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Short communication

Phasmarhabditis californica in Germany

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Phasmarhabditis hermaphrodita is a malacopathogenic nematode that can kill several species of pestiferous slugs and snails (Wilson *et al*., 1993). It has been formulated as a biological control agent (Nemaslug®) and used broadly throughout Europe (Rae *et al*., 2007). It has been isolated outside Europe in Egypt (Genena *et al*., 2011), Chile (France & Gerding, 2000), the USA (Tandingan De Ley *et al*., 2014) and New Zealand (Wilson *et al*., 2012).

When *P. hermaphrodita* is applied to soil, the nematodes are attracted to slug mucus and faeces (Rae *et al*., 2006) and penetrate the pore at the back of the slug's mantle where they are thought to release a bacterium (*Moraxella osloensis*) that causes bacterial septicaemia and kills the slug within 4-21 days (Tan & Grewal, 2001a). *Phasmarhabditis hermaphrodita* reproduces on the cadaver and once the food supply is depleted new infective juveniles go in search of slug hosts in the soil. *Phasmarhabditis hermaphrodita* has been shown to provide significant protection against slug damage when applied to a range of crops including lettuce, wheat and Chinese cabbage (see Rae *et al*., 2007). *Phasmarhabditis hermaphrodita* has been used to control slugs across northern Europe for over 25 years, yet there is little knowledge about the biology and worldwide distribution of other members of the genus *Phasmarhabditis* including: *P. apuliae*, *P. papillosa*, *P. neopapillosa*, *P. valida*, *P. nidrosiensis*, *P. californica*, *P. tawfiki*, *P. bonaquaense*, *P. bohemica*, *P. huizhouensis*, *P. circassica*, *P. clausiliae*, *P. quinamensis*, *P. meridionalis*, *P. kenyaensis* and *P. zhejiangensis* (Andrássy, 1983; Hooper *et al*., 1999; Azzam, 2003; Tandingan De Ley *et al*., 2014, 2016; Huang *et al*., 2015; Nermut' *et al*., 2016a, b, c; Ivanova *et al*., 2017, 2020; Pieterse *et al*., 2020; Zhang & Liu, 2020; Ivanova & Spiridonov, 2021). An understanding of the biology, behaviour, distribution and diversity of these nematodes has the potential to develop new biological control agents based on the information gleaned from decades of research on *P. hermaphrodita* (see Rae *et al*., 2007 for further information). This is especially pertinent as *P. hermaphrodita* is unable to kill larger, more problematic, pest species such as *Arion lusitanicus* (Grimm, 2002), and there are reports of *P. hermaphrodita* failing to provide protection against slugs in field trials (*e.g.*, Wilson *et al*., 1995).

Other than *P. hermaphrodita* the most studied member of the *Phasmarhabditis* genus is *P. californica*. It was first discovered in California, USA (Tandingan De Ley *et al*., 2016) and seems to have a cosmopolitan distribution, having been found in Wales (Andrus & Rae, 2019), New Zealand (Wilson *et al*., 2016), Canada (Brophy *et al*., 2020) and Ireland (Carnaghi *et al*., 2016). As well as its distribution being recorded, garden experiments have demonstrated that the USA strains of *P. californica* can parasitise and kill the grey field slug (*Deroceras reticulatum*) (McDonnell *et al*., 2020) and snails (*Theba pisana*) (Tandingan De Ley *et al*., 2020), much like *P. hermaphrodita*. Apart from the isolation of *P. californica* in Wales and Ireland there are no other records of these

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nematodes being found in northern Europe. Hence, we conducted a survey of 993 slugs and snails collected from Germany, a country where *Phasmarhabditis* nematodes have frequently been isolated (Mengert, 1953) and where *P. hermaphrodita* was first isolated and described (Schneider, 1859). Our main aim was to discover what the most abundant *Phasmarhabditis* species were in Germany that commonly infect slugs and snails.

Slugs and snails (see Table 1 for species) were collected early evening from three locations including a private garden in Hassloch/Pfalz, Rhineland-Palatinate, 49°21′43.5″N, 8°14′39.6″E (n = 482; 12 snails and 470 slugs); a public green in Kiel, Schleswig-Holstein, 54°19′22.9″N, 10°07′47.2″E (n = 243; ten snails and 233 slugs), and a private garden in Plau am See, Mecklenburg-Vorpommern 53°27′22.1″N, 12°16′03.0″E (n = 268; 262 snails and six slugs). They were stored in non-airtight containers with moist tissue paper and fed with lettuce *ad libitum*. They were kept at 15°C and left for 1 week to ensure there were no symptoms of nematode infection of slugs before dissection *e.g.*, swollen mantle, moribund appearance.

Slugs and snails were crudely dissected using protocols by Wilson *et al*. (2016) and placed on separate 5 cm or 10 cm (for larger slugs or snails) diam. inverted Petri dishes and stored at 20°C for 5 days. *Phasmarhabditis* nematodes are facultative parasites that reproduce on the carcass of the slug; any other slug or snail parasites that are obligate will die, *e.g.*, nematodes from the genus *Agfa* and *Angiostoma*. Furthermore, these nematodes are morphologically different from *Phasmarhabditis* nematodes and easily distinguishable. After 3 and 5 days each slug and snail was examined for the presence of *Phasmarhabditis*-like nematodes *e.g.*, approximately 1.5-2.0 mm long and hermaphroditic. Any *Phasmarhabditis*-like nematodes were individually moved to separate modified White traps (see Andrus & Rae, 2019 for further details). Briefly, a slice of frozen slug (*Limax flavus*) was used as a food source for the individual nematodes. Once devoured, the infective juveniles (IJ) nematodes moved into the nearby water and were stored at 10°C for molecular analysis.

Approximately 20-50 IJ were added to a 5 cm diam. Petri dish half filled with 1% nutrient agar and seeded with a lawn of 50 μl *M. osloensis* (the bacterium on which commercial *P. hermaphrodita* are grown). After 5-7 days growth at 20°C the nematodes had exited the infective juvenile stage and had grown to adults, which were then picked into 20 μl of sterile water and frozen at –80°C. The following day the nematodes were thawed and DNA was extracted using a Qiagen DNA extraction kit and the 18S rRNA gene was amplified using the following primers: 18A (5′-AAAGATTAAGCCATGCATG-3′) and 26R (5′-CATTCTTGGCAAATGCTTTCG-3′) (Blaxter *et al*., 1998; Tandingan De Ley *et al*., 2014). The PCR cycling conditions consisted of the following: 3 min at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 55°C, 1.5 min at 72°C and a final step of 8 min at 72°C. Successful PCR amplification was confirmed using gel electrophoresis and each product underwent PCR purification. Each PCR product was sequenced in forward and reverse directions by Eurofins and NCBI BLAST was used to determine species using similarity matches of 99%.

Fifteen gastropods from 993 slugs and snails collected from Plau am See, Hassloch/Pfalz and Kiel had *Phasmarhabditis*-like nematodes present (Table 1). Through molecular verification we found 13 strains of *P. californica* from the three locations (six from Kiel, five from Plau am See and two from Hassloch/Pfalz) and 13 strains of *P. hermaphrodita* but only from Plau am See (Table 1) (see NCBI GenBank accession numbers OL762419-OL762443 for sequences). We examined the sequence variation between strains and for *P. hermaphrodita* there are only three single nucleotide polymorphisms (SNPs) between the 13 strains and a similar number for *P. californica*.

In general, snails were more successful hosts for finding *P. hermaphrodita* and *P. californica* compared to slugs. From 680 *D. invadens* and *D. reticulatum* only one slug was found with two strains of *P. californica* and a single *A. rufus* was infected with one *P. californica*. This is in contrast to snails, where the most *Phasmarhabditis*-like nematodes were found infecting *Cepaea hortensis* snails. Eight individual *C. hortensis* snails were infected with eight different strains of *P. hermaphrodita*. Also a single *Discus rotundatus* snail was infected with four *P. californica* and one *P. hermaphrodita* and one *Oxychilus draparnaudi* was infected with six individual *P. californica*.

Phasmarhabditis species have been isolated from terrestrial gastropods from around the world including France (Maupas, 1900; Coupland, 1995), Egypt (Azzam, 2003; Genena *et al*., 2011), Norway (Ross *et al*., 2015), Chile (France & Gerding, 2000), New Zealand (Wilson *et al*., 2012), South Africa (Ross *et al*., 2012; Pieterse *et al*., 2017), China (Huang *et al*., 2015; Zhang and Liu, 2020), Italy (Nermut' *et al*., 2016a), Czech Republic (Ner-

Table 1. List of locations, slug and snail species dissected and molecular identification of *Phasmarhabditis* species.

Location in Germany	Slug or snail species	Number collected	Number of individuals infected with *Phasmarhabditis*-like nematodes	Number of isogenic lines	Number of strains of each *Phasmarhabditis* species isolated	Genbank Accession Study Sequences	NCBI species match	Genbank Accession number
Plau am See	*Cepaea nemoralis*	49	1	1	1 × *P. hermaphrodita*	OL762439	*Phasmarhabditis hermaphrodita*	MK214808
	Cepaea hortensis	153	8	8	8 × *P. hermaphrodita*	OL762431, OL762432, OL762433, OL762434, OL762440, OL762441, OL762442, OL762443	*Phasmarhabditis hermaphrodita*	MK214808
	Arianta arbustorum	23	2	3	3 × *P. hermaphrodita*	OL762435, OL762436, OL762437	*Phasmarhabditis hermaphrodita*	MK214808
	Succinea putris	8	0	0	0		N/A	N/A
	Monacha cantiana	20	0	0	0		N/A	N/A
	Helix pomatia	1	0	0	0		N/A	N/A
	Discus rotundatus	8	1	5	4 × *P. californica* 1 × *P. hermaphrodita*	OL762424, OL762425, OL762426, OL762427, OL762438	*Phasmarhabditis* sp. 1 ITDL-2014; *Phasmarhabditis hermaphrodita*	KM510210 for *P. californica* MK214808 for *P. hermaphrodita*
	Limax maximus	4	0	0	0		N/A	N/A
	Arion rufus	2	1	1	1 × *P. californica*	OL762428	*Phasmarhabditis* sp. 1 ITDL-2014	KM510210
Kiel	*Oxychilus draparnaudi*	10	1	6	6 × *P. californica*	OL762419, OL762420, OL762421, OL762422, OL762423, OM475722	*Phasmarhabditis* sp. 1 ITDL-2014	KM510210
	Deroceras invadens	99	0	0	0		N/A	N/A
	Deroceras reticulatum	134	0	0	0		N/A	N/A
Hassloch	*Deroceras reticulatum*	447	1	2	2 × *P. californica*	OL762429, OL762430	*Phasmarhabditis* sp. 1 ITDL-2014	KM510210
	Limax maximus	3	0	0	0		N/A	N/A
	Arion hortensis	17	0	0	0		N/A	N/A
	Cepaea nemoralis	2	0	0	0		N/A	N/A
	Oxychilus draparnaudi	1	0	0	0		N/A	N/A
	Hygromia cinctella	3	0	0	0		N/A	N/A
	Candidula intersecta	5	0	0	0		N/A	N/A
	Milax budapestensis	1	0	0	0		N/A	N/A
	Arion vulgaris	2	0	0	0		N/A	N/A
	Cornu aspersum	1	0	0	0		N/A	N/A

mut' *et al*., 2010, 2016b, c), the USA (Tandingan De Ley *et al*., 2016), Russia, Vietnam (Ivanova & Spiridonov, 2017, 2021; Ivanova *et al*., 2021), Switzerland (Jaffuel *et al*., 2019), Germany (Schneider, 1859; Mengert, 1953), England (Wilson *et al*., 1993), Ireland (Carnaghi *et al*., 2017) and Wales (Andrus & Rae, 2019). However, only *P. hermaphrodita*, *P. neopapillosa* and *P. papillosa* have been isolated in Germany (Schneider, 1859; Mengert, 1953), and this is the first time *P. californica* has been isolated and identified from several locations in Germany.

Our success rate of isolating *Phasmarhabditis* is slightly higher than other studies. From 993 gastropods, we found 15 individual gastropods infected with 26 *Phasmarhabditis* nematodes (13 *P. hermaphrodita* and 13 *P. californica*). Tandingan De Ley *et al*. (2014) isolated four isolates of *P. hermaphrodita* from three slugs (*D. laeve*, *D. reticulatum* and *Lehmannia valentiana*) out of 693 gastropods from California. Brophy *et al*. (2020) found a single *P. californica* isolate from *A. rufus* from 2406 slugs. Perhaps concentrating on snails may be more successful than slugs as the majority of the *Phasmarhabditis* strains we isolated (n = 26) were from 13 separate snails from five species, whereas only one *P. californica* was isolated from the slug *A. rufus* and two *P. californica* from an individual *D. reticulatum*.

Phasmarhabditis californica has been isolated from Wales (Andrus & Rae, 2019), Ireland (Carnaghi *et al*., 2016), the USA (Tandingan De Ley *et al*., 2016; Mc Donnell *et al*., 2020), New Zealand (Wilson *et al*., 2016) and recently Canada (Brophy *et al*., 2020). The biology of the nematode is relatively unknown but it is a lethal parasite (like *P. hermaphrodita*) as USA strains of *P. californica* have been shown to kill the grey field slug (*D. reticulatum*) (Mc Donnell *et al*., 2020) and snails (*T. pisana*) (Tandingan De Ley *et al*., 2020). The virulence of our strains isolated from Germany warrants further investigation as natural variation in *P. hermaphrodita* strains has been demonstrated (Cutler & Rae, 2020).

Of the gastropods that were infected with *Phasmarhabditis* nematodes half had infections of 1-2 nematodes, but some of the snails were heavily infected. For example, one snail (*O. draparnaudi*) from Kiel was infected with six *P. californica*. It is surprising that such a small snail (<1 cm) can tolerate such a heavy infection as it has been shown a single nematode can kill the grey field slug *D. reticulatum* (Tan & Grewal, 2001b). In general, snails are thought to be less susceptible to infection by *P. hermaphrodita* than slugs due to the ability of snails to trap, encase and kill nematodes using their shell, often hundreds at a time (Rae, 2017). However, as we did not examine the shells during the dissection process it is unknown how many individuals attempted to infect the snails but were encased and killed.

We found evidence of co-infection of *Phasmarhabditis* species. One *D. rotundatus* was infected with four *P. californica* and one *P. hermaphrodita*. From our understanding there are no reports of co-infection of terrestrial gastropods with different *Phasmarhabditis* species. The only example of co-infection with *Phasmarhabditis* is with members of the genera *Angiostoma*, *Agfa* and *Alloinema*, which were found infecting one slug (Ross *et al*., 2015). How co-infection of two *Phasmarhabditis* species could influence the pathogenicity process and how they outcompete each other for nutrients when the slug dies is unknown and warrants further study.

In summary, our survey of Germany has revealed, for the first time, the presence of *P. californica* in three locations and that this nematode seems to be as abundant as *P. hermaphrodita*.

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