

# Survey of nematodes associated with terrestrial slugs in Norway

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## Abstract

A survey of nematodes associated with terrestrial slugs was conducted for the first time in Norway. A total of 611 terrestrial slugs were collected from 32 sample sites. Slugs were identified by means of morphological examination, dissection of genitalia and molecular analysis using mitochondrial DNA. Twelve slug species were identified, representing four different slug families. Internal nematodes were identified by means of morphological analysis and the sequencing of the 18S rRNA gene. Of the sample sites studied, 62.5% were found to be positive for nematode parasites, with 18.7% of all slugs discovered being infected. Five nematode species were identified in this study: *Alloionema appendiculatum*, *Agfa flexilis*, *Angiostoma limacis*, *Angiostoma* sp. and *Phasmarhabditis hermaphrodita*. Of these species, only one nematode was previously undescribed (*Angiostoma* sp.). This is the first record of the presence of *A. appendiculatum*, *A. flexilis* and *A. limacis* in Norway.

## Introduction

Current understanding of nematodes associated with terrestrial slugs is based on surveys conducted in Germany (Mengert, 1953), France (Morand, 1988), Slovenia (Laznik *et al.*, 2009), the Crimea (Ivanova *et al.*, 2013), Bulgaria (Ivanova *et al.*, 2013), the USA (Gleich *et al.*, 1977; Ross *et al.*, 2010a, b), Australia (Charwat & Davies, 1999), Africa (Ross *et al.*, 2011, Andersen *et al.*, 2012) and the UK (Ross *et al.*, 2010a, b). A total of seven nematode

families have been found to parasitize terrestrial slugs, including Agfidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogasteridae, Mermithidae and Rhabditidae; however, *Phasmarhabditis hermaphrodita* (Rhabditidae) is the only nematode to have been developed commercially as a biological molluscicide (Rae *et al.*, 2007). *Phasmarhabditis hermaphrodita* is pathogenic to a range of slug families, including Agriolimacidae, Arionidae, Limacidae, Milacidae and Vagnulidae (Rae *et al.*, 2007). The infectious nematode larvae seek out slugs and enter through natural openings. Once inside, slug feeding is inhibited, with death usually following 4–21 days after nematode infection (Tan & Grewal, 2001).

The invasive slug *Arion vulgaris* (also regarded as *Arion lusitanicus* Mabille, 1968) originates from south-western Europe, and was first reported in Norway in 1988 (Von Proschwitz & Winge, 1994). Since then, it has become a

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major pest in ornamental and vegetable plots, as well as being a serious nuisance pest (Hofsvang *et al.*, 2008). Of major concern is the spread of *A. vulgaris* to cultivated agricultural and horticultural crops such as strawberries and vegetables (Andersen *et al.*, 2012). Once established, populations of *A. vulgaris* are difficult to manage, especially in years of favourable slug weather, where mild winters are followed by a wet spring and summer. The reasons for successful colonization of *A. vulgaris* are not fully understood; however, typical for invasive organisms is their flexible behaviour that allows for relatively easy adaptation to a new environment. Ross *et al.* (2010a) have also demonstrated that parasite release plays an important role in successful mollusc invasions. This paper presents new data on the diversity and distribution of nematode parasites that are associated with terrestrial slugs in Norway.

## Materials and methods

### Collection and identification of slugs

In the current study, adult slugs were collected from suitable localities that were found in cooperation with local gardeners, growers and agricultural advisory services. Coordinates and land use were recorded. Slugs were collected during late August, September and October 2011. Slugs were identified through morphological examination and dissection of their genitalia (Hatteland *et al.*, 2015; Von Proschwitz, pers. comm.). For slugs identified as *A. vulgaris* and *Arion ater*, the mitochondrial DNA was analysed (Folmer *et al.*, 1994; Hatteland *et al.*, 2015).

### Nematode identification

The slugs were dissected and examined for nematode parasites. Nematodes were identified using a combination of morphological and molecular techniques. Morphological identification was conducted using a Zeiss Jenaval microscope (Zeiss, Germany). The adult nematodes, after being heat killed at 60°C in 5% formaldehyde, were processed using anhydrous glycerol for purposes of mounting and identification (Ross *et al.*, 2012). To obtain the adult stages of rhabditid juveniles for morphological identification, juvenile bacterial-feeding nematodes were cultured on modified kidney medium (Ross *et al.*, 2012), pre-inoculated with the bacterium *Moraxella osloensis*. This method is known to be effective for rearing *Phasmarhabditis* spp. (Ross *et al.*, 2012). For purposes of molecular identification, the nematodes, after storage in 70% ethanol, were transferred to Chelex/Proteinase K mix to allow for DNA extraction (Ross *et al.*, 2010b). Characterization of the partial 18S rRNA gene was then conducted, using the methods described by Ross *et al.* (2010b). Raw sequences were trimmed and assembled using Sequencher 4.1 (Genes Codes Corp., Ann Arbor, Michigan, USA). Sequences were used for searching GenBank with the BLASTn search tool (Altschul *et al.*, 1990).

## Results

In total, 611 terrestrial slugs were collected from 32 sample sites around Norway. Twelve slug species were

identified, representing four different slug families (table 1). Nematodes were found to have parasitized seven of the 12 slug species identified (table 1). Slugs that were free from nematode parasites included *Deroceras panormitanum*, *Arion circumscriptus*, *Arion distinctus*, *Limax cinereoniger* and *Milax gagates* (table 1). The two most abundant slug species collected in Norway were *Deroceras reticulatum* (260 slugs) and *A. vulgaris* (204 slugs). Of these, 3.1% of *D. reticulatum* were found to be infected with nematodes, compared to 34.8% of *A. vulgaris* (table 1).

Nematodes were found to be parasitizing slugs at 20 of the 32 sites examined, i.e. 62.5% of the sample sites used. Of all the slugs, 18.7% were found to be infected with nematodes. A total of five nematode species were identified using morphological characteristics and 18S sequencing. As sequences of the same species were found to be identical, only one representative sequence was submitted for each taxon involved (table 2). The identified species were *Alloionema appendiculatum* Schneider, 1859; *Agfa flexilis* Dujardin, 1845; *Angiostoma limacis* Dujardin, 1845; *Angiostoma* sp., and *Phasmarhabditis hermaphrodita* (Schneider, 1859) Andrassy, 1983. Of the 20 positive sample sites, nine sites were found to be infected by *A. appendiculatum*, three sites by *A. flexilis*, four sites by *A. limacis*, seven sites by *Angiostoma* sp. and 14 sites by *P. hermaphrodita*. The most heavily infected sites were Avaldsnes (59°17'29"N, 05°13'39"E), Haukedalen (60°27'49"N, 05°17'58"E), Leirvik (59°46'24"N, 05°29'44"E), Melsum Vik (59°15'04"N, 10°21'13"E) and Utne (60°55'54"N, 06°62'47"E). Of all species collected, only one nematode was previously undescribed. (*Angiostoma* sp.). This is the first record of *A. appendiculatum*, *A. flexilis* and *A. limacis* in Norway.

## Discussion

This paper presents the findings of a new survey on the diversity and distribution of slug-parasitic nematodes in Norway. Five nematode species were identified in Norway – *A. appendiculatum* Schneider, 1859, *A. flexilis* Dujardin, 1845, *A. limacis* Dujardin, 1845, *Angiostoma* sp. and *P. hermaphrodita* (Schneider, 1859) Andrassy, 1983. These nematodes represent four different families: Alloionematidae, Agfidae, Angiostomatidae and Rhabditidae.

*Alloionema appendiculatum* was found at nine of the 32 sample sites examined, and is the first record in Norway. The nematode was found to parasitize the foot sole of arionids (*A. ater*, *A. fuscus*, *A. rufus*, *A. vulgaris*), and this is the first record of its association with an *A. rufus*/*A. ater* hybrid. The family Alloionematidae is known from two genera, *Rhabitophanes* and *Alloionema*. *Rhabitophanes* commonly associates with insects, while the monotypic genus, *Alloionema*, is represented by the mollusc-parasitic nematode, *A. appendiculatum* (Schuurmans-Stekhoven, 1950). Charwat & Davies (1999) demonstrated that *A. appendiculatum* is non-pathogenic to mollusc hosts, while more recent work illustrates that the nematode is capable of significant mortality in *A. lusitanicus* (Laznik *et al.*, 2009). Cabaret & Morand (1990) also suggested that the nematode is pathogenic to several snail species.

Table 1. The prevalence (%) and intensity of infection of terrestrial slugs with nematode species from Norway; *N*, number of slugs examined.

Slug family/species	<i>N</i>	Nematode species	Site of infection	%	Mean intensity
<b>Agriolimacidae</b>					
<i>Deroceras reticulatum</i>	260	<i>Angiostoma limacis</i> *†	cp, oe	0.4	1.0
		<i>Phasmarhabditis hermaphrodita</i>	mc	2.3	1.7
<i>Deroceras panormitanum</i>	4	–	–	–	–
<b>Arionidae</b>					
<i>Arion ater</i>	33	<i>Alloionema appendiculatum</i> †	fs	12.1	16.8
		<i>Angiostoma</i> sp.*†	cp, oe	12.1	28.0
		<i>Phasmarhabditis hermaphrodita</i>	in, mc, sg, sp	18.2	2.3
<i>Arion circumscriptus</i>	4	–	–	–	–
<i>Arion distinctus</i>	5	–	–	–	–
<i>Arion fasciatus</i>	1	<i>Angiostoma</i> sp.*†	cp	100	4.0
<i>Arion fuscus</i>	70	<i>Alloionema appendiculatum</i> †	fs	15.7	19.5
		<i>Angiostoma limacis</i> †	cp, mc, oe	4.3	1.0
		<i>Angiostoma</i> sp.*†	cp	1.4	2.0
		<i>Phasmarhabditis hermaphrodita</i>	sg	1.4	1.0
		<i>Alloionema appendiculatum</i> *†	fs	33.3	14.0
<i>Arion rufus</i> /A. <i>ater</i> hybrid	6	<i>Angiostoma</i> sp.*†	bm, cp, oe	16.7	14.0
		<i>Phasmarhabditis hermaphrodita</i>	mc, rs, sp	33.3	2.5
		<i>Alloionema appendiculatum</i> †	fs	18.6	56.0
<i>Arion vulgaris</i>	204	<i>Agfa flexilis</i> *†	cp, sg	0.5	2.0
		<i>Alloionema appendiculatum</i> †	fs	18.6	56.0
		<i>Angiostoma limacis</i> *†	cp, mc, sg	2.9	1.8
		<i>Angiostoma</i> sp.*†	bm, cp, oe	12.3	28.1
		<i>Phasmarhabditis hermaphrodita</i>	sg, in, rs, sp	13.2	3.9
<b>Limacidae</b>					
<i>Limax maximus</i>	16	<i>Agfa flexilis</i> †	sg	6.3	15.0
		<i>Angiostoma limacis</i> †	cp	6.3	4.0
<i>Limax cinereoniger</i>	1	–	–	–	–
<b>Milacidae</b>					
<i>Milax gagates</i>	7	–	–	–	–

bm, buccal mass; cp, crop; fs, foot sole; in, intestine; mc, mantle cavity; oe, oesophagus; rs, reproductive system; sg, salivary glands; sp, spermatheca.

\*New host association.

†First record in Norway.

In the current study, *A. flexilis* associated with *A. vulgaris* and *Limax maximus*, and was found to be present at three of the 32 sample sites. This is the first record of the presence of *A. flexilis* in Norway, as well as being the first time that it has been associated with *A. vulgaris*. Three species of the family Agfidae are known: *A. flexilis*, *Agfa morandi* and *Agfa tauricus* (Morand *et al.*, 2004). *Agfa morandi* is known by a single record in the French Pyrenees, while *A. tauricus* was found in several localities in the Crimea and Bulgaria (Ivanova *et al.*, 2013). However, *A. flexilis* has been found further afield in Europe, the

USA and Africa (Morand *et al.*, 2004; Ross *et al.*, 2010a, b, 2012). All three species parasitize limacid hosts; however, *A. tauricus* has also been recorded from agriolimacid slugs and a zonitid snail. All three nematodes are obligate parasites of molluscs and little is known about their life cycles, apart from their association with the salivary gland or genital tract (Morand *et al.*, 2004), albeit the localization of the nematodes in the reproductive tract of a mollusc has been subject to dispute (Ivanova *et al.*, 2013).

Two angiotomatids, *A. limacis* and *Angiostoma* sp., were identified. This is the first record of the presence of

Table 2. The partial 18S rRNA gene accession numbers of five nematode species from slugs in Norway with NCBI matches (EU573704, EU573707, EU573705, EU196008, DQ639980); ranges in identity and coverage are 97–100%.

Nematode family/species	GenBank no.	NCBI match	Source
<b>Family: Agfidae</b>			
<i>Agfa flexilis</i>	KC883636	<i>Agfa flexilis</i> ; EU573704	Ross <i>et al.</i> , 2010b
<b>Family: Alloionematidae</b>			
<i>Alloionema appendiculatum</i>	KC883638	<i>Alloionema appendiculatum</i> ; EU573707	Ross <i>et al.</i> , 2010b
<b>Family: Angiotomatidae</b>			
<i>Angiostoma limacis</i>	KC883639	<i>Angiostoma limacis</i> ; EU573705	Ross <i>et al.</i> , 2010b
<i>Angiostoma</i> sp.	KC883640	<i>Phasmarhabditis</i> sp. EM434; EU196008	Kiontke <i>et al.</i> , 2007
<b>Family: Rhabditidae</b>			
<i>Phasmarhabditis hermaphrodita</i>	KC883637	<i>Phasmarhabditis hermaphrodita</i> ; DQ639980	MacMillan <i>et al.</i> , 2006

*A. limacis* in Norway. Generally, its distribution is based on the presence of hosts from the Arionidae, and, rarely, the Agriolimacidae, family. The latter, representing the new species, is currently undergoing description. However, it is known to closely resemble *A. limacis* morphologically. The Angiostomatidae family has two known genera, *Angiostoma* with 17 species, and *Aulacnema*, which is monotypic. The latter genus is a parasite of a tropical terrestrial snail. Molluscan angiostomatids are generally obligate parasites of the intestine, hepatopancreas (Ivanova & Spiridonov, 2010) and oesophagus (Ross *et al.*, 2011). Currently, 13 angiostomatids have been described as possessing mollusc hosts, with eight of the former having been reported from Europe and the UK. The remaining four species have been isolated from the intestine and bronchi of amphibian and reptile hosts (Falcon-Ordaz *et al.*, 2008). Falcon-Ordaz *et al.* (2008) suggest that the salamander parasite *Angiostoma lamotheargumedoi* could have resulted from the host-switching event of a gastropod. Grewal *et al.* (2003) define angiostomatids as being parasites of vertebrates, using molluscs as obligate intermediate hosts. However, no angiostomatid species has yet been reported as being from both an invertebrate (supposedly intermediate) and a vertebrate (definitive) host. In both kinds of hosts, parasites have presented in their adult stages, thus showing the finalization of their development in a single host. A clear indication of the presence of separate, directly transmitted parasites has been found (Poulin, 2011), leading to the need for each case of invasion to be regarded separately. To date, the molecular affiliations of the nematode species from vertebrates are still unknown, thus preventing the resolution of the relationships between the two parts of the genus.

*Phasmarhabditis hermaphrodita* (Rhabditidae family) was also identified in the survey. Several genera within the Rhabditidae family, including *Rhabditis*, *Caenorhabditis* and *Phasmarhabditis*, are known to associate with slugs. However, *Phasmarhabditis* is the only genus that is considered to be truly parasitic towards slugs (Morand *et al.*, 2004). *Phasmarhabditis* spp. parasitize the mantle cavity of slugs, in close association with the shell, where present (in Limacoidea slugs). However, unlike the obligate parasites, *Agfa* and *Angiostoma*, *Phasmarhabditis* spp. are facultative parasites that are known to live on leaf litter and slug faeces (Tan & Grewal, 2001; MacMillan *et al.*, 2009). *Phasmarhabditis* spp. usually display a remarkably high level of pathogenicity against their hosts; however, for *A. vulgaris*, the nematode can only control slugs under the weight of 1 g, indicating that the nematode is only effective against juvenile slug stages (Speiser *et al.*, 2001; Grimm, 2002). However, this study has identified multiple new strains of *P. hermaphrodita*, whose pathogenicity, it is recommended, requires examination and testing against *A. vulgaris*.

Apart from the generalist nematode, *A. appendiculatum* (Laznik *et al.*, 2009), no specialist parasitic nematode of *A. vulgaris* has yet been recorded. A large number of such slugs that were collected by Ivanova (unpubl.) from the East Flanders province of Belgium were found to be free of infection, contrary to the state of many other local slug species. However, the results of the present study showed that, of all the slug species detected in Norway, nematode

diversity, prevalence and intensity was highest in *A. vulgaris* (table 1). The enemy-release hypothesis suggested an explanation for the success of an invasive species by the lack of its co-evolved natural enemies on the invaded territory (Torchin *et al.*, 2001; Ross *et al.*, 2010a). Although the parasite burden is high for *A. vulgaris* in Norway, studies conducted by Ross & Haukeland (unpubl.) have witnessed prevalence levels of 80–90% in the native range.

The role of parasites in the successful invasion of *A. vulgaris* has not been discussed in the current paper, because nematodes, such as angiostomatids, have little pathogenic impact on their hosts. Obviously, other traits of invasiveness, such as vigour and abundance of *A. vulgaris*, are to be considered as being of relatively high significance (Hatteland *et al.*, 2015). However, a tendency to be less affected by enemies is considered as being one of the few consistent characteristics of invasiveness (Colautti *et al.*, 2006). Whether *A. vulgaris* is the exception to the rule remains to be determined.

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### Conflict of interest

None.

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