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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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Research Paper

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Abstract

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

Introduction

Nematodes have evolved to parasitize 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 in coping with stressful conditions such as host enzymes (Poulin, 1998; Weischer & Brown, 2000). Several parasitic species have been developed as genetic models but they are unsuitable to answer this question as they require intermediate and/or definitive mammal hosts (e.g. *Brugia* sp.) to complete their life cycle, 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, whereby it infects the host, waits for it to die and reproduces on its cadaver (Morgan *et al.*, 2012). However, neither species is parasitic (Herrmann *et al.*, 2006; Rae & Sommer, 2011), and therefore they provide little information about the underlying evolution of genetic mechanisms that are used to infect, parasitize and even kill their hosts. They are, however, formidable at unravelling genes associated with a plethora of biologically 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, kept in culture and grown in large numbers (on nematode growth media (NGM) plates fed *Escherichia coli* OP50), and they can be mutagenized and mated easily (Brenner, 1974; Sommer *et al.*, 2000). Furthermore, as well as routine full genome sequencing (*C. elegans* Sequencing Consortium, 1998; Dieterich *et al.*, 2008), post-genomic tools, such as reverse genetic techniques – first RNAi (Fire *et al.*, 1998; Cinkornpumin & Hong, 2011) and now CRISPR-Cas9 (Lo *et al.*, 2013; Witte *et al.*, 2015) – can be carried out in both species to understand gene function, while transgenic techniques facilitate the analysis of gene expression (Chalfie *et al.*, 1994; Schlager *et al.*, 2009). Similar

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techniques can be carried out in mammalian parasites, e.g. *Brugia malayi*, *Nippostrongylus brasiliensis* and *Ascaris suum*, but the efficiency is variable and only a selection of genes can be inhibited (Geldhof *et al.*, 2006, 2007). A promising genetic model to study nematode parasitism would combine the ease of keeping and growing *C. elegans* and *P. pacificus* en masse in the lab with the ability to collect different species and strains easily to facilitate micro and macro evolutionary studies. Also, it would be closely related to other parasitic and necromenic species that would allow genomic comparison of the evolution of potential parasitism genes from different parasitic lifestyles. Furthermore, it could be genetically manipulated, which would facilitate an in-depth analysis of gene function.

A nematode that theoretically meets all these criteria is the terrestrial gastropod parasite *Phasmarhabditis hermaphrodita* (fig. 1a). *Phasmarhabditis hermaphrodita* can complete its life cycle in several ways. First, it can infect and kill several susceptible slug species (e.g. *Deroceras reticulatum*) (Wilson *et al.*, 1993; Rae *et al.*, 2009). Second, it can infect and remain inside larger slug and snail species, where it waits for the host to die and then reproduces on the decaying cadaver (termed 'necromeny') (Rae *et al.*, 2009). Third, it can reproduce on decomposing organic matter such as leaf litter, dead earthworms and slug faeces (Tan & Grewal, 2001a; MacMillan *et al.*, 2009). Therefore, it is not an obligate parasite that requires a host to survive but a bacterivorous nematode that can be grown in the lab without slugs but is still able to retain is pathogenicity towards slugs. Due to its pathogenic potential it has been formulated into a biological control agent (Nemaslug®, BASF, Germany) for farmers and gardeners to control slugs and snails (Rae *et al.*, 2007). Nematodes are applied to soil, where they actively seek out slugs and infect and kill them 4–21 days later (Wilson *et al.*, 1993; Tan & Grewal, 2001a). *Phasmarhabditis hermaphrodita* has been shown to provide protection against slug damage in many agriculturally important crops (Wilson & Rae, 2015). Although *P. hermaphrodita* has received considerable attention as an agricultural biopesticide, it is also interesting from a fundamental evolutionary perspective and has been proposed as an excellent candidate as a genetic model to elucidate how parasitism has arisen in free-living species (Wilson *et al.*, 2015; Rae, 2017). It was even a potential candidate as Sydney Brenner's nematode of choice instead of *C. elegans* (Cold Spring Harbor Laboratory Archives, 2017; http://libgallery.cshl.edu/items/show/75709). *Phasmarhabditis hermaphrodita* is the only nematode of an estimated 1 million (Lambshead, 1993) that has evolved to parasitize and kill gastropods. There are over 108 species of nematodes that parasitize molluscs, and four of five clades of the Nematoda have members that parasitize gastropods (Blaxter *et al.*, 1998; Grewal *et al.*, 2003). Parasitism of gastropods is therefore a very important lifestyle choice amongst nematodes; however, the genes involved in infecting and surviving in these hosts are unknown.

The majority of research on *P. hermaphrodita* has focused on optimizing application techniques in the field (see Rae *et al.*, 2007), host range studies (Wilson *et al.*, 1993; Grewal *et al.*, 2003; Rae *et al.*, 2009), taxonomic descriptions, and surveys charting abundance and diversity of *Phasmarhabditis* in various countries (Ross *et al.*, 2012, 2016; Wilson *et al.*, 2012; Tandingan De Ley *et al.*, 2014, 2016; Nermut *et al.*, 2016a, b, 2017). There are few details and no reported methods regarding how to keep this nematode under lab conditions, like *C. elegans*, and whether it could be amenable to genetic manipulation. There is little information about its life history traits and how they change with bacterial diet or temperature. Also, there have been few experiments investigating natural variation in any ecological traits of the genus *Phasmarhabditis*; all studies so far have focused on the commercial strain of *P. hermaphrodita* (DMG0001), which has been in culture for over 20 years (Rae *et al.*, 2007).

Here we provide information on how to grow, maintain, mutagenize and mate not only *P. hermaphrodita* but also several other species of the *Phasmarhabditis* genus under lab conditions. We

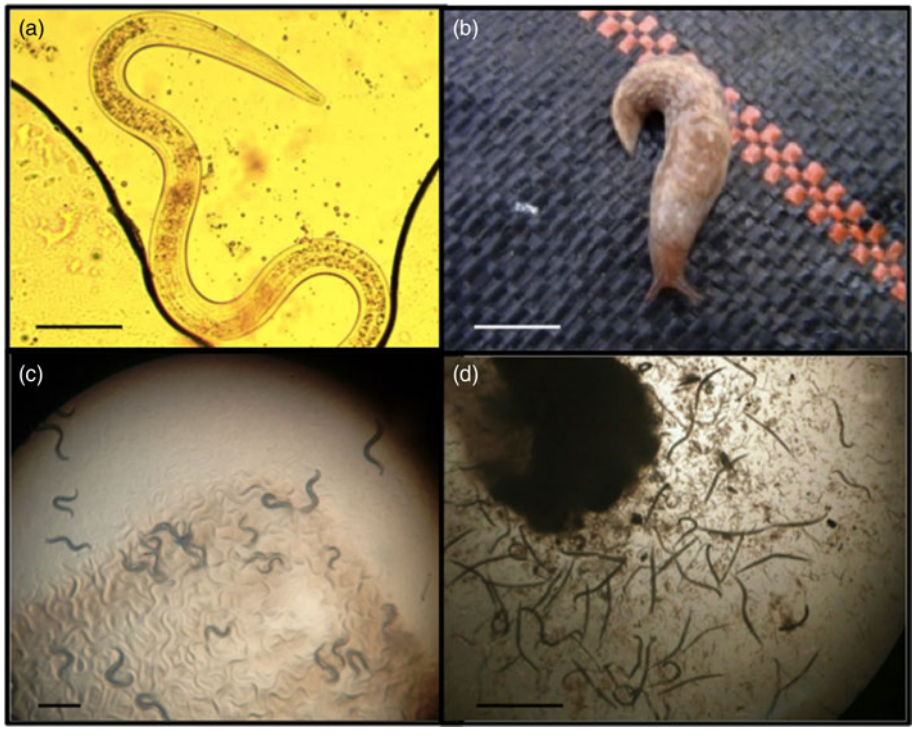

Fig. 1. (a) The nematode *Phasmarhabditis hermaphrodita* is a parasite of several slug species, including (b) *Deroceras reticulatum*. It can be kept under lab conditions on NGM agar, feeding on (c) *Pseudomonas* sp. 1 and on (d) rotting *Limax flavus* in a White trap. Scale bars: (a) 100 µm; (b) 1 cm; (c, d) 1 mm.

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also provide information on how to isolate, identify and make isogenic lines of *P. hermaphrodita*. Taken together, these results show that many of these species can be maintained easily under lab conditions and could make excellent candidates as genetic models to understand the evolution of parasitism in the Nematoda.

Materials and methods

Terrestrial gastropod survey and molecular identification of parasitic nematodes

Slugs (*Deroceras panormitanum*, *D. reticulatum* (fig. 1b), *Arion subfuscus*, *A. ater*, *A. hortensis*, *Limax flavus*, *L. maximus*, *Lehmannia valentiana*, *Milax budapestensis* and *M. sowerbyi*) and snails (*Cepaea nemoralis*, *Cornu aspersum* and *Oxychilus draparnaudi*) were collected from several locations in Liverpool, UK, including Priory Wood near St Michael's station (Grid reference number SJ3673586862) (n = 107), Sefton Park (SJ3787187058) (n = 195) and Otterspool (SJ3707686321) (n = 57). Slugs were also collected from the Cruickshank Botanic Garden at the University of Aberdeen (NJ9376008556) (n = 48) and from Dale, Wales (SM809057) (n = 19). Once collected they were transported back to the lab, where they were chopped in half and placed in a 5 cm Petri dish with a few drops of distilled water and stored at room temperature (Wilson *et al.*, 2016). Over four days the slugs were examined for presence of nematodes. Any nematodes that morphologically resembled *Phasmarhabditis* nematodes (fig. 1), e.g. hermaphrodites, females or males over 1 mm, were transferred individually to modified White traps (White, 1927) (see below for description) to make isogenic lines. After 21 days, when the food was exhausted and the nematodes had reached the dauer stage, they were present in the surrounding water and were removed and centrifuged at 16,000 rpm to concentrate, and their DNA was extracted using a DNA extraction kit (Qiagen, Hilden, Germany). Using polymerase chain reaction (PCR), three genes were then amplified (ITS1, 18SrRNA and the D2-D3 domain of large subunit (LSU) rDNA) (Blaxter *et al.*, 1998; Tandingan De Ley *et al.*, 2014) and sequenced in both directions for species identification. For the ITS1 gene the primers used were N93 (5′-TTGAACCGGGTAAAAGTCG-3′) and N94 (5′-TTAGTTTCTTTTCCTCCGCT-3′). The 18SrRNA gene was amplified using 18A (5′-AAAGATTAAGCCATGCATG-3′) and 26R (5′-CATTCTTGGCAAATGCTTTCG-3′). The D2-D3 LSU rDNA primers were D2 (5′-AGCGGAGGAAAAGAAA CTAA-3′) and D3 (5′-TCGGAAGGAACCAGCTACTA-3′). As well as these three genes *P. hermaphrodita*-specific primers were also used, which were based on 150–200 bp of the Cytochrome Oxidase I gene created by Read *et al.* (2006), which consisted of Ph-F-1754 (5′-TGGGTGCCCCTGATATAAGAT-3′) and Ph-R-1887 (5′-CGGATGACCAAGGGTACTTAAT-3′). These primers were used to investigate their potential use as a rapid and cheap method for identifying *P. hermaphrodita* without DNA sequencing, as they have been used previously to determine if *P. hermaphrodita* was present in mites and collembolans (Read *et al.*, 2006). PCR cycling conditions consisted of the following: 3 minutes at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 55°C, 1.5 minutes at 72°C and a final step of 8 minutes at 72°C. The PCR products were then purified and sequenced in both forward and reverse directions for each gene (ITS1, 18SrRNA and the D2-D3 domain of LSU rDNA). Gene sequences of nematodes were then compared with National Center for Biotechnology Information (NCBI) database sequences using BLASTN searches, using similarity matches of 99%. For PCR using primers designed by Read *et al.* (2006) the 200 bp product was visualized after gel electrophoresis, where a positive band indicated presence of *P. hermaphrodita*, and no sequencing was carried out.

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

Any potential *Phasmarhabditis*-like nematodes observed growing on the collected rotting slugs and snails were transferred to modified White traps (White, 1927) for maintenance, to make isogenic lines and to quantify the number of dauers that were produced per individual hermaphrodite mother. Modified White traps consisted of a 10 cm Petri dish half filled with tap water. A 5 cm lid of a Petri dish was then placed on top of the water, with a 4.5 cm diameter piece of pre-moistened Whatman No. 1 filter paper (Whatman, Maidstone, UK) inside. A 3 mm slice of *L. flavus* was added to each White trap as a food source. *Limax flavus* were collected from Liverpool John Moores University greenhouses and frozen at −80°C for 1 h to kill any nematodes present. *Limax flavus* was used as food for *Phasmarhabditis* for several reasons: (1) it is a common slug species that is easily collected; (2) it is a large slug, and therefore many White traps can be made at one time; and (3) from preliminary experiments it consistently produces large numbers of nematodes. Also, a previous study has shown that feeding on this slug species in White traps produces consistently virulent *P. hermaphrodita* (Rae *et al.*, 2010). A single hermaphrodite mother was then transferred via a worm pick to each White trap, which was then sealed with Parafilm® (Bemis, Neenah, Wisconsin, USA) and stored at 20°C for 21 days, after which dauer stage nematodes were found in the surrounding water. This procedure was repeated for all of the *Phasmarhabditis* collection to make isogenic lines.

In a separate experiment the number of dauer juveniles produced by one self-fertilizing hermaphrodite mother was quantified for two strains of *P. hermaphrodita* (the commercial strain DMG0001 and the naturally isolated strain DMG0007) and one strain of *P. californica* (DMG0017) to understand the brood size of these nematodes. These strains were chosen to investigate if there was a difference between the growth of the commercial strain (*P. hermaphrodita* DMG0001; Hooper *et al.*, 1999) and natural strains of *P. hermaphrodita*, as the commercial strain has been in culture for over 20 years and fed solely on the monoxenic diet consisting of the bacterium *Moraxella osloensis* (Wilson *et al.*, 1995a, b). The number of dauer juveniles produced by a single hermaphrodite mother of *P. hermaphrodita* DMG0001, DMG0007 or *P. californica* DMG0017 was quantified by setting five White traps containing either 0.025 g or 0.25 g of *L. flavus*, left for 21 days at 20°C (fig. 1d). The experiment was repeated twice.

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

We investigated whether *Phasmarhabditis* nematodes could grow on several different bacteria, including *Escherichia coli* OP50 (the food of *C. elegans* and *P. pacificus*; Brenner, 1974; Sommer *et al.*, 2000), *E. coli* BR (an easily available strain used for cloning in molecular biology) and two naturally isolated bacterial species found to be associated with *Pristionchus entomophagus* called

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Pseudomonas sp. 1 and *Bacillus* sp. 1 (Rae *et al.*, 2008) (fig. 1c). Bacteria were grown in nutrient broth at 30°C overnight. The following morning 100 µl of each bacterium was spread onto five 5 cm NGM plates (Hope, 1999), which were then incubated at 30°C overnight. An individual dauer stage nematode was transferred to each plate via a worm pick and incubated at 20°C. The numbers of offspring per plate were then recorded after three and six days. The experiment used *P. hermaphrodita* DMG0001 and DMG0007 and *P. californica* DMG0017. This experiment was repeated three times with all four bacteria and with all three nematode isolates.

To understand the feeding behaviour of *Phasmarhabditis* nematodes in more detail we also recorded the pharyngeal pumping rate whilst they were eating. This has been recorded easily in both *C. elegans* and *P. pacificus* (Kroetz *et al.*, 2012) but never for any *Phasmarhabditis* species. The pharyngeal pumping rate of an individual of each of three *Phasmarhabditis* species (*P. hermaphrodita* DMG0007, *P. neopapillosa* DMG0012 and DMG0016, and *P. californica* DMG0017) was counted for 60 s and repeated ten times with different worms.

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

To ascertain the optimum temperature for growth of *Phasmarhabditis* nematodes under laboratory conditions, 15 5-cm NGM plates were seeded with 100 µl of *Pseudomonas* sp. 1 and then incubated at 30°C overnight. *Pseudomonas* sp. 1 was chosen from the four bacterial species tested because it resulted in *Phasmarhabditis* nematodes producing a large number of offspring, which were easy to see in the bacterial lawn because of its translucent nature. A single L4 hermaphrodite was placed onto each NGM plate and groups of five plates were incubated at either 10°C, 15°C or 20°C for six days. On days 3 and 6 the numbers of offspring were recorded. This experiment was repeated three times using the same nematode species and strains as above.

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

It is imperative that a genetic model nematode can be mated under laboratory conditions and it is unknown how commonly males are produced in *P. hermaphrodita* collected from the wild. Five NGM plates (5 cm) were spread with 50 µl of *Pseudomonas* sp. 1 and incubated overnight at 30°C. The following morning dauer juveniles (1000–7000 per strain) were added to each plate and incubated at 20°C for two to three days. The numbers of males present were then recorded. The species and strains used (and numbers of dauer observed) were *P. hermaphrodita* DMG0001 (n = 4040), DMG0002 (n = 6771), DMG0010 (n = 4581), DMG0009 (n = 3108), DMG0003 (n = 2503) and DMG0007 (n = 3572); *P. californica* DMG0017 (n = 1098) and DMG0019 (n = 1127); and *P. neopapillosa* (DMG0012, DMG0015 and DMG0016; n = 750 for each strain).

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

Genetic crosses of Phasmarhabditis *species under lab conditions*

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

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

To assess whether there was natural genetic variation in the ability of the *Phasmarhabditis* nematodes to cope with extreme pH and temperatures, the following experiments were carried out. For the thermotolerance experiment, three 1.5 ml Eppendorf tubes containing 4500–6000 nematodes per 1 ml were placed into a heat block set at 33°C, 37°C or 41°C for 15 minutes. At time 0 and after 15 minutes the numbers of nematodes were quantified. The Eppendorf containing the nematodes was vortexed every 2.5 minutes to avoid clumping. Eppendorfs containing the same numbers of nematodes but kept at room temperature were used as the control. The experiment was repeated three times for each temperature. The following species and strains were used: *P. hermaphrodita* (DMG0001, DMG0007, DMG0010, DMG0006 and DMG0008), *P. californica* (DMG0017 and DMG0019) and *P. neopapillosa* (DMG0013, DMG0015 and DMG0016).

To assess natural variation of pH tolerance, 10 individual dauer stage nematodes were added to 80 wells containing 60 µl of water adjusted to pH 4, 5, 6, 7, 8, 9 and 10, as well as a control of distilled water. The correct pH was obtained by addition of either 1 M NaOH or 1 M HCl and confirmed using a pH meter and indicator paper. There were ten wells per pH and the whole experiment was repeated twice. The 96 well plates were then incubated at 20°C and survival was recorded daily for four days. The same *Phasmarhabditis* species and strains were used as in the thermotolerance experiment.

Formaldehyde mutagenesis of P. hermaphrodita *DMG0001*

To investigate if *P. hermaphrodita* can be mutated using formaldehyde mutagenesis (like *C. elegans* and *P. pacificus*) we used similar methods to those developed by Johnsen & Baillie (1988) for *C. elegans*. *P. hermaphrodita* DMG0001 (L4 and young adult stage) were grown on several NGM plates with *Pseudomonas* sp. 1 for four days. They were then washed in distilled water, concentrated to a pellet and exposed to 0.1% formaldehyde for four hours, after which the P0's were washed several times in water to remove any residual formaldehyde and 100 individual mothers were separated out and placed on individual NGM

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plates seeded with *Pseudomonas* sp. 1. They were stored at 20°C for three to four days and allowed to produce offspring, and then 300 F1's were separated out (three individuals were picked randomly from each plate of P0 mothers) and the F2's were screened for any morphological abnormalities after five to seven days.

Data analysis

The difference between the numbers of dauers produced by *P. hermaphrodita* DMG0001 and DMG0007 and *P. californica* DMG0017 grown on 0.025 g and 0.25 g of *L. flavus* was analysed using a one-way analysis of variance (ANOVA) with Tukey's post-hoc test. These tests were also used to analyse the data on pumping rate, number of offspring produced on different bacteria and at different temperatures, and the numbers of surviving nematodes exposed to 33, 37 and 41°C and pH 4–10. The body length of *P. hermaphrodita* DMG0001 WT and *sma* mutants was compared using a Student's t test. SPSS v. 23 (IBM, Armonk, USA) was used for data analysis.

Results

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

From 426 slugs and snails collected from around the UK we found 12 isolates of *P. hermaphrodita*, three isolates of *P. californica* and five isolates of *P. neopapillosa* (table 1). These isolates were all from separate slugs apart from *P. californica*, of which three isolates were found in a single *O. draparnaudi*. We had initially identified many of these *Phasmarhabditis* species as *P. hermaphrodita* by using species-specific primers developed by Read *et al.* (2006) (supplementary fig. S1). However, we found that these primers amplify not only *P. hermaphrodita* but also other members of the *Phasmarhabditis* genus and even diverse insect-associated and free-living species such as *Steinernema feltiae*, *Panagrellus redivivus*, *Aphelenchus avenae*, *Pelodera teres* and *Pristionchus entomophagus* (supplementary fig. S1). Hence, they are not suitable for identification of *P. hermaphrodita* specifically and should be used with caution. We therefore amplified and sequenced three genes (ITS1, 18SrRNA and the D2-D3 domain of LSU rDNA) for species identification. These *P. hermaphrodita* strains and *Phasmarhabditis* species are the start of an ongoing effort to make a collection of *P. hermaphrodita* strains and *Phasmarhabditis* species to study the genetic evolution of parasitism, and we have categorized them using *C. elegans* nomenclature (table 1).

Growth of Phasmarhabditis *species using semi-natural conditions*

We made isogenic lines by growing single hermaphrodites of *P. hermaphrodita* DMG0001 and DMG0007 and *P. californica* DMG0017 on 0.025 g (fig. 2a) and 0.25 g of *L. flavus* (fig. 2b). The numbers of dauer juveniles that were produced by one *P. hermaphrodita* DMG0001, DMG0007 and *P. californica* DMG0017

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

Phasmarhabditis species	Slug/snail species isolated from	Location	Code
P. hermaphrodita	*Arion* spp.	Priory Wood, Liverpool	DMG0002
P. hermaphrodita (Deceased)	*Deroceras panormitanum*	Sefton Park, Liverpool	
P. hermaphrodita	*Deroceras panormitanum*	Sefton Park, Liverpool	DMG0003
P. hermaphrodita	*Deroceras panormitanum*	Sefton Park, Liverpool	DMG0004
P. hermaphrodita	*Arion subfuscus*	Sefton Park, Liverpool	DMG0005
P. hermaphrodita	*Arion subfuscus*	Sefton Park, Liverpool	DMG0006
P. hermaphrodita	*Limax flavus*	Sefton Park, Liverpool	DMG0007
P. hermaphrodita	*Deroceras panormitanum*	Festival Gardens, Liverpool	DMG0008
P. hermaphrodita	*Deroceras panormitanum*	Festival Gardens, Liverpool	DMG0009
P. hermaphrodita	*Milax budapestensis*	Festival Gardens, Liverpool	DMG0010
P. hermaphrodita	*Milax budapestensis*	Festival Gardens, Liverpool	DMG0011
P. neopapillosa	*Deroceras reticulatum*	Cruickshank Botanic Garden, University of Aberdeen	DMG0012
P. neopapillosa	*Deroceras reticulatum*	Cruickshank Botanic Garden, University of Aberdeen	DMG0013
P. hermaphrodita (Deceased)	*Limax flavus*	Sefton Park, Liverpool	
P. neopapillosa	*Limax flavus*	Sefton Park, Liverpool	DMG0014
P. neopapillosa	*Limax flavus*	Sefton Park, Liverpool	DMG0015
P. neopapillosa	*Limax flavus*	Sefton Park, Liverpool	DMG0016
P. californica	*Oxychilus draparnaudi*	Dale, Pembrokeshire	DMG0017
P. californica	*Oxychilus draparnaudi*	Dale, Pembrokeshire	DMG0018
P. californica	*Oxychilus draparnaudi*	Dale, Pembrokeshire	DMG0019

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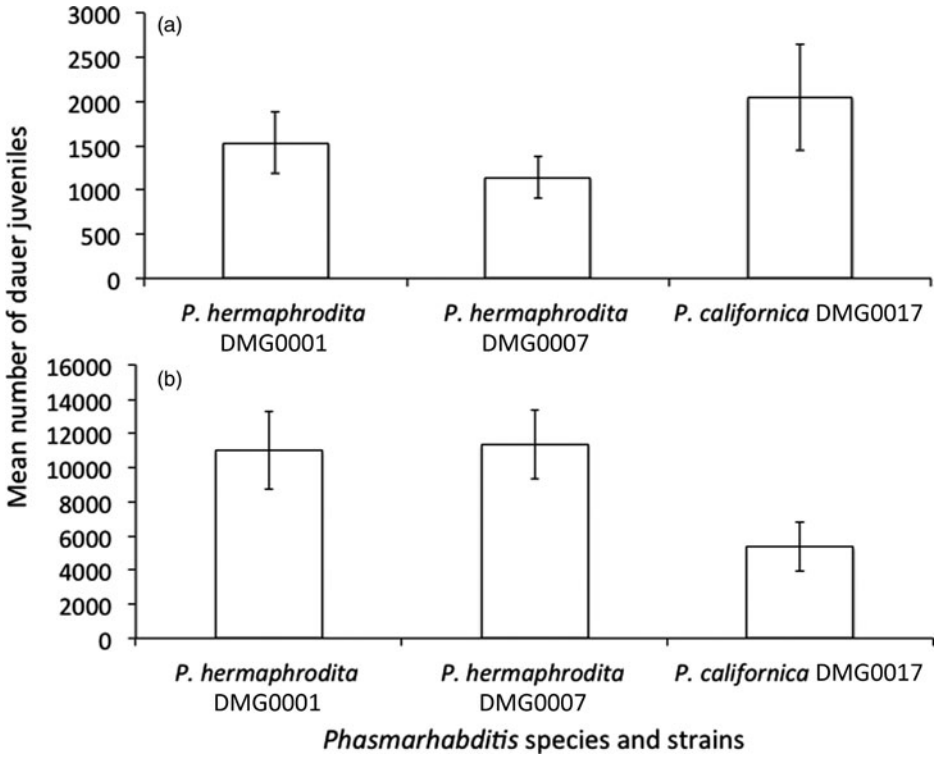

Fig. 2. The mean (± SE) number of offspring that were produced by *P. hermaphrodita* DMG0001, *P. hermaphrodita* DMG0007 and *P. californica* DMG0017 when fed (a) 0.025 g and (b) 0.25 g of *L. flavus*.

on 0.025 g of *L. flavus* after 21 days ranged from 43 to 6166 dauers per White trap (fig. 2a) and did not differ significantly (F (2, 36) = 1.369; P = 0.268). The numbers of dauer juveniles that were produced by single mothers of *P. hermaphrodita* DMG0001 and DMG0007 and *P. californica* DMG0017 fed on 0.25 g of *L. flavus* after 21 days ranged from 417 to 27,750 dauers per plate (fig. 2b) and also did not differ significantly (F (2, 38) = 2.832; P = 0.072). Therefore, *Phasmarhabditis* spp. can be grown easily under semi-natural conditions using *L. flavus* White traps and in large numbers for experiments.

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

Phasmarhabditis hermaphrodita DMG0001 and DMG0007 and *P. californica* DMG0017 were fed two nematode-associated bacteria (*Pseudomonas* sp. 1 and *Bacillus* sp. 1) and two strains of *E. coli* that have been used historically in *C. elegans* culture and molecular biology (*E. coli* OP50 and *E. coli* BR, respectively). Over six days both nematode species were able to grow on each of these bacteria, and laid F1 eggs that developed to offspring and were quantified (fig. 3). There was a significant difference between

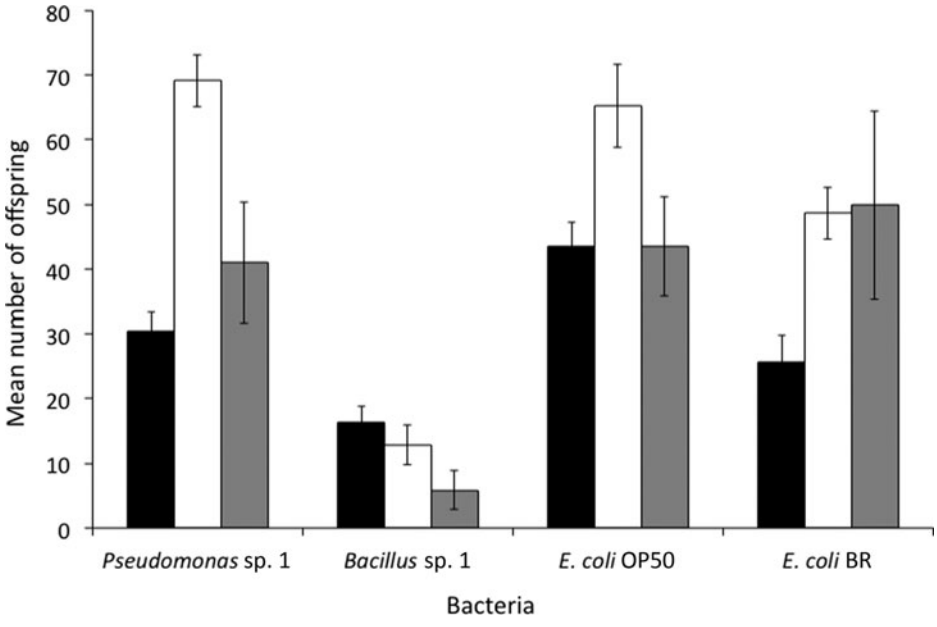

Fig. 3. The mean (± SE) number of offspring that were produced by *P. hermaphrodita* DMG0001 (black bars), *P. hermaphrodita* DMG0007 (white) and *P. californica* DMG0017 (grey) when fed *Pseudomonas* sp. 1, *Bacillus* sp. 1, *Escherichia coli* OP50 and *E. coli* BR at 20°C.

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the numbers of offspring produced by *P. hermaphrodita* DMG0001 when fed *Pseudomonas* sp. 1, *Bacillus* sp. 1, *E. coli* OP50 and *E. coli* BR (F (3, 29) = 11.101; P = 0.000). Specifically, the numbers of offspring produced by *P. hermaphrodita* DMG0001 was highest on *Pseudomonas* sp. 1, *E. coli* OP50 and *E. coli* BR, and lowest on *Bacillus* sp. 1 ($P < 0.05$). This was also the case for *P. hermaphrodita* DMG0007 and *P. californica* DMG0017. Therefore, the laboratory bacteria (*E. coli* OP50 and *E. coli* BR) and naturally isolated *Pseudomonas* sp. 1 can be used for growing *Phasmarhabditis* species.

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

When feeding on *Pseudomonas* sp. 1 there was a significant difference between the pumping rates of *P. hermaphrodita* DMG0001 and DMG0007, *P. neopapillosa* DMG0012 and DMG0016 and *P. californica* DMG0017 (F (4, 19) = 18.577; P = 0.000) (fig. 5). Specifically, there was no difference between the pumping rates of *P. hermaphrodita* DMG0001 and DMG0007 and *P. neopapillosa* DMG0012 and DMG0016 ($P > 0.05$) but all differed significantly from *P. californica* DMG0017, which had the lowest number of pumps per minute ($P < 0.05$) (fig. 5).

Natural variation in thermotolerance of Phasmarhabditis

There was a significant difference in the survival of the *Phasmarhabditis* isolates when exposed to 30°C (F (10, 98) = 18.389; $P < 0.001$) (fig. 6a). Specifically, the survival of the commercial strain of *P. hermaphrodita* (DMG0001) was significantly lower than that of *P. hermaphrodita* DMG0007 and DMG0008 ($P < 0.001$) but not *P. hermaphrodita* DMG0010 and DMG0006 ($P > 0.05$). When the survival of *P. hermaphrodita* DMG0001 was compared to other species of *Phasmarhabditis* there was a significant difference between it and *P. neopapillosa* DMG0015 and DMG0016 ($P < 0.001$) but not DMG0013 ($P > 0.05$). Also, there was a significant difference between the survival of *P. hermaphrodita* DMG0001 and that of *P. californica* DMG0019 ($P < 0.05$) but not DMG0017 ($P > 0.05$).

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

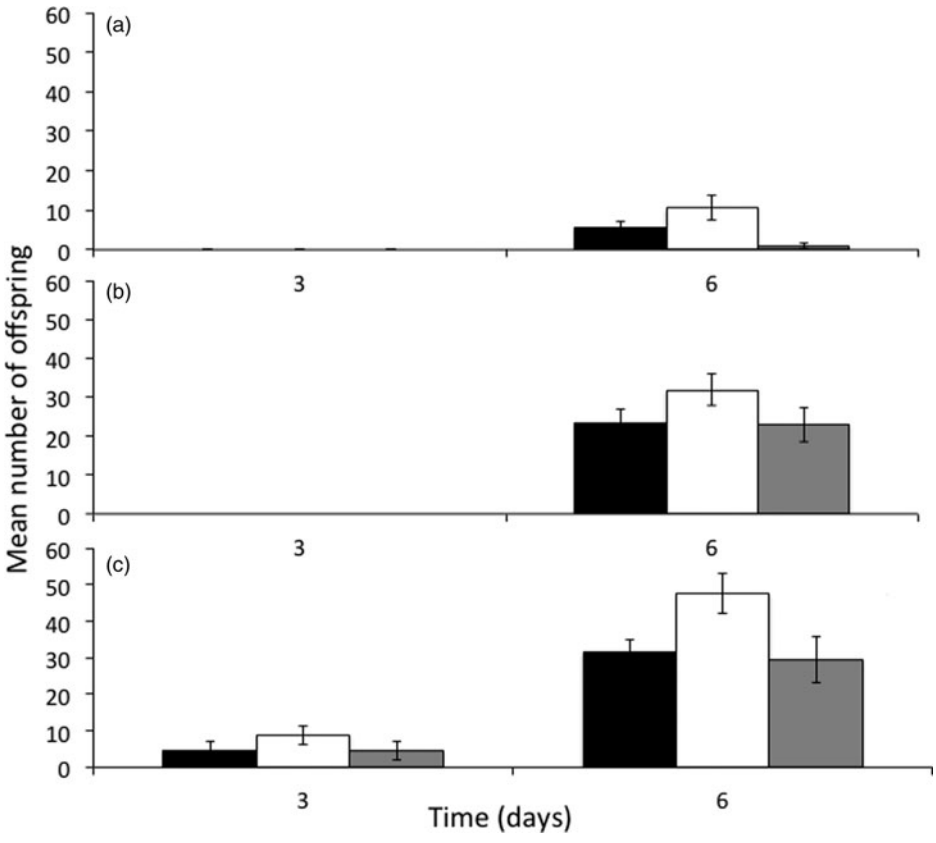

Fig. 4. The mean (± SE) number of offspring that were produced by *P. hermaphrodita* DMG0001 (black bars), *P. hermaphrodita* DMG0007 (white) and *P. californica* DMG0017 (grey) at (a) 10°C, (b) 15°C and (c) 20°C.

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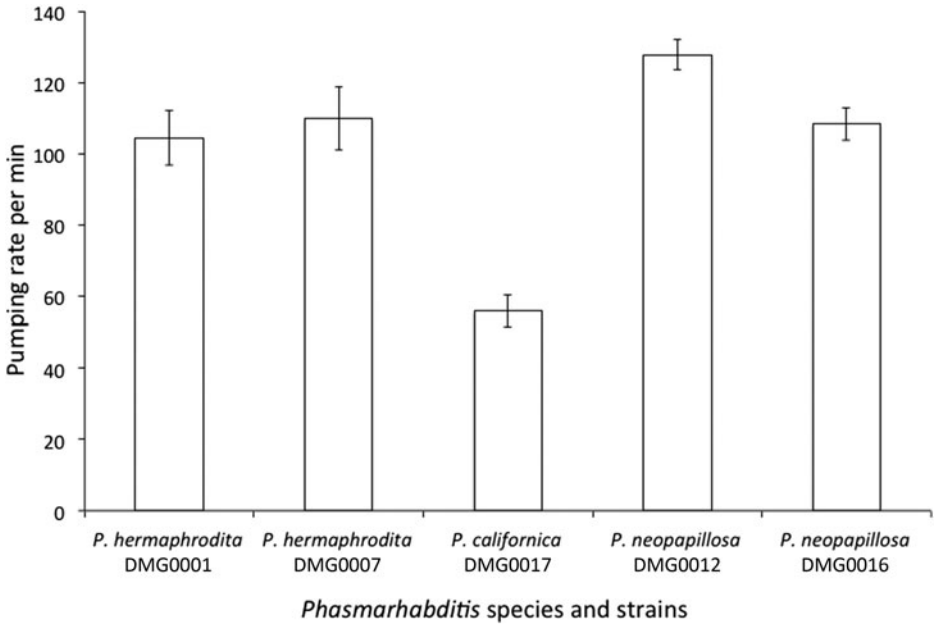

Fig. 5. The mean (± SE) pumping rate over 60 s of *P. hermaphrodita* DMG0001 and DMG0007, *P. californica* DMG0017 and *P. neopapillosa* DMG0012 and DMG0016 fed *Pseudomonas* sp. 1 at 20°C.

When the *Phasmarhabditis* isolates were exposed to 41°C there was a significant difference in their survival (F (10, 98) = 19.546; P = 0.000) (fig. 6c). The survival of *P. hermaphrodita* DMG0001 was significantly greater than that of all other species and isolates (P < 0.05).

Natural variation in pH resistance in Phasmarhabditis

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

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

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

At pH 9 (F (9, 29) = 3.378; P = 0.011) and pH 10 (F (9, 29) = 5.481; P = 0.001) survival was significantly different. In both cases the survival of *P. neopapillosa* DMG0015 was significantly lower than that of *P. californica* DMG0017 and DMG0019 (P < 0.05). Also, the survival of *P. hermaphrodita* DMG0007 was

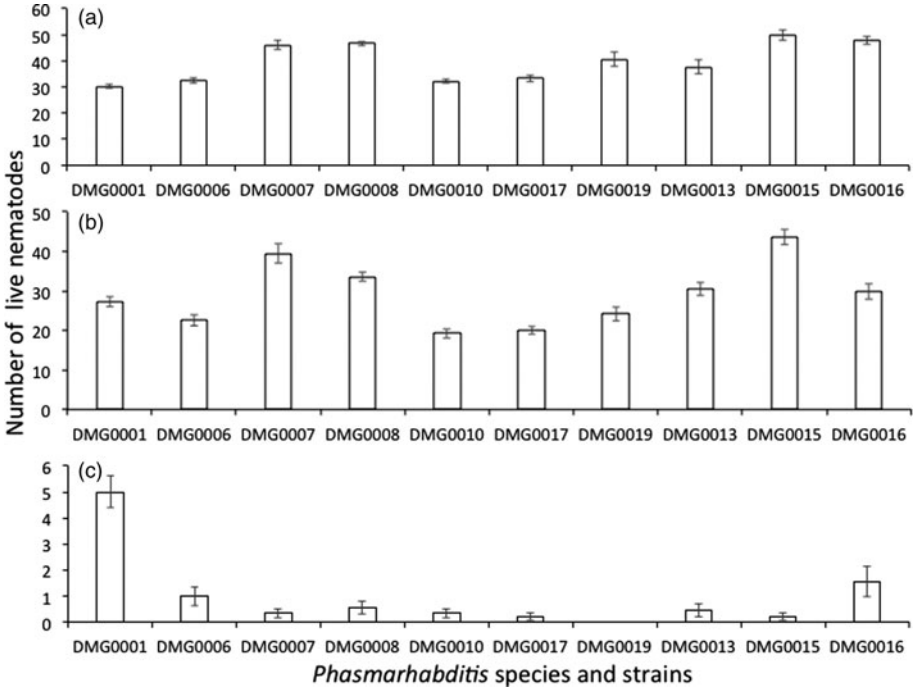

Fig. 6. The mean (± SE) number of live *P. hermaphrodita* DMG0001, DMG0007, DMG0010, DMG0006 and DMG0008, *P. californica* DMG0018 and DMG0019 and *P. neopapillosa* DMG0013, DMG0015 and DMG0016 exposed to (a) 31°C, (b) 37°C and (c) 41°C.

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Table 2. The mean (± SE) number of live individuals of *Phasmarhabditis hermaphrodita*, *P. californica* and *P. neopapillosa* after four days of exposure to pH 4–10.

			pH						
Species	Strain	Control	4	5	6	7	8	9	10
P. hermaphrodita	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
P. californica	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
P. neopapillosa	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

significantly lower than that of *P. californica* DMG0017 and DMG0019 when exposed to pH 10 ($P < 0.05$).

Rate of spontaneous male production, heat shocking and genetic crosses using Phasmarhabditis *species*

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

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

To understand whether *Phasmarhabditis* species could be mated under lab conditions we concentrated on using the gonochoristic species *P. neopapillosa* DMG0012 and DMG0016. Both strains were crossed using standard procedures based on *C. elegans* and were fed *Pseudomonas* sp. 1. Under these conditions we could show that two males to one female placed together resulted in 47.3 ± 6.7 *P. neopapillosa* DMG0016 and 83.9 ± 5.6 *P. neopapillosa* DMG0012 offspring six days later. Therefore, successful crossing of these two strains could be carried out to demonstrate inheritance of recessive and dominant mutations and to aid mapping of mutations.

Formaldehyde mutagenesis of Phasmarhabditis *species*

From 300 F1 *P. hermaphrodita* DMG0001 hermaphrodite mothers (fig. 7a) several mutants were isolated. Specifically, two small (*sma*) mutants (fig. 7b, c) and three uncoordinated (*unc*) mutants (fig. 7d, e) were found. *sma* mutants were significantly smaller than *P. hermaphrodita* WT ($P < 0.05$), and *unc* mutants strongly resembled *C. elegans unc* phenotype as they were

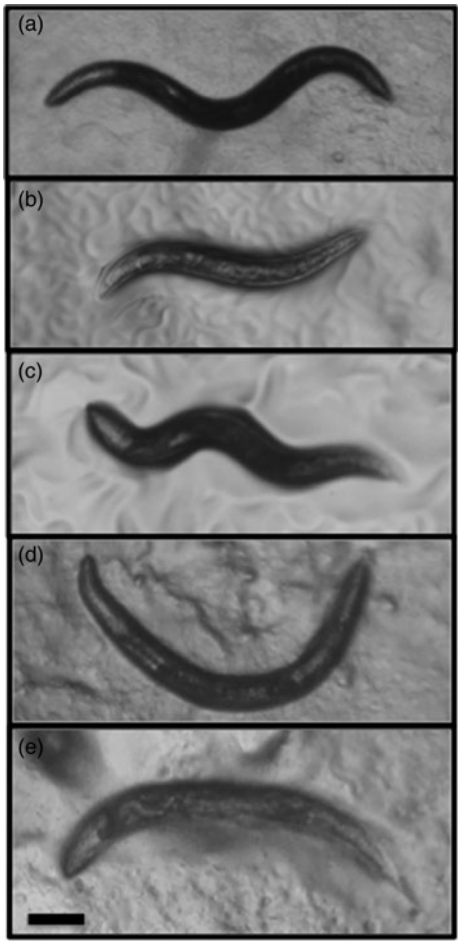

Fig. 7. (a) *P. hermaphrodita* DMG0001 were mutagenized with 0.1% formaldehyde, and (b, c) *sma* (small) mutants and (d, e) *unc* (uncoordinated) mutants were found in the F2 generation. Scale bar: 100 µm.

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lethargic, stationary and the underlying body wall muscle produced a constant twitch (Waterston *et al.*, 1980). Therefore, *P. hermaphrodita* can be mutagenized using formaldehyde, allowing forward genetic screens to be carried out.

Discussion

The genus *Phasmarhabditis* contains 11 species: *P. hermaphrodita*, *P. apuliae*, *P. papillosa*, *P. neopapillosa*, *P. valida*, *P. nidrosiensis*, *P. californica*, *P. tawfiki*, *P. bonaquaense*, *P. bohemica* and *P. huizhouensis* (Andrássy, 1983; Hooper *et al.*, 1999; Azzam, 2003; Tandingan De Ley *et al.*, 2014, 2016; Huang *et al.*, 2015; Nermut *et al.*, 2016a, b, 2017). *Phasmarhabditis hermaphrodita* and other *Phasmarhabditis* species have been isolated from slugs from around the world, including the UK (Wilson *et al.*, 1993; this study), Germany (Schneider, 1859; Mengert, 1953), France (Maupas, 1900; Coupland, 1995), Iran (Karimi *et al.*, 2003), Egypt (Azzam, 2003; Genena *et al.*, 2011), Norway (Ross *et al.*, 2016), Chile (France & Gerding, 2000), New Zealand (Wilson *et al.*, 2012), South Africa (Ross *et al.*, 2012; Pieterse *et al.*, 2017a, b), China (Huang *et al.*, 2015), Japan (Waki, 2017), Italy (Nermut *et al.*, 2016a) and the Czech Republic (Nermut *et al.*, 2010, 2016b, 2017), and *P. californica* has been found in the USA (Tandingan De Ley *et al.*, 2016), Ireland (Carnaghi *et al.*, 2017) and Wales (this study). From these studies it is apparent that members of the *Phasmarhabditis* genus have a cosmopolitan distribution and are very easy to isolate from gastropod hosts. We found 20 separate *Phasmarhabditis* isolates from 426 slugs and snails from three locations around the UK. From our survey the success rate of collecting *Phasmarhabditis* (4.7%) seems small, yet we have found a wealth of different species, including *P. californica* (which had never been identified in the UK previously) and several strains of *P. neopapillosa*. Therefore, it seems that the UK has an underappreciated diversity of *Phasmarhabditis* species.

Although *P. hermaphrodita* is a parasite, we have shown that it can be maintained and cultured under laboratory conditions using a combination of NGM agar and naturally isolated bacteria (*Pseudomonas* sp. 1). For laboratory genetic model nematodes to be used successfully their bacterial food must be nutritious enough to facilitate the growth of hundreds of offspring, transparent (so nematodes are visible) and easy to grow (but does not grow too much when added to the NGM plate). This was why *E. coli* OP50 was selected to grow and maintain *C. elegans* (Brenner, 1974). However, regular passage of hundreds of thousands of generations of nematodes using the same culture conditions (constant temperature, lots of food and same species of food) with no interaction or variation in the environment can severely affect genetic make up (Huey & Rosenzweig, 2009). For example, *C. elegans* N2 (wild type) has lost the ability to perform thermoregulatory behaviour when exposed to a temperature gradient as a result of being reared at the same temperature for over 40 years (Anderson *et al.*, 2007). We propose the use of *Pseudomonas* sp. 1, a bacterium found in the intestine of *P. entomophagus* from Tübingen, Germany (Rae *et al.*, 2008), and not an unusual food source such as *E. coli* OP50. Yet continual culturing on NGM plates is an unnatural culture method for these nematodes, as *Phasmarhabditis* are necromenic and parasitic nematodes used to reproducing on rotting cadavers of molluscs (Wilson *et al.*, 1993; Rae *et al.*, 2009). To this end we propose growing *Phasmarhabditis* on decaying slugs in 'semi-natural' conditions using White traps, which is a more realistic environment. This method means that they can be stored at 10–15°C for months as dauers (Grewal & Grewal, 2003) and cultured infrequently (every four to five months), and therefore the effect of accumulating any deleterious mutations will be reduced. Furthermore, future research will focus on the development of cryopreservation techniques for *Phasmarhabditis*, which will allow access to a library of 'unevolved' strains and species as well as mutants.

Once *P. hermaphrodita* has killed a slug it feeds on the bacteria growing on the rotting cadaver, and when this is depleted it turns to the dauer stage and searches for more slugs in the soil. These dauers associate with a rich diversity of bacteria that are carried in their intestines (Rae *et al.*, 2010). Previous studies have shown that the bacteria isolated from the intestine of *P. hermaphrodita*, from xenic cultures of *P. hermaphrodita* and from swabs of slugs that died from infection of *P. hermaphrodita* can affect the number of dauers produced as well as their virulence towards slugs (Wilson *et al.*, 1995a, b). These studies showed that *P. hermaphrodita* can grow on an array of bacterial species, such as *Pseudomonas fluorescens*, *Sphingobacterium spiritivorum*, *M. osloensis*, *Serratia proteamaculans*, *Aeromonas* sp. and *Providencia rettgeri* (Wilson *et al.*, 1995b), and *P. hermaphrodita* grown on bacteria such as *P. fluorescens*, *M. osloensis* and *P. rettgeri* can produce high yields of pathogenic nematodes that kill slugs (Wilson *et al.*, 1995a). However, the commercial isolate of *P. hermaphrodita* (DMG0001) is grown on *M. osloensis*, as it can produce consistently high yields of highly pathogenic nematodes (Tan & Grewal, 2001b; Wilson *et al.*, 1995a, b). It has been shown that when introduced into the shell cavity of *D. reticulatum*, *M. osloensis* produces a lipopolysaccharide (LPS) that acts as an endotoxin, causing rapid mortality (Tan & Grewal, 2002). By utilizing this collection of naturally isolated *P. hermaphrodita* and *Phasmarhabditis* species the co-evolution of these tritrophic interactions between bacteria (such as *M. osloensis*), nematodes and slug hosts could be analysed at the molecular level.

As well as established genetic model nematodes (*C. elegans* and *P. pacificus*) there are several other nematodes that have been proposed, including *Poikilolaimus oxycercus* (Hong *et al.*, 2005), *Oscheius tipulae* (Félix, 2006) and *Meloidogyne hapla* (to study plant parasitism) (Opperman *et al.*, 2008). For these nematodes (as well as *P. pacificus*) to be used under laboratory conditions, information is needed about appropriate bacterial food as well as methods for genetic crosses, mutagenesis and long-term storage. We have shown that logistically and financially, nematodes such as *Phasmarhabditis* are easy to maintain. There is little difference in the equipment needed to keep *C. elegans* (Stiernagle, 2006), e.g. simple reagents and microbiological media, and incubators and freezers for growth and long-term storage. As well as these factors, another important point about model nematodes is that the ability to be isolated easily can allow tens if not hundreds of strains to be studied to investigate natural phenotypic variation, which can lead to an understanding of the underlying genotype using approaches such as RADseq (restriction site-associated DNA sequencing) (Davey & Blaxter, 2010) and GWAS (genome-wide association studies) (Cook *et al.*, 2016). In global sampling efforts, several hundred *C. elegans* strains and 26 *Caenorhabditis* species have been collected (Frézal & Félix, 2015), which are available from the *Caenorhabditis* Genetics Center at the University of Minnesota, USA. Studying natural variation has been successful in understanding genes involved with foraging behaviour, thermal tolerance and outcrossing (De Bono & Bargmann, 1998; Teotónio *et al.*, 2006; Harvey &

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Viney, 2007). A similar approach has been taken utilizing natural strains and investigating variation in behaviour, cold tolerance and dauer formation in *P. pacificus* (Hong *et al.*, 2008; Mayer & Sommer, 2011; McGaughran & Sommer, 2014). In total there are 28 species of *Pristionchus* (Ragsdale *et al.*, 2015) and hundreds of strains of *P. pacificus*, which are available from the Sommer lab, Tübingen, Germany (Morgan *et al.*, 2012). We have shown that, like both *C. elegans* and *P. pacificus*, *P. hermaphrodita* and a selection of *Phasmarhabditis* species can be isolated and maintained in the lab easily. We have shown that there is natural variation within *P. hermaphrodita* and *Phasmarhabditis* species in terms of surviving different temperatures and pHs. This means that with the development of appropriate sequencing and genomic techniques (e.g. RADseq) macroevolutionary and microevolutionary processes could potentially be unravelled at the genetic level.

The isolation of mutants via forward genetic screens using mutagenesis is a powerful technique that can identify genes responsible for specific phenotypes. The first step for any proposed genetic model nematode is to show it can be mutagenized. Here we showed *P. hermaphrodita unc* and *sma* mutants could be isolated by using similar protocols to those used for *C. elegans* (Johnsen & Baillie, 1988). If *P. hermaphrodita* can be mutated then there is no reason why unbiased forward genetic screens could not be carried out to investigate an array of evolutionary and ecologically important traits. These include finding mutants that are defective in killing slugs, inducing slug avoidance (Wilson *et al.*, 1999; Wynne *et al.*, 2016) or failing to chemotax towards host cues such as slug mucus (Rae *et al.*, 2006, 2009). As *P. hermaphrodita* is one of the candidates for the 959 Nematode Genomes project (Kumar *et al.*, 2012) and several species are currently undergoing full genome sequencing, this will facilitate genomic comparison with closely related free-living nematodes, arthropod and mammalian parasites present in Clade 5.

We found that *P. neopapillosa* could be mated under lab conditions using similar procedures as for *C. elegans*. However, generating enough males for genetic crosses with *P. hermaphrodita* proved difficult. This is not unusual for hermaphroditic nematodes that are able to produce males spontaneously. *Caenorhabditis elegans* produces only 0.1–0.2% males in culture (Hodgkin & Doniach, 1997). Maupas (1900) noted that only one male was found in 14,888 *P. hermaphrodita*. Our strains under lab conditions seem not to produce males, even when heat shocked for 1–4 h at 30°C. When these strains were first isolated one was found to have males present (DMG007) (R. Rae, unpubl. data) but when grown on rotting slug and NGM plates the ability to produce males seemed to diminish rapidly over time. Future research will focus on methods used to generate males, including exposing hermaphrodites to ethanol (Lyons & Hecht, 1997), and isolating a mutant (using forward genetics) that has a high incidence of males (*him* mutant) (Hodgkin *et al.*, 1979).

In conclusion, we have outlined the methods used to work with *P. hermaphrodita* and other *Phasmarhabditis* species under laboratory conditions. We believe that *P. hermaphrodita* (and other *Phasmarhabditis* species) could be used to identify genes that are essential for pathogenicity towards slugs. The most logical way to achieve this would be to take a natural variation approach to isolate as many *P. hermaphrodita* strains as possible and grow them on rotting slugs (as we have outlined here), which does not affect their virulence (Rae *et al.*, 2010), and assess their pathogenic potential towards slugs. The main aim would be to identify a strain that is more or less virulent than DMG0001 (an approach that is currently ongoing; R. Rae, unpubl. data). Through genome sequencing, potential parasitism genes could be identified and confirmed by reverse genetics, e.g. RNAi and/or CRISPR-Cas9. This information could provide deep insight into the evolution of parasitism in other Clade 5 animal, plant and invertebrate nematode parasites, and allow comparison with *C. elegans* and *P. pacificus*.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X18000305

Conflict of interest. None.

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