

11

12 **Heat shocking and spontaneous male rate of several *Phasmarhabditis* species**

13

14 It is imperative that a genetic model nematode can be mated under laboratory
15 conditions and it is unknown how common males are produced in *P. hermaphrodita*
16 collected from the wild. Five NGM plates (5 cm) were spread with 50 µl of
17 *Pseudomonas* sp. 1 and incubated overnight at 30°C. The following morning dauer
18 juveniles (between 1,000 to 7,000 per strain) were added to each plate and they were
19 incubated at 20°C for 2-3 days. The numbers of males present was then recorded. The
20 species and strains used (and numbers of dauer observed) were: *P. hermaphrodita*
21 DMG0001 (n = 4040), DMG0002 (n = 6771), DMG0010 (n = 4581), DMG0009 (n =
22 3108), DMG0003 (n = 2503) and DMG0007 (n = 3572); *P. californica* DMG0017 (n
23 = 1098) and DMG0019 (n = 1127) and *P. neopapillosa* (DMG0012, DMG0015 and
24 DMG0016; n = 750 for each strain).

1 In *C. elegans* the number of males can be increased by exposing
2 hermaphrodite mothers to 30°C for 4 hours (Hope et al., 1999). We examined
3 whether the same was true for *P. hermaphrodita* (DMG0001, DMG0007 and
4 DMG0009). Fifteen to twenty L4 hermaphrodites were added to 5 separate 5 cm
5 NGM plates seeded with *Pseudomonas* sp. 1 and placed in a 30°C incubator for 1, 3,
6 4.5, 5 and 6 hours. After which the plates were placed at 20°C to recover and the
7 number of males in the offspring was recorded after 4 days. The experiment was
8 repeated 3 times.

9

10 **Genetic crosses of *Phasmarhabditis* species under lab conditions**

11

12 Some parasitic nematodes are difficult to mate under lab conditions using agar
13 plates e.g. the free-living generation of *Strongyloides ratti* (Nemetschke et al., 2010)
14 and it is unknown if *P. hermaphrodita* or any other *Phasmarhabditis* species can be
15 mated which is essential to monitor the inheritance of mutations and to facilitate
16 mapping of mutated genes. Therefore, we used methods that are commonly used to
17 mate *C. elegans*. Specifically, five 5 cm NGM plates with 50 µl of *Pseudomonas* sp. 1
18 and incubated at 30°C overnight. One L4 hermaphrodite was added to each plate with
19 2 young males and the plates were incubated at 20°C for 6 days. After two days of
20 mating the males were removed with a worm pick and killed. After 6 days the sex and
21 number of offspring were recorded. We used *P. neopapillosa* (DMG0012 and
22 DMG0016) a gonochoristic species which produces almost 50% males, as we had
23 difficulties in finding males from *P. hermaphrodita* even after heat shocking. The
24 experiment was repeated three times.

1

2 **Natural genetic variation in thermotolerance and pH tolerance of**
3 ***Phasmarhabditis* species**

4

5 To assess whether there was natural genetic variation in the ability of the
6 collection of *Phasmarhabditis* nematodes to cope with extreme pH and temperatures
7 the following experiments were carried out. For the thermotolerance experiment three
8 1.5ml Eppendorf tubes containing between 4,500-6,000 nematodes per 1ml were
9 placed into a heat block set at 33°C, 37°C or 41°C for 15 mins. At time 0 min and
10 after 15 mins the numbers of nematodes were quantified. The Eppendorf containing
11 the nematodes was vortexed every 2.5 minutes to avoid clumping. Eppendorfs
12 containing the same numbers of nematodes but kept at room temperature were used as
13 the control. The experiment was repeated three times for each temperature. The
14 following species and strains were used: *P. hermaphrodita* (DMG0001, DMG0007,
15 DMG0010, DMG0006 and DMG0008), *P. californica* (DMG0017 and DMG0019)
16 and *P. neopapillosa* (DMG0013, DMG0015 and DMG0016).

17 To assess natural variation of pH tolerance 10 individual dauer stage
18 nematodes were added to eighty wells containing 60µl of water adjusted to pH 4, 5, 6,
19 7, 8, 9 and 10 as well as a control of distilled water. The correct pH was obtained by
20 addition of either 1 M NaOH or 1 M HCl and confirmed a pH meter and indicator
21 paper. There were 10 wells per pH and the whole experiment was repeated twice. The
22 96 well plate was then incubated at 20°C and survival was recorded observed daily
23 for 4 days. The same *Phasmarhabditis* species and strains were used as in the
24 thermotolerance experiment.

25

1 **Formaldehyde mutagenesis of *P. hermaphrodita* DMG0001**

2

3 To investigate if *P. hermaphrodita* can be mutated using formaldehyde
4 mutagenesis (like *C. elegans* and *P. pacificus*) we used similar methods developed by
5 Johnson & Baillie (1988) for *C. elegans*. *P. hermaphrodita* DMG0001 (L4 and young
6 adult stage) were grown on several NGM plates with *Pseudomonas* sp. 1 for 4 days.
7 They were then washed in distilled water and concentrated to a pellet and exposed to
8 0.1% formaldehyde for 4 hours. After which the P0's were washed several times in
9 water to remove any residual formaldehyde and 100 individual mothers were
10 separated out and placed on individual NGM plates seeded with *Pseudomonas* sp. 1.
11 They were stored at 20°C for 3-4 days and allowed to produce offspring and then 300
12 F1's were separated out (3 individuals were randomly picked from each plate of P0
13 mothers) and the F2's were screened for any morphological abnormalities after 5-7
14 days.

15

16 **Data analysis**

17

18 The difference between the numbers of dauers produced by *P. hermaphrodita*
19 DMG0001, DMG0007 and *P. californica* DMG0017 grown on 0.025 g and 0.25 g of
20 *L. flavus* was analysed using a One Way ANOVA with Tukey's post hoc test. These
21 tests were also used to analyse the data on pumping rate, number of offspring
22 produced on different bacteria and at different temperatures, as well as the numbers of
23 surviving nematodes exposed to 33, 37 and 41°C and pH 4 to 10. The body length of
24 *P. hermaphrodita* DMG0001 WT and *sma* mutants was compared using a Student's t
25 test. SPSS version 23 was used for analysing data.

1

2 **Results**

3

4 ***Phasmarhabditis* species can be easily isolated and identified from gastropods**

5

6 From 426 slugs and snails collected from around the U.K. we found 12
7 isolates of *P. hermaphrodita*, 3 isolates of *P. californica* and 5 isolates of *P.*
8 *neopapillosa* (Table 1). These isolates were all from separate slugs apart from *P.*
9 *californica* where 3 isolates were found in a single *O. draparnaudi*. We had initially
10 identified many of these *Phasmarhabditis* species as *P. hermaphrodita* by using
11 species-specific primers developed by Read et al. (2006) (Supplementary Fig 1).
12 However, we found that these primers do not just amplify *P. hermaphrodita* but also
13 other members of the *Phasmarhabditis* genus and even diverse insect associated and
14 free-living species such as *Steinernema feltiae*, *Panagrellus redivivus*, *Aphelenchus*
15 *avenae*, *Pelodera teres* and *Pristionchus entomophagus* (Supplementary Fig 1).
16 Hence, they are not suitable for identification of *P. hermaphrodita* specifically and
17 should be used with caution. We therefore amplified and sequenced three genes
18 (ITS1, 18SrRNA and the D2-D3 domain of LSU rDNA) for species identification.
19 These *P. hermaphrodita* strains and *Phasmarhabditis* species are the start of an on-
20 going effort to make a collection of *P. hermaphrodita* strains and *Phasmarhabditis*
21 species to study the genetic evolution of parasitism and we have categorised them
22 using *C. elegans* nomenclature (Table 1).

23

24 **Growth of *Phasmarhabditis* species using semi-natural conditions**

25

1 We made isogenic lines by growing single hermaphrodites of *P.*
2 *hermaphrodita* DMG0001, DMG0007 and *P. californica* DMG0017 on 0.025 g (Fig
3 2A) and 0.25 g of *L. flavus* (Fig 2B). The numbers of dauer juveniles that were
4 produced by one *P. hermaphrodita* DMG0001, DMG0007 and *P. californica*
5 DMG0017 on 0.025 g of *L. flavus* after 21 days ranged from 43 to 6,166 dauers per
6 White trap (Fig 2A) and did not differ significantly ($F(2, 36) = 1.369$; $p = 0.268$).
7 The numbers of dauer juveniles that were produced by single mothers of *P.*
8 *hermaphrodita* DMG0001, DMG0007 and *P. californica* DMG0017 fed on 0.25 g of
9 *L. flavus* after 21 days ranged from 417 to 27,750 dauers per plate (Fig 2B) and also
10 did not differ significantly ($F(2, 38) = 2.832$; $p = 0.072$). Therefore, *Phasmarhabditis*
11 sp. can be easily grown under semi-natural conditions using *L. flavus* White traps and
12 in large numbers for experiments.

13

14 **Growth of *Phasmarhabditis* species on different bacteria at different** 15 **temperatures**

16

17 *P. hermaphrodita* DMG0001, DMG0007 and *P. californica* DMG0017 were
18 fed two nematode associated bacteria (*Pseudomonas* sp. 1 and *Bacillus* sp. 1) and two
19 strains of *E. coli* that have been used historically in *C. elegans* culture and molecular
20 biology (*E. coli* OP50 and *E. coli* BR, respectively). Over 6 days both nematode
21 species were able to grow on each of these bacteria and laid F1 eggs that developed to
22 offspring and were quantified (Fig 3). There was a significant difference between the
23 numbers of offspring produced by *P. hermaphrodita* DMG0001 when fed
24 *Pseudomonas* sp. 1; *Bacillus* sp. 1; *E. coli* OP50 and *E. coli* BR ($F(3, 29) = 11.101$; p
25 $= 0.000$). Specifically, the numbers of offspring produced by *P. hermaphrodita*

1 DMG0001 was highest on *Pseudomonas* sp. 1, *E. coli* OP50 and *E. coli* BR but
2 produced the least number of offspring on *Bacillus* sp. 1 ($p < 0.05$). This was also the
3 case for *P. hermaphrodita* DMG0007 and *P. californica* DMG0017. Therefore, the
4 laboratory bacteria (*E. coli* OP50 and *E. coli* BR) and naturally isolated *Pseudomonas*
5 sp. 1 can be used for growing *Phasmarhabditis* species.

6 When grown at specific temperatures (10°C, 15°C and 20°C) and fed
7 *Pseudomonas* sp. 1 to investigate the optimum conditions for growth and brood size
8 of *P. hermaphrodita* DMG0001, *P. hermaphrodita* DMG0007 and *P. californica*
9 DMG0017 it was found that 20°C was best for growth for both species (Fig 4). There
10 was no significant difference between the numbers of offspring of both species when
11 fed *Pseudomonas* sp. 1 at 20°C after 3 days ($F(2,35) = 0.917$; $p = 0.41$). However,
12 after 6 days at 20°C *P. hermaphrodita* DMG0007 produced significantly more
13 offspring than *P. hermaphrodita* DMG0001 and *P. californica* DMG0017 ($F(2,31) =$
14 5.067 ; $p = 0.013$). Production of offspring of both species was low at 10°C after 6
15 days. Eggs were laid in small numbers but they did not develop into live offspring.
16 There was no significant difference between the numbers of viable offspring
17 produced by both species after 6 days at 15°C ($F(2,28) = 1.649$; $p = 0.212$). When
18 grown at temperatures higher than this (25°C) the hermaphrodite mothers died rapidly
19 (Andrus and Rae, unpublished observation) so this seems to represent the upper limit
20 for growth of these natural strains.

21 When feeding on *Pseudomonas* sp. 1 there was a significant difference
22 between the pumping rates of *P. hermaphrodita* DMG0001, *P. hermaphrodita*
23 DMG0007, *P. neopapillosa* DMG0012 and DMG0016 and *P. californica* DMG0017
24 ($F(4, 19) = 18.577$; $p = 0.000$) (Fig 5). Specifically, there was no difference between
25 the pumping rate of *P. hermaphrodita* DMG0001 and DMG0007, *P. neopapillosa*

1 DMG0012 and DMG0016 ($p > 0.05$) but all differed significantly with *P. californica*
2 DMG0017, which had the lowest number of pumps per minute ($p < 0.05$) (Fig 5).

3

4 **Natural variation in thermotolerance of *Phasmarhabditis***

5

6 There was a significant difference between the survival of the
7 *Phasmarhabditis* isolates when exposed to 30°C ($F(10, 98) = 18.389$; $p < 0.001$) (Fig
8 6A). Specifically, the survival of the commercial strain *P. hermaphrodita*
9 (DMG0001) was significantly lower than *P. hermaphrodita* DMG0007 and
10 DMG0008 ($p < 0.001$) but not *P. hermaphrodita* DMG0010 and DMG0006 ($p >$
11 0.05). When the survival of *P. hermaphrodita* (DMG0001) was compared to other
12 species of *Phasmarhabditis* there was a significant difference between *P.*
13 *neopapillosa* DMG0015 and DMG0016 ($p < 0.001$) but not DMG0013 ($p > 0.05$).
14 Also there was a significant difference between the survival of *P. hermaphrodita*
15 (DMG0001) compared to *P. californica* DMG0019 ($p < 0.05$) but not DMG0017 ($p >$
16 0.05).

17 When the *Phasmarhabditis* isolates were exposed to 37°C there was a
18 significant difference in their survival ($F(10, 98) = 24.017$; $p = 0.000$) (Fig 6B). The
19 survival of *P. hermaphrodita* (DMG0001) was significantly lower than *P.*
20 *hermaphrodita* DMG0007 but significantly higher than DMG0010 ($p < 0.05$) but not
21 any other *P. hermaphrodita* strain. The survival of *P. hermaphrodita* DMG0001
22 differed from *P. neopapillosa* DMG0015 ($p < 0.05$) but no other species or isolate.

23 When the *Phasmarhabditis* isolates were exposed to 41°C there was a
24 significant difference in their survival ($F(10, 98) = 19.546$; $p = 0.000$) (Fig 6C). The

1 survival of *P. hermaphrodita* (DMG0001) was significantly greater than all other
2 species and isolates ($p < 0.05$).

3

4 **Natural variation in pH resistance in *Phasmarhabditis***

5

6 When the *Phasmarhabditis* species and strains were exposed to pH 4 there
7 was a significant difference in survival ($F(9, 29) = 6.060$; $p = 0.000$) (Table 2) with
8 the numbers of surviving *P. hermaphrodita* (DMG0001) being significantly greater
9 than that of *P. neopapillosa* DMG0015 and DMG0016 ($p < 0.05$).

10 When exposed to pH 5 the survival of *Phasmarhabditis* species and strains
11 was significantly different ($F(9, 29) = 2.706$; $p = 0.031$) (Table 2) with the survival
12 of *P. hermaphrodita* DMG0007 being significantly lower than *P. californica*
13 DMG0017 ($p < 0.05$). There was no significant difference between the survival of
14 *Phasmarhabditis* species and strains exposed to pH 6 ($F(9, 29) = 1.937$; $p = 0.105$) or
15 pH 8 ($F(9, 29) = 1.956$; $p = 0.101$).

16 The survival of *Phasmarhabditis* also differed at pH 7 ($F(9, 29) = 3.778$; $p =$
17 0.006) with the numbers of alive *P. hermaphrodita* DMG0007 and *P. neopapillosa*
18 DMG0015 being significantly lower than *P. californica* DMG0019 ($p < 0.05$).

19 At pH 9 ($F(9, 29) = 3.378$; $p = 0.011$) and pH 10 ($F(9, 29) = 5.481$; $p =$
20 0.001) survival was significantly different. In both cases there the survival of *P.*
21 *neopapillosa* DMG0015 was significantly lower than that of *P. californica* DMG0017
22 and DMG0019 ($p < 0.05$). Also the survival of *P. hermaphrodita* DMG0007 was also
23 significantly lower than *P. californica* DMG0017 and DMG0019 when exposed to
24 pH 10 ($p < 0.05$).

25

1 **Spontaneous male rate, heat shocking and genetic crosses using *Phasmarhabditis***
2 **species**

3

4 We observed the numbers of *Phasmarhabditis* dauer juveniles that developed
5 into males when grown on NGM agar and fed *Pseudomonas* sp. 1 for 4 days. From 6
6 strains of *P. hermaphrodita* (DMG0001, DMG0002, DMG0010, DMG0009,
7 DMG0003 and DMG0007) and 2 strains of *P. californica* (DMG0017 and
8 DMG0019) no males were observed and only hermaphrodites were produced. All
9 gonochoristic species produced males in varying amounts *P. neopapillosa* DMG0012
10 (50% males to 50% females) and DMG0015 (25% males to 75% females), and
11 DMG0016 (46% males to 54% females).

12 We investigated if the number of males could be increased by heat shocking
13 *P. hermaphrodita* hermaphrodites (DMG0001, DMG0007 and DMG0009) for 1, 3,
14 4.5, 5 and 6 hours at 30°C. No offspring were produced by hermaphrodites that had
15 been exposed to 30°C for 5 and 6 hours and the number of offspring produced was
16 low for heat treatment for 4.5 hours (11 to 30 animals); 3 hours (11 to 33 animals) but
17 increased when exposed for 1 hour (16 to 115 animals). However, no males were
18 observed in any of the offspring. Therefore, it is problematic to find *P. hermaphrodita*
19 males when grown under these conditions.

20 To understand whether *Phasmarhabditis* species could be mated under lab
21 conditions we concentrated on using the gonochoristic species *P. neopapillosa*
22 DMG0012 and DMG0016. Both strains were crossed using standard procedures
23 based on *C. elegans* and were fed *Pseudomonas* sp. 1. Under these conditions we
24 could show that two males to one females placed together using *P. neopapillosa*
25 DMG0016 and DMG0012 resulted in between 47.3 ± 6.7 *P. neopapillosa* DMG0016

1 and 83.9 ± 5.6 *P. neopapillosa* DMG0012 offspring 6 days later. Therefore,
2 successful crossing of these two strains could be carried out to demonstrate
3 inheritance of recessive and dominant mutations and to aid mapping of mutations.

4

5 **Formaldehyde mutagenesis of *Phasmarhabditis* species**

6

7 From 300 F1 *P. hermaphrodita* DMG0001 hermaphrodite mothers (Fig 7A)
8 several mutants were isolated. Specifically, 2 small (*sma*) mutants (Fig 7B,C) and 3
9 uncoordinated (*unc*) mutants (Fig 7D,E) were found. *sma* mutants were significantly
10 smaller than *P. hermaphrodita* WT ($p < 0.05$) and *unc* mutants strongly resembled *C.*
11 *elegans unc* phenotype as they were lethargic, stationary and the underlying body
12 wall muscle produced a constant twitch (Waterson et al., 1980). Therefore, *P.*
13 *hermaphrodita* can be mutagenized using formaldehyde permitting forward genetic
14 screens to be carried out.

15

16 **Discussion**

17

18 The genus *Phasmarhabditis* contains eleven species including *P.*
19 *hermaphrodita*, *P. apuliae*, *P. papillosa*, *P. neopapillosa*, *P. valida*, *P. nidrosiensis*,
20 *P. californica*, *P. tawfiki*, *P. bonaquaense*, *P. bohémica* and *P. huizhouensis*
21 (Andrássy, 1983; Azzam, 2003; Hooper et al., 1999; Huang et al., 2015; Tandingan
22 De Ley et al. 2014; 2016; Nermut et al., 2016a,b,c). *P. hermaphrodita* and other
23 *Phasmarhabditis* species have been isolated from slugs from around the world
24 including the U.K. (Wilson et al., 1993; this study), Germany (Schneider, 1859;
25 Mengert, 1953), France (Coupland, 1995; Maupas, 1900), Iran (Karimi et al., 2003),

1 Egypt (Azzam, 2003; Genena et al., 2011), Norway (Ross et al., 2015), Chile (France
2 & Gerding, 2000), New Zealand (Wilson et al., 2012), South Africa (Ross et al.,
3 2012; Pieterse et al., 2017a,b). China (Huang et al., 2015), Japan (Waki, 2017), Italy
4 (Nermut et al., 2016a), Czechoslovakia (Nermut et al., 2010; 2016b,c) and *P.*
5 *californica* has been found in the USA (Tandingan De Ley et al., 2016), Ireland
6 (Carnaghi et al., 2017) and Wales (this study). From these studies it is apparent that
7 members of the *Phasmarhabditis* genus have a cosmopolitan distribution and are very
8 easy to isolate from gastropod hosts. We found 20 separate *Phasmarhabditis* isolates
9 from 426 slugs and snails from three locations around the U.K. From our survey the
10 success rate of collecting *Phasmarhabditis* (4.7%) seems small yet we have
11 discovered a wealth of different species including *P. californica* (which had never
12 been identified in the U.K. before) and several strains of *P. neopapillosa*. Therefore,
13 it seems that the U.K. has an underappreciated diversity of *Phasmarhabditis* species.

14 Although *P. hermaphrodita* is a parasite we have shown that it can be
15 maintained and cultured under laboratory conditions using a combination of NGM
16 agar and naturally isolated bacteria (*Pseudomonas* sp. 1). For laboratory genetic
17 model nematodes to be used successfully their bacterial food must be nutritious
18 enough to facilitate the growth of hundreds of offspring; transparent (so nematodes
19 are visible); easy to grow and does not grow too much when added to the NGM plate.
20 This was why *E. coli* OP50 was selected to grow and maintain *C. elegans* (Brenner,
21 1974). However, regular passage of hundreds of thousands of generations of
22 nematodes using the same culture conditions (constant temperature, lots of food and
23 same species of food) with no interaction or variation in the environment can severely
24 affect genetic make up (Huey & Rosenzweig, 2009). For example, *C. elegans* N2
25 (wild type) has lost the ability to perform thermoregulatory behaviour when exposed

1 to a temperature gradient due to being reared at the same temperature for over 40
2 years (Anderson et al., 2007). We propose to use *Pseudomonas* sp. 1. - a bacterium
3 found in the intestine of *P. entomophagus* from Tuebingen, Germany (Rae et al.,
4 2008) and not an unusual food source like *E. coli* OP50. Yet continual culturing on
5 NGM plates is an unnatural culture method for these nematodes as *Phasmarhabditis*
6 are necromenic and parasitic nematodes used to reproducing on rotting cadavers of
7 molluscs and not agar plates (Wilson et al., 1993; Rae et al., 2009). To this end we
8 propose to grow *Phasmarhabditis* on decaying slugs in ‘semi-natural’ conditions
9 using White traps, which is a more realistic environment. This method means that
10 they can be stored at 10 to 15°C for months as dauers (Grewal & Grewal, 2003), and
11 cultured infrequently (every 4-5 months) and therefore the effect of accumulating any
12 deleterious mutations will be reduced. Furthermore, future research will focus on the
13 development of cryopreservation techniques for *Phasmarhabditis* which will allow
14 access to a library of ‘un-evolved’ strains and species as well as mutants.

15 Once *P. hermaphrodita* has killed a slug it feeds on the bacteria growing on
16 the rotting cadaver and this is depleted it turns to the dauer stage and searches for
17 more slugs in the soil. These dauers associate with a rich diversity of bacteria that are
18 carried in their intestine (Rae et al., 2010). Previous studies have shown that the
19 bacteria isolated from the intestine of *P. hermaphrodita*, xenic cultures of *P.*
20 *hermaphrodita* and from swabs of slugs that died from infection of *P. hermaphrodita*
21 can affect the number of dauers produced as well as their virulence towards slugs
22 (Wilson et al., 1995a,b). These studies showed that *P. hermaphrodita* can grow on an
23 array of bacterial species such as *Pseudomonas fluorescens*, *Sphingobacterium*
24 *spintovorum*, *M. osloensis*, *Serratia proteamaculans*, *Aeromonas* sp. and *Providencia*
25 *rettgeri* (Wilson et al., 1995b), and *P. hermaphrodita* grown on bacteria such as *P.*

1 *fluorescens*, *M. osloensis* and *P. rettgeri* can produce high yields of pathogenic
2 nematodes that kill slugs (Wilson et al., 1995a). However, the commercial isolate of
3 *P. hermaphrodita* DMG0001 is grown on *M. osloensis* as it can produce consistent
4 high yields of highly pathogenic nematodes (Tan and Grewal, 2001b; Wilson et al.,
5 1995a,b). It has been shown that when introduced into the shell cavity of *D.*
6 *reticulatum*, *M. osloensis* produces a lipopolysaccharide (LPS) that acts an endotoxin,
7 causing rapid mortality (Tan and Grewal, 2002). By utilizing this collection of
8 naturally isolated *P. hermaphrodita* and *Phasmarhabditis* species the co-evolution of
9 these tri-trophic interactions between bacteria (such as *M. osloensis*), nematodes and
10 slug hosts could be analysed at the molecular level.

11 As well as established genetic model nematodes (*C. elegans* and *P. pacificus*)
12 there are several other nematodes that have been proposed including *Poikilolaimus*
13 *oxycercus* (Hong et al., 2005), *Oscheius tipulae* (Félix, 2006) and *Meloidogyne hapla*
14 (to study plant parasitism) (Opperman et al., 2008). In order for these nematodes (as
15 well as *P. pacificus*) to be used under laboratory conditions information is needed
16 about appropriate bacterial food as well as methods to allow genetic crosses,
17 mutagenesis and long term storage. We have shown that logistically and financially,
18 nematodes such as *Phasmarhabditis* are not difficult to keep and are easy to maintain.
19 There is little difference in the equipment needed to keep *C. elegans* (Stiernagle,
20 2006) e.g. simple reagents and microbiological media, incubators and freezers for
21 growth and long term storage. As well as these factors, another important point about
22 model nematodes is the ability to be isolated easily can allow tens if not hundreds of
23 strains to be studied to investigate natural phenotypic variation, which can lead to an
24 understanding of the underlying genotype using approaches such as RAD seq
25 (Restriction site Associated DNA Sequencing) (Davey & Blaxter, 2010) and GWAS

1 (Genome Wide Association Studies) (Cook et al., 2016). In *C. elegans* global
2 sampling efforts have collected several hundred *C. elegans* strains and 26
3 *Caenorhabditis* species (Frezal & Félix, 2015); which are available from the
4 *Caenorhabditis* Genetic Stock Centre, US. Studying natural variation has been
5 successful in understanding genes involved with foraging behaviour, thermal
6 tolerance and outcrossing (De Bono & Bargmann, 1998; Harvey & Viney, 2007;
7 Teotonio et al., 2006). A similar approach utilising natural strains and investigating
8 variation in behaviour, cold tolerance and dauer formation in *P. pacificus* has been
9 taken (Hong et al., 2008; Mayer & Sommer, 2011; McGaughran & Sommer, 2014).
10 In total there are 28 species of *Pristionchus* (Ragsdale et al., 2015) and hundreds of
11 strains of *P. pacificus* that are available from the Sommer lab, Tuebingen, Germany
12 (Morgan et al., 2012). We have shown that, like both *C. elegans* and *P. pacificus*, *P.*
13 *hermaphrodita* and a selection of *Phasmarhabditis* species can be isolated and
14 maintained in the lab easily. We have shown that there is natural variation within *P.*
15 *hermaphrodita* and *Phasmarhabditis* species surviving different temperatures and
16 pHs. This means that with the development of appropriate sequencing and genomic
17 techniques (e.g. RAD seq) macro-evolutionary and micro-evolutionary processes
18 could potentially be unravelled at the genetic level.

19 The isolation of mutants via forward genetic screens using mutagenesis is a
20 powerful technique that can identify genes responsible for specific phenotypes. The
21 first step of any proposed genetic model nematode is to show it can be mutagenized.
22 Here we showed *P. hermaphrodita unc* and *sma* mutants could be isolated by using
23 similar protocols as *C. elegans* (Johnson & Baillie, 1988). If *P. hermaphrodita* can be
24 mutated then there is no reason why full-unbiased forward genetic screens could not
25 be carried out to investigate an array of evolutionary and ecologically important traits.

1 These include finding mutants that are defective in killing slugs, inducing slug
2 avoidance (Wilson et al., 1999, Wynne et al., 2016) or failing to chemotax towards
3 host cues such as slug mucus (Rae et al., 2006; 2009). As *P. hermaphrodita* is one of
4 the candidates for the 959 nematode genome project (Kumar et al., 2012) and several
5 species are currently undergoing full genome sequencing, this will allow genomic
6 comparison to closely related free-living nematodes, arthropod and mammalian
7 parasites present in Clade 5.

8 We found that *P. neopapillosa* could be mated under lab conditions using
9 similar procedures as *C. elegans*. However, generating enough males for genetic
10 crosses with *P. hermaphrodita* proved difficult due to scarcity. This is not unusual for
11 hermaphroditic nematodes that are able to spontaneously produce males. *C. elegans*
12 only produces 0.1-0.2% of males in culture (Hodgkin & Doniach, 1997). Maupas
13 (1900) noted that from 14,888 *P. hermaphrodita* only 1 male was found. Our strains
14 under lab conditions seem to not produce males, even when heat shocked for 1-4
15 hours at 30°C. Strangely, when these strains were first isolated one was found with
16 males present (DMG007) (Rae, unpublished) but when grown on rotting slug and
17 NGM plates this ability seemed to diminish rapidly over time. Future research will
18 focus on methods used to generate males including exposing hermaphrodites to
19 ethanol (Lyons & Hecht, 1997) or isolating a mutant (using forward genetics) that has
20 a high incidence of males (*him* mutants) (Hodgkin et al., 1979).

21 In conclusion, we have outlined the methods used to work with *P.*
22 *hermaphrodita* and *Phasmarhabditis* species under laboratory conditions. We believe
23 that *P. hermaphrodita* (and other *Phasmarhabditis* species) could be used to identify
24 genes that are essential for pathogenicity towards slugs. The most logical way to
25 achieve this would be to take a natural variation approach to isolate as many *P.*

1 *hermaphrodita* strains as possible and grow them on rotting slugs (as we have
2 outlined here which does not affect their virulence, Rae et al. (2010) and assess their
3 pathogenic potential towards slugs. The main aim would be to identify a strain that is
4 more or less virulent than DMG0001 (an approach that is currently ongoing, Rae,
5 unpublished). Through genome sequencing potential parasitism genes could be
6 identified and confirmed by reverse genetics e.g. RNAi and/or CRISPR-Cas9. This
7 information may allow in-depth insight into the evolution of parasitism in other Clade
8 5 animal, plant and invertebrate nematode parasites as well as comparison to *C.*
9 *elegans* and *P. pacificus*.

10

11 **Figure legends**

12

13 Figure 1: The slug parasitic nematode *P. hermaphrodita* (A) is a parasite of several
14 slug species such as *D. reticulatum* (B). It can also be kept under lab conditions on
15 NGM agar feeding on *Pseudomonas* sp. 1 (C) and on rotting *L. flavus* in a White trap
16 (D). Scale bars represent 100 μ m in A, 1 cm in B and 1 mm in C and D.

17

18 Figure 2: The mean number of offspring that were produced by *P. hermaphrodita*
19 DMG0001, *P. hermaphrodita* DMG0007 and *P. californica* DMG0017 when fed
20 0.025 g (A) and 0.25 g (B) of *L. flavus*. Bars represent \pm one standard error.

21

22 Figure 3: The mean number of offspring that were produced by *P. hermaphrodita*
23 DMG0001 (black bars), *P. hermaphrodita* DMG0007 (white) and *P. californica*
24 DMG0017 (grey) when fed *Pseudomonas* sp. 1, *Bacillus* sp. 1, *E. coli* OP50 and *E.*
25 *coli* BR at 20°C. Bars represent \pm one standard error.

1

2 Figure 4: The mean number of offspring that were produced by *P. hermaphrodita*
3 DMG0001 (black bars), *P. hermaphrodita* DMG0007 (white) and *P. californica*
4 DMG0017 (grey) at 10°C (A), 15°C (B) and 20°C (C). Bars represent \pm one standard
5 error.

6

7 Figure 5: The mean pumping rate over 60 seconds of *P. hermaphrodita* (DMG0001
8 and DMG0007), *P. californica* DMG0017 and *P. neopapillosa* (DMG0012 and
9 DMG0016) fed *Pseudomonas* sp. 1 at 20°C. Bars represent \pm one standard error.

10

11 Figure 6: The number of alive *P. hermaphrodita* (DMG0001, DMG0007, DMG0010,
12 DMG0006 and DMG0008); *P. californica* (DMG0018 and DMG0019) and *P.*
13 *neopapillosa* (DMG0013, DMG0015 and DMG0016) exposed to 31°C (A), 37°C (B)
14 and 41°C (C). Bars represent \pm one standard error.

15

16 Figure 7: *P. hermaphrodita* DMG0001 (A) were mutagenized with 0.1%
17 formaldehyde and *sma* (small) mutants (B, C) and *unc* (uncoordinated) mutants (D,
18 E) were found in the F2 generation. Scale bar represents 100 μ m.

19

20 **Supplementary Figures**

21

22 Supplementary Figure 1: PCR product of COI gene based on primers by Read et al.
23 (2006) from DNA that was extracted and amplified from *P. hermaphrodita*
24 DMG0001 (lane 1), DMG0002 (2), B30 (3), DMG0003 (4), DMG0004 (5),
25 DMG0005 (6), DMG0006 (7), DMG0007 (8), DMG0008 (9), DMG0009 (10),

1 DMG0010 (11), DMG0011 (12), *P. neopapillosa* DMG0012 (13), DMG0013 (14), *P.*
2 *hermaphrodita* AB46 (15), *P. neopapillosa* DMG0014 (16), DMG0015 (17),
3 DMG0016 (18), *P. californica* DMG0017 (19), DMG0018 (20), DMG0019 (21), *C.*
4 *elegans* N2 (22), *Acrobeloides* sp. (23), *Cervidellus vexilliger* (24), *Aphelenchus*
5 *avenae* (25), *Pelodera teres* (26), *Pristionchus entomophagus* (27), *Pristionchus*
6 *uniformis* (28), *Steinernema feltiae* (29), *Steinernema carpocapsae* (30), *Panagrellus*
7 *redivivus* (31), *Heterorhabditis bacteriophora* (32), negative control (water as
8 template) (33) and positive control (*P. hermaphrodita* DMG0001 DNA) (34). L = 1
9 kb DNA Ladder.

10

11

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Code	<i>Phasmarhabditis</i> species	Slug/snail species isolated from	Location	Code
A75	<i>P. hermaphrodita</i>	<i>Arion</i> spp.	Priory Wood, Liverpool	DMG0002
B30	<i>P. hermaphrodita</i> (Deceased)	<i>Deroceras panormitanum</i>	Sefton Park, Liverpool	
B33	<i>P. hermaphrodita</i>	<i>Deroceras panormitanum</i>	Sefton Park, Liverpool	DMG0003
B36	<i>P. hermaphrodita</i>	<i>Deroceras panormitanum</i>	Sefton Park, Liverpool	DMG0004
B93	<i>P. hermaphrodita</i>	<i>Arion subfuscus</i>	Sefton Park, Liverpool	DMG0005
B113	<i>P. hermaphrodita</i>	<i>Arion subfuscus</i>	Sefton Park, Liverpool	DMG0006
B178	<i>P. hermaphrodita</i>	<i>Limax flavus</i>	Sefton Park, Liverpool	DMG0007
C11	<i>P. hermaphrodita</i>	<i>Deroceras panormitanum</i>	Festival gardens, Liverpool	DMG0008
C25	<i>P. hermaphrodita</i>	<i>Deroceras panormitanum</i>	Festival gardens, Liverpool	DMG0009
MG2	<i>P. hermaphrodita</i>	<i>Milax budapestensis</i>	Festival gardens, Liverpool	DMG0010
MG6	<i>P. hermaphrodita</i>	<i>Milax budapestensis</i>	Festival gardens, Liverpool	DMG0011
AB9	<i>P. neopapillosa</i>	<i>Deroceras reticulatum</i>	Cruickshank gardens, University of Aberdeen	DMG0012
AB10	<i>P. neopapillosa</i>	<i>Deroceras reticulatum</i>	Cruickshank gardens, University of Aberdeen	DMG0013
AB46	<i>P. hermaphrodita</i> (Deceased)	<i>Limax flavus</i>	Sefton Park, Liverpool	
B170	<i>P. neopapillosa</i>	<i>Limax flavus</i>	Sefton Park, Liverpool	DMG0014
B190	<i>P. neopapillosa</i>	<i>Limax flavus</i>	Sefton Park, Liverpool	DMG0015
B175B	<i>P. neopapillosa</i>	<i>Limax flavus</i>	Sefton Park, Liverpool	DMG0016
P19B	<i>P. californica</i>	<i>Oxychilus draparnaudi</i>	Dale, Pembrokeshire	DMG0017
P19D	<i>P. californica</i>	<i>Oxychilus draparnaudi</i>	Dale, Pembrokeshire	DMG0018
P19E	<i>P. californica</i>	<i>Oxychilus draparnaudi</i>	Dale, Pembrokeshire	DMG0019

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2 Table 1: The *Phasmarhabditis* species that were isolated from slugs and snails collected from Aberdeen, Liverpool and Pembrokeshire and
3 nomenclature of isolated *Phasmarhabditis* species based on *C. elegans* rules.

Species	Strain	Control	pH						
			4	5	6	7	8	9	10
<i>P. hermaphrodi ta</i>	DMG0001	9.3 ± 0.3	7.7 ± 0.3	7.7 ± 0.3	9.7 ± 0.3	9.3 ± 0.7	7.3 ± 1.2	7.3 ± 0.7	5.7 ± 0.9
	DMG0006	8.7 ± 0.3	6.7 ± 0.3	7.7 ± 0.3	9.3 ± 0.3	8.7 ± 0.3	7.3 ± 0.3	6.7 ± 0.3	5.7 ± 0.3
	DMG0007	8 ± 0.6	6 ± 0.6	6.7 ± 0.9	8.3 ± 0.3	7.7 ± 0.3	7.7 ± 0.3	7.7 ± 0.3	4.3 ± 0.3
	DMG0008	9 ± 0.6	7.3 ± 0.3	8.3 ± 0.3	9 ± 0.3	8.3 ± 0.3	8.3 ± 0.3	7.3 ± 0.3	5.7 ± 0.3
	DMG0010	8.3 ± 0.3	6.7 ± 0.3	8 ± 0	9 ± 0.6	8 ± 0.6	8 ± 0.6	7.3 ± 0.3	5.7 ± 0.3
<i>P. californica</i>	DMG0017	9.7 ± 0.3	8 ± 0	9 ± 0	9.7 ± 0.3	9.3 ± 0.3	9 ± 0	8.7 ± 0.3	7.3 ± 0.3
	DMG0019	9.3 ± 0.3	7.3 ± 0.3	8.3 ± 0.3	9.7 ± 0.3	9.7 ± 0.3	8.7 ± 0.3	8.3 ± 0.3	7 ± 0
<i>P. neopapillosa</i>	DMG0013	8.3 ± 0.3	6.7 ± 0.3	8.3 ± 0.3	8.7 ± 0.3	8 ± 0	7.3 ± 0.3	7.3 ± 0.3	5.3 ± 0.3
	DMG0015	7.3 ± 0.3	5 ± 0.6	7.3 ± 0.3	8.3 ± 0.3	7.7 ± 0.3	6 ± 1	6 ± 0.6	4.3 ± 0.3
	DMG0016	8.7 ± 0.3	5.7 ± 0.3	7.7 ± 0.3	9.7 ± 0.3	9.3 ± 0.3	8 ± 0.6	7.3 ± 0.3	5.7 ± 0.3

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2 Table 2: The mean number of alive *Phasmarhabditis* ± one standard error after 4 days exposure to pH 4 to 10.

