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Nematodes associated with fig wasps

Bachelor thesis

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Annotation

Figs and their pollinating wasps engage in obligate mutualism. Their relationship is exploited by various antagonists, one of such parasitic group being nematodes. Main aim of this thesis is to review current literature on this topic and provide wider context. In the experimental part of my work, I attempted to screen fig wasp samples collected at tropical elevational gradient (Mt. Wilhelm, Papua New Guinea) using molecular methods for nematode presence, identification, and quantification.

Declaration

I hereby declare that I worked on this Bachelor thesis independently and used only the sources listed in the bibliography.

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České Budějovice, 2018

Kateřina Pužejová

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And last but not least, my gratitude belongs to all kind people who were around me and who supported me during the crucial time of writing this thesis.

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1. Aims of the thesis

A pilot aim of my thesis was to gather and review available literature related to nematodes associated with fig wasps. I tried to provide wider context for this issue, because majority of current studies focuses only on new species description (morphological characteristics and taxonomy systematic) leaving the ecological and evolutionary aspects behind. As any complex overview is still missing, first aim of my thesis was:

I) To review the current literature on nematodes in fig wasps: their diversity, host specificity and costs to fig wasps.

For an experimental part of my thesis I tried to screen several fig wasp samples for nematode presence and identification. Since my thesis experiment was part of a larger project focused on evolutionary issues of fig wasps assemblages, I worked with fig samples collected along a tropical elevational gradient. Most of these samples were in form of extracted DNA (for other analysis) therefore I used mainly molecular methods for nematode detections. Aims of the experimental part of my thesis were:

II) To detect and identify nematodes in fig wasps using molecular markers.

III) To quantify rates of nematode infection across the elevation gradient.

2. Review - Nematodes associated with fig wasps

2.1 Mutualism in insect-plant interactions

Plants and insects exist alongside for millions of years and during that time a wide range of interactions developed between them. These interactions scale from antagonistic; such as herbivory, when insects feed on plants causing them damage and plants fight back with various defences or opposite example of carnivorous plants, up to commensalism and mutualistic relationships; for instance providing shelter or food in trade for seed dispersal or pollination.

As an obligate mutualism is considered a relationship between two organisms in which reproduction of both partners depends on each other. And this is also the case of the close relationship between trees of the genus *Ficus* and their wasp pollinators. Their association, which includes also many other interactions, is well studied for many years and therefore can serve as a complex and unique model of mutualism (Herre, 1989; Bronstein, Alarcón and Geber, 2006; Jander and Herre, 2010)

2.2 Figs and fig wasps

2.2.1 Ficus spp. and its mutualism with wasps from family Agonidae

Ficus spp. is a woody plant from Moraceae family. With over 750 of described species belongs to the most numerous genera of flowering plants. It is divided into 4 subgenera and 18 sections and demonstrates pantropical distribution. Figs grow in forms of trees, shrubs or climbers and occupy wide range of tropical and subtropical forest biotopes.

One of the main characteristics of the genus is its unique type of fruit called syconium. It is a round shaped, almost enclosed inflorescence containing from tens to thousands (depending on the species) of individual unisexual flowers. Figs occur in both monoecious and dioecious breeding systems, thought lineage of dioecious fig species is distributed in the Old World only (Berg, 1989; Rønsted *et al.*, 2008).

Figs play very important role in the ecosystem food webs, because their trees fruit asynchronously and so provide valued nutrition source for high number of vertebrate frugivores throughout the year. These frugivores in return insure fig seed dispersal (Shanahan *et al.*, 2001)

Figs pollination is completely dependent on wasps from family Agonidae and simultaneously pollinator larvae can evolve only in their fig host. Hence we can truly speak about an obligate mutualism. The origin of their association is dated 75 million years ago. Such an ancient cohabitation provided plenty of time for common evolution, diversification, and adaptation, whose results can be observed today (Berg, 1989; Janzen, 1995; Cruaud *et al.*, 2012).

2.2.2 Life cycle figs and their pollinating fig wasps

Flowering figs release blend of volatiles that attract female wasps of their pollinating species (Grison-Pigé *et al.*, 2002; Chen *et al.*, 2009). One (or a few) pollinator foundress enters the syconium through a terminal pore called ostiole, which closes soon after the female entry, imprisoning her inside. Monoecious fig species produce uniform syconia with both male and female flowers. Foundress pollinates female fig flowers while laying eggs into some of them. For doing so she must insert her ovipositor down the flower style and reach its ovary.
As the flowers differ in the style length, pollinator can oviposit only those short-styled, leaving long-styled flowers to develop into seeds. After insuring of the reproduction for both fig and itself, the foundress dies inside the syconium. Wasp larvae form galls and feed on the endosperm of developing seeds. Few weeks later wingless males hatch out and mate females which are still in their galls. Then they start to bite escape holes in the fig wall, dying soon after that. When winged females eclose, they either actively or passively collect pollen, as they are looking for the escape holes. Then fly away searching for a new receptive fig, while abandoned ripe syconium waits for its frugivor to disperse seeds (Janzen, 1995; Weiblen, 2002; Cook and Rasplus, 2003; Borges, Bessière and Hossaert-McKey, 2008).

In dioecious fig the situation is a little bit different. Male trees produce syconia containing both male and female flowers, but as all of the female flowers are short-styled and so easily accessible, foundress oviposits all of them and therefore the male syconia give rise only to wasps. In contrast, female trees bear only syconia full of long-styled female flowers – future seeds (Cook and Rasplus, 2003; Borges, Bessière and Hossaert-McKey, 2008).

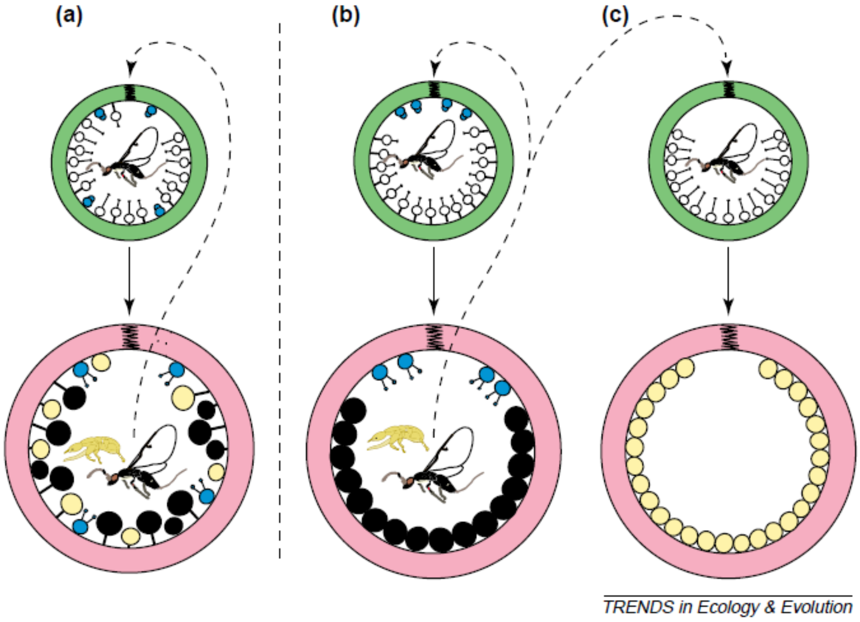

Figure 1: Difference between monoecious and dioecious figs; monoecious trees produce uniform syconia (a) containing both male (blue) and female flowers of different style length and give rise to both seeds (yellow) and wasps (black). Male syconia (b) of dioecious figs give rise to wasps, while female syconia (c) as they contain long styled flowers give rise to seeds only. Picture reproduced from (Cook and Rasplus, 2003).

2.2.3 Pollinating fig wasps and their coevolution with figs

Almost 400 species from 20 genera of pollinating fig wasps are described all belonging to the family Agonidae (Hymenoptera: Chalcidoidea), which seems to be monophyletic (Cook and Rasplus, 2003; Cruaud *et al.*, 2012).

Because only female disperse pollen, diversification of roles led to significant sexual dimorphism as well as shift in sex ratio in favour of females. Males are wingless and significantly smaller than females. They also have reduced eyes and antennae. Females are winged and have specifically flattened head with mandibular appendages, which helps her enter the syconium. Some species of pollinators are also equipped with coxal combs and actively collect pollen to their thoracic pockets. Pollinator females have very short lifespan (about 48 hours) and are proovigenic (once they are adult, all of their eggs are already mature) (Cook and Rasplus, 2003; Cruaud *et al.*, 2012).

Pollinating wasps are highly host specific. For a long time it was assumed that each one of the fig species associates with just single one specialised pollinator species. However, later studies suggest that this “one to one” rule might not be valid in almost one third of all cases. Although majority of figs is pollinated by single wasp species, it is quite often to find more than one wasp species hosted by one fig and in some cases also more of fig species share their pollinator (Rasplus, 1996; Weiblen *et al.*, 2002; Cook and Rasplus, 2003).

This close host specificity essentially led to coevolution and cospeciation among figs and their pollinators. Various adaptations generated by the cospeciation process can be observed in many aspects of this association (Wiebes, 1979).

Firstly, the indispensable synchronization of already mentioned life cycles and development of fig seeds and wasp larvae can be reminded. Further, a mixture of volatiles that attract female pollinators to receptive syconia is highly species specific. In view of her short lifespan, the pollinator female must be very effective in search and recognition of the right syconium to enter, as reproduction of both partners depend on its success (Grison-Pigé *et al.*, 2002; Chen *et al.*, 2009).

Body size of the pollinator female and shape of her head are also important, because depending on the species, figs vary in syconium size and ostiole diameter. If the pollinator wants to enter the syconium, she needs to squeeze through narrow and scaly ostiole, which therefore serves as a mechanical filter letting enter only well adapted wasps.

And once inside thy syconium also the length of the ovipositor comes in consideration as the pollinator needs to reach the ovary of the florets. There is a conflict in interests of fig and the pollinator, because both of them naturally want to maximize their offspring.

Although some discrepancies like host shifts and duplications of wasp lineage occurs, according to several studies, phylogenies of pollinators and figs (at least in some genera) almost perfectly fit one to another (Weiblen, 2004; Rønsted *et al.*, 2008).

2.3 Antagonists of the mutualism

Almost every kind of mutualistic relationship host its own antagonists – organisms which do not contribute to the common interest of the mutualists, but only exploit their partnership. Such antagonists exist as competitors, parasites or predators and may negatively influence fitness of their host. (Bronstein, 2001; Cook and Rasplus, 2003).

Nonetheless, antagonists always face a dilemma between the extent of exploiting the mutualism and endangering its stability. Over-exploited mutualisms can lead to extinction of all associated species.

In case of fig – fig wasps mutualism are well known and studied wasps which don’t pollinate figs and further parasites of fig wasps such as mites or nematodes.

2.3.1 Non pollinating fig wasps

These wasps are relatives of pollinators and together with them belong to the clade of Chalcidoidea. Their affiliation to several diverse lineages suggests that his type of parasitism arose independently several times (Heraty *et al.*, 2013; Borges, 2015). Non pollinating fig wasps have longer life span than pollinator and are synovigenic.

3-30 species of non pollinating fig wasps were recorded to associate with syconium of certain fig species (Compton and Hawkins, 1992; Compton, Rasplus and Ware, 1994).

They represent several different ecological approaches across at least three trophical levels. Most of the non pollinating wasps are gallers - either primary or secondary (inquilines). Further also seed eaters, hyper-parasites, and kleptoparasites occur.

Main differences are whether they enter the syconium or oviposit from the exterior.

From the external parasites we can distinguish group of wasps (both gallers and their parasitoids) which are significantly larger than pollinators and occur in few syconia, usually in low numbers. Another group includes small parasitic wasp (also gallers and their parasitoids). They occur in many syconia in medium-high numbers (Cook and Rasplus, 2003; Cook and Segar, 2010; Chen *et al.*, 2013).

Internal parasitic wasps occur only in few syconia, but when present, it is usually in high numbers. As these wasps need to enter the fig, convergence with pollinators in head shape can be observed. This type of parasites may have evolved from pollinator wasps by cheating (Noort and Compton, 1996; Zhao *et al.*, 2014).

2.3.2 Nematodes associated with fig syconia and fig wasps

In the case of fig-fig wasp association figures also a community of nematode parasites. They are much less studied then already mentioned non pollinating fig wasps. Most of the studies focus mainly on morphology and molecular description of individual species rather than on their role in the community structure or on their ecological and evolutionary effects on the fig – fig wasp association. Furthermore in last few years we are experiencing a boom in describing of new species. In table 1 an overview of described genera is shown.

The first records of observations of nematode infection in fig syconia come from Martin *et al.* (1973). They noticed that nematodes are very common in figs and that they occur in high numbers. Up to 50,000 nematode individuals were found in a single syconium. They also recognized more than 20 morphospecies, but these species were not further described (till 2015) (Martin, Owen and Way, 1973; Kanzaki *et al.*, 2015)

Since then, further observations were recorded from figs all over the world and also some studies focused on nematode descriptions, virulence rates and affects on the fig wasps were conducted.

All of the nematodes occupying fig syconia are phoretic – due to their own low mobility capability, they use pollinator fig wasps as transport to new figs. Their development therefore has to be well synchronised with the life cycle of figs and fig wasps and also their distribution is dependent on the pollinator (Krishnan *et al.*, 2010).

Therefore also certain degree of host specificity can be expected to find (especially in plant-parasitic species, eg. *Schistonchus*) and also probably a specific assemblage of nematodes exist for each fig species. It is still questioned how close this specificity actually is.

There were only few studies attempting to clarify the influence of nematodes on the fig-fig wasp mutualism. Some studies suggest that high rates of nematode infections may actually reduce pollinator offspring and dispersal abilities of pollinator females (Herre, 1995).

So far nematodes from several families, yet only few genera are described. In table 1 I attempted to summarize all known genera with number of described species and reference of introducing the genus as fig wasp associate (in some cases the genus was described earlier from other organisms).

Table 1: Genera of nematodes recorded from fig syconia.

Nematode family	**Described genera (number of species)**	**Year of description**
Aphelenchoididae:	*Schistonchus* (20)	1927
	Ficophagus (6)	2015
	Martininema (2)	2015
Diplogastridae	*Parasitodiplogaster* (16)	1979
	Teratodiplogaster (3)	2009
Anguinidae	*Ficotylus* (2)	2009
Parasitaphelenchidae	*Bursaphelenchus* (1)	2014

Best studied are definitely genera *Shistonchus* and *Parasitodiplogaster*, as they are also described and observed for longer time.

Schistonchus

Schistonchus is a genus of plant-parasitic nematodes from family Aphelenchoididae. According to latest taxonomy review, this genus seems to by polyphyletic, comprising of three groups.

Only mated *Schistonchus* females are carried by pollinating fig wasps. Once the female nematodes exit wasp body, they find male florets. They spent in there their whole life cycle, feeding on the plant tissue causing damage to them. Females of next generation leave the floret at the time when pollinator females emerge, disperse inside the syconium and actively attach to pollinator female body and enter its cavities (Giblin-Davis *et al.*, 1995; Kanzaki *et al.*, 2015).

In 2015 two new nematode species were separated from Schistonchus species (*Martininema* sp. and *Ficophagus* sp.)(Kanzaki *et al.*, 2015).

Parasitodiplogaster

Entomopathogenous *Parasitodiplogaster* belongs to family of Diplogastridae. Pollinating female wasps carry nematode juveniles (as the third stage - J3) into the fig syconium. Still inside the body of pollinator female, young nematodes molt into J4 stage and increase in size. After leaving the dead body of the foundress they molt to adults, mate and lay eggs. Next generation J3 infect young pollinator wasps (Giblin-davis *et al.*, 1995).

In 2009 new genus of *Teratodiplogaster* was separated from this genus (Kanzaki *et al.*, 2009).

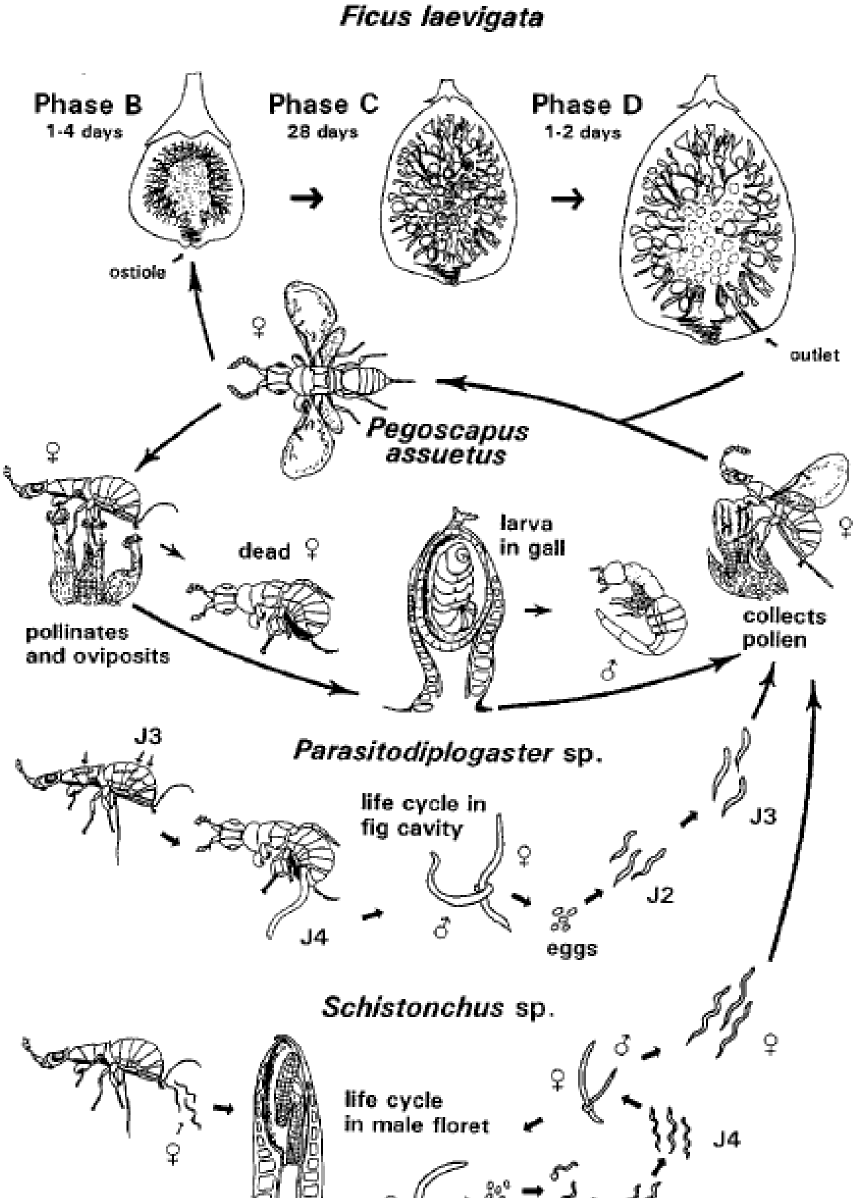

Figure 2: Description of life cycles of *Parasitodiplogaster* and *Schistonchus*. Picture reproduced from (Giblin-Davis *et al.*, 1995).

Ficotylus

First records of tylenchid nematodes associated with ficus sycons found in *Ficus congesta* in Australia. Yet it is not clear if they parasite an invertebrate host (fig wasp) or whether they are understory nematodes (Davies *et al.*, 2009; Giblin-Davis *et al.*, 2014).

Bursaphelenchus

In 2014 a new species of nematode (*Bursaphelenchus sycophilus*) from *Ficus variegata* was described, belonging to mostly mycophagus genus. It showed intriguing morphological convergent evolution with Schistonchus (Kanzaki *et al.*, 2014).

3. Experiment report

3.1 Introduction

Ecosystems are made up of highly complex and complicated networks and if we want to understand their structure, we must begin to uncover individual relationships first. Interactions within networks vary in their strength and direction, the first step is to identify and quantify all involved species. Later other ecological and environmental questions can be addressed.

The influence of antagonistic nematodes on fig-fig wasp interactions is still not well known. In this pilot study I attempted to detect and identify nematodes in DNA samples of fig wasp pollinators from a tropical elevational gradient using molecular methods. I also intended to explore whether the nematode infection rates differ across the gradient. The main aim for the experimental section of my thesis was:

II) To detect and identify nematodes in fig wasps using molecular markers.

III) To quantify rates of nematode infection across the elevation gradient.

3.2 Material and Methods

3.2.1 Study site and sampling design

Field sample collection was conducted on a previously established elevational gradient situated on the slopes of Mount Wilhelm (4, 509 m a.s.l.), the highest peak of Papua New Guinea. Along the transect six sites are placed 500 elevational meters apart, with the lowest station located at 200 m a.s.l. and highest at 2,700 m a.s.l.. Six highly abundant fig species present along the whole gradient were sampled. At each elevation and for each species, 10-15 near ripe figs were collected and placed into breathable plastic pots, wasps were allowed to emerge naturally (thus becoming infected with nematodes) and stored in 99% ethanol upon emergence.

DNA from some of the wasps was extracted and stored in freezer (-30°C). Fifty one samples from all six fig species (across the whole gradient) were screened for nematode presence.

Table 2: List of sites on the elevational gradient and sampled fig species.

Elevation (m a.s.l.)	**Site**	**abbreviation**	***Ficus* species**	**abbreviation**
200	Kausi	Kau	*F. afarkensis*	Afa
700	Numba	Num	*F. microdyctia*	Mic
1,200	Memeku	Mem	*F. Itoana*	Ito
1,700	Degenumbu	Deg	*F. Itoana-microdyctia* (hybrid)	Imi
2,200	Sinopass	Sin	*F. trichocerasa*	Tri
2,700	Bruno Sawmill	Bru	*F. wassa*	Was

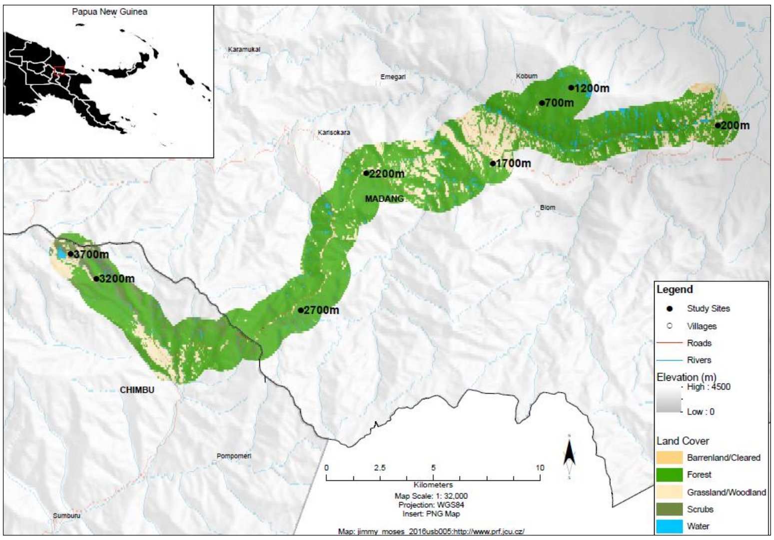

Figure 3: Map of the elevational gradient at Mt. Wilhelm, Papua New Guinea.

3.2.2 Molecular methods

3.2.2.1 PCR

At first, nematodes were detected in the fig wasp samples as part of routine barcoding using COI primers (Folmer *et al.*, 1994), see table 3 and 5.

Later, for targeted screening ‘nematode specific’ primers apparently suitable for detecting nematode DNA even in mixed samples were used (Floyd *et al.*, 2005). These primers targeted the 18S region of the small ribosomal subunit. PCR reactions were conducted in total volume of 25 µL and thermocycler conditions were set according to recommendation of the authors (table 3, 4 and 5).

Table 3: Used primers COI and 18S.

HC02198	5’ TAAACTTCAGGGTGACCAAAAAATCA 3’ (26 bases)
LCO1490	5’ GGTCAACAAATCATAAAGATATTGG 3’ (25 bases)
Nem_18S_F	5’ CGCGAATRGCTCATTACAACAGC 3’ (23 bases)
Nem_18S_R	3’ GGGCGGTATCTGATCGCC 5’(18 bases)

Table 4: Compounds of the PCR for 18S primers.

compound	**volume**
PPP MasterMix	12.5 µL
Forward primer	1µL
Reverse primer	1µL
Template DNA	1 µL
PCR Water	9.5 µL
	25 µL

Table 5: Thermocycler conditions.

			COI			18S	
			temperature	time		temperature	time
Initial denaturation			94°C	5min		94°C	5 min
Amplification	Denaturation	40 cycles	94°C	30 s	35 cycles	94°C	30 s
	Annealing of primers		50°C	30 s		54°C	30 s
	Extension		72°C	1 min		72°C	1 min
Final extension			72°C	7 min		72°C	10 min
Cooling			14°C	pause		22°C	pause

3.2.2.2 Gel electrophoresis

The yield of each PCR reaction was assessed by running PCR product on a 1% agarose gel stained with GelRed™ (in concentration 1:10 000) for 30 min and 120 V. A 100 bp ladder was used for approximate estimate and comparison of the length of amplified DNA fragments. Results of electrophoresis were visualised, pictures taken and the rest of the sample was stored in freezer with temperature of -30°C.

3.2.2.3 DNA sequencing and BLAST

A probe of 16 samples was sent to Macrogen commercial service for DNA Sanger sequencing. The DNA sequences were processed with Geneious 11.1.2. and compared with the GenBank nr (non-redundant) nucleotide database using BLAST (Basic Local Alignment Tool). A table of 10 closes hits (according to Bit-score and pairwise identity) for each sample was built.

Also sequences from the previous COI primers were included for this analysis.

3.2.2.4 Comparison of PCR product length using gel electrophoresis

Samples were run again on 2% gel for a longer time (60min, 90V) with aim to clearly separate products of similar lengths and compare them with already sequenced samples and so verify amplification of nematode DNA before sending samples for sequencing.

(see Results; figure 6 , table 10).

3.2.2.5 Temperature gradient during PCR primer annealing

In order to find optimal temperature for primer annealing and thus increasing their specificity, temperature gradient 54-58°C (7 wells per sample) during annealing phase of PCR was set. (see Results; figure 7, table 11)

3.2.3 Microdissections and optical microscopy

As an additional technique to the molecular methods, some of the wasps (preserved in ethanol) from the same syconia which showed nematode presence were placed under a stereo microscope (resolution 20x4,5) and visually checked for nematode presence. At first the wasp exterior was examined and then also the body was microdissected.

3.3 Results

3.3.1 Gel electrophoresis

Gel visualisation of the results showed that the majority of the samples run in PCR reactions were amplified. Lengths of the products and strengths of the bands were variable. Figure 4 shows six samples gained by use of 18S primers (table 6). All of these samples were later sent for sequencing (see Results, table 8) .Only sample in lane 5 significantly proved nematode presence.

Table 6: Gel electrophoresis of PCR products gained by use of 18S primers.

Lane	sample	sequence length (bp)	BLAST results	sequence quality
1	Mem Afa 9	160	-	very low (too short)
2	Kau Was 8	65	-	very low (too short)
3	Deg Afa 60	1012	wasp + possibly nematode	double peaks
4	Deg Was 75	905	possibly nematode (*Schistonchus*)	very low (weak peaks)
5	Deg Imi 6	996	nematode (*Ficotylus*)	clear
6	Deg Imi 009	976	wasp + possibly nematode	double peaks

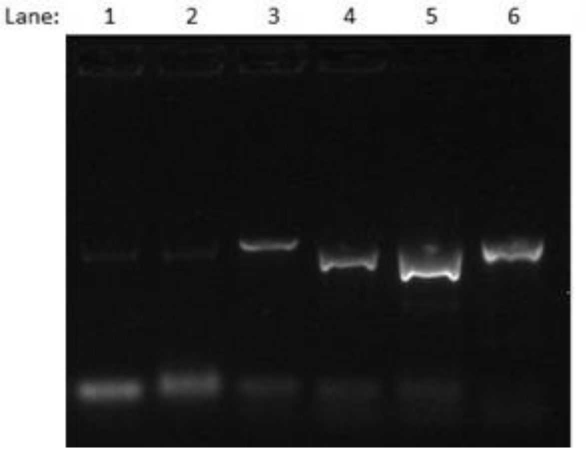

Figure 4: Gel electrophoresis of PCR products gained by use of 18S primers. Visualisation of six samples (Table 6) showing variability in PCR product lengths and band strengths.

3.3.2 DNA Sanger sequencing and BLAST

Previous use of COI primers provided nematode presence in eleven samples and suggests belonging to different families. Their closer taxonomic classification is however not reliable. Table 7 shows three closes hits (according to Bit-score) for each sample (see complete table of 10 closes hits in attachments, table12).

Table 7: Results of comparing COI sequences with GenBank database (using BLAST) showing 3 closest hits for each sample.

Sample	Organism	Pairwise Identity	Bit-Score	Taxonomy
BruMic 036	Pristionchus pacificus	93.2%	363,999	Chromadorea; Diplogasterida; Neodiplogasteridae
	Oscheius chongmingensis	91.8%	360,533	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
	Nematodirus oiratianus	91.8%	360,147	Chromadorea; Rhabditida; Strongylida; Trichostrongyloide
BruMic 038	Pristionchus pacificus	95.3%	358,992	Chromadorea; Diplogasterida; Neodiplogasteridae
	Oscheius chongmingensis	93.8%	355,91	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
	Phasmarhabditis sp.	94.8%	352,443	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
BruMic 045	Pristionchus pacificus	96.7%	364,77	Chromadorea; Diplogasterida; Neodiplogasteridae
	Oscheius chongmingensis	95.2%	361,303	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
	Nematodirus oiratianus	94.7%	360,918	Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea
DegWas 023	Nematoda sp.	86.5%	382,874	
	Ortleppascaris sp.	82.7%	382,104	Chromadorea; Ascaridida; Ascaridoidea; Ascarididae
	Steinernema feltiae	85.6%	381,333	Chromadorea; Rhabditida; Panagrolaimoidea; Steinernematidae
DegWas 075	Nematoda sp.	86.6%	374,015	
	Ortleppascaris sp.	84.7%	373,629	Chromadorea; Ascaridida; Ascaridoidea; Ascarididae
	Steinernema feltiae	85.6%	372,859	Chromadorea; Rhabditida; Panagrolaimoidea; Steinernematidae
NumArf 006	Parelaphostrongylus andersoni	92.2%	361,303	Chromadorea; Rhabditida; Strongylida; Metastrongyloidea
	Parelaphostrongylus andersoni	92.2%	361,303	Chromadorea; Rhabditida; Strongylida; Metastrongyloidea
	Parelaphostrongylus andersoni	92.2%	361,303	Chromadorea; Rhabditida; Strongylida; Metastrongyloidea
SnoMic 046	Pristionchus pacificus	95.4%	373,244	Chromadorea; Diplogasterida; Neodiplogasteridae
	Nematodirus oiratianus	94.0%	370,548	Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea
	Oscheius chongmingensis	94.0%	370,163	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
SnoMic 048	Pristionchus pacificus	94.9%	363,999	Chromadorea; Diplogasterida; Neodiplogasteridae
	Oscheius chongmingensis	93.5%	360,533	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
	Phasmarhabditis sp.	94.4%	356,681	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
SnoMic 049	Pristionchus pacificus	94.0%	364,385	Chromadorea; Diplogasterida; Neodiplogasteridae
	Oscheius chongmingensis	92.6%	361,303	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
	Phasmarhabditis sp.	95.7%	355,91	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
SnoMic 050	Necator sp.	95.8%	322,013	Chromadorea; Rhabditida; Strongylida; Ancylostomatoidea
	Phasmarhabditis sp.	96.8%	320,857	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
	Phasmarhabditis sp.	97.4%	320,857	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
SnoMic 051	Pristionchus pacificus	95.3%	364,385	Chromadorea; Diplogasterida; Neodiplogasteridae
	Oscheius chongmingensis	93.9%	361,303	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
	Phasmarhabditis sp.	94.9%	357,451	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
SnoMic 055	Pristionchus pacificus	95.7%	350,903	Chromadorea; Diplogasterida; Neodiplogasteridae
	Phasmarhabditis sp.	96.1%	344,354	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae
	Phasmarhabditis sp.	96.6%	344,354	Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae

From 16 samples sent to Macrogen (gained by the use of 18S primers), sequences were obtained, but only 6 of them were of high enough quality for further analysis. 7 samples showed multiple peaks which indicates the presence of multiple PCR templates (e.g. both fig wasp and nematode). The rest of the gained sequences were of very low quality or too short. Comparison of chromatograms of clear and double peaked sequence is shown in Figure 5.

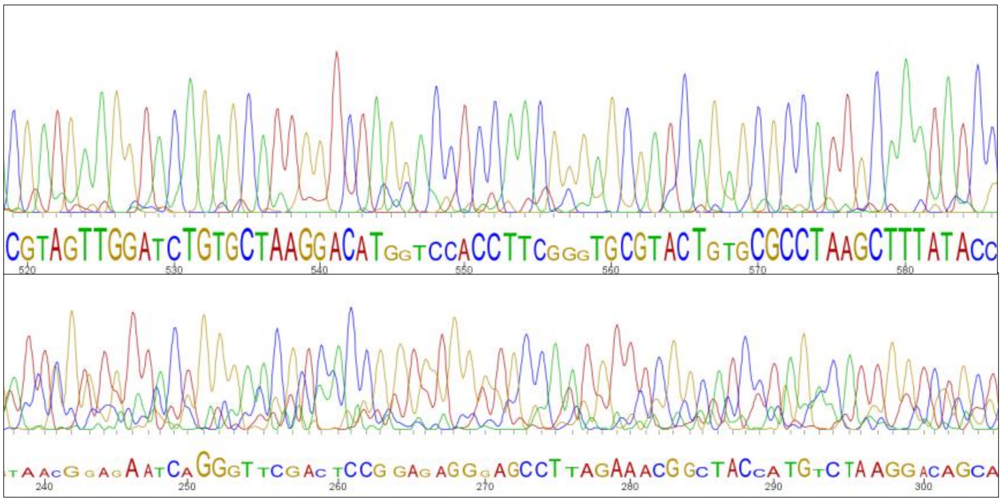

Figure 5: Visualisation of chromatogram of sequences in Geneious 11.1.2. Upper picture presents high quality sequence (DegImi 6), identified as *Ficotylus* sp.. Lower picture shows sequence (DegWas 23) with multiple peaks.

Table 8 shows three closes hits (similarly as for COI primers above) for each of high-quality sequences gained by the use of 18S primers.

It appeared that used 18S primers (Floyd *et al.*, 2005) were not enough specific for detecting nematodes in mixed samples. Five of six sequences contained amplified DNA of fig wasps (Chalcidoidea) and only one sequence (sample DegImi 6) clearly provided presence of nematode (*Schistonchus*, Tylenchida; see full record of 10 closes hits in attachments, table 13).

Table 8: Results of comparing 18S sequences with GenBank database (using BLAST) showing 3 closest hits for each sample.

Sample	Organism	Pairwise Identity	Bit-Score	Taxonomy
NumAfa 38	Rileya grisselli	96.7%	1557,85	Chalcidoidea; Eurytomidae; Rileyinae
	Callocleonymus sp.	96.7%	1557,85	Chalcidoidea; Pteromalidae; Cleonyminae
	Eupelmus sp.	96.6%	1550,46	Chalcidoidea; Eupelmidae; Eupelminae
DegImi 1	Callocleonymus sp.	97.8%	1576,31	Chalcidoidea; Pteromalidae; Cleonyminae
	Rileya grisselli	97.7%	1570,77	Chalcidoidea; Eurytomidae; Rileyinae
	Liepara sp.	97.6%	1565,23	Chalcidoidea; Pteromalidae; Diparinae
DegImi 001	Callocleonymus sp.	94.8%	1351,02	Chalcidoidea; Pteromalidae; Cleonyminae
	Rileya grisselli	94.7%	1345,48	Chalcidoidea; Eurytomidae; Rileyinae
	Liepara sp.	94.6%	1338,09	Chalcidoidea; Pteromalidae; Diparinae
DegAfa 51	Rileya grisselli	98.1%	1633,56	Chalcidoidea; Eurytomidae; Rileyinae
	Liepara sp.	98.0%	1628,02	Chalcidoidea; Pteromalidae; Diparinae
	Eupelmus sp.	97.9%	1622,48	Chalcidoidea; Eupelmidae; Eupelminae
DegImi 011	Callocleonymus sp.	96.4%	1533,84	Chalcidoidea; Pteromalidae; Cleonyminae
	Rileya grisselli	96.3%	1528,3	Chalcidoidea; Eurytomidae; Rileyinae
	Liepara sp.	96.2%	1522,76	Chalcidoidea; Pteromalidae; Diparinae
DegImi 6	**Ficotylus congestae**	99.0%	1038,94	Nematoda; Chromadorea; Tylenchida
	Uncultured nematode	89.2%	725,007	Nematoda; environmental samples
	Ditylenchus ferepolitor	89.1%	723,16	Nematoda; Chromadorea; Tylenchida

Sequences showing double peaks or low quality were also compared with the GenBank Database, but values that determine quality of sequences (HQ, pairwise identity, bit-score) were so low that these data cannot be considered as reliable. Nevertheless, in four samples certain level of match with several nematode species (which are associated with fig wasps) was found (Table 9).

Table 9: Results of comparison of low quality sequences with GenBank Database.

Sample	HQ%	BLAST results
Deg Was 75	0,4	nematode - *Schistonchus*
Deg Arf 76	0,1	nematode - *Ficotylus*
Deg Was 23	0,6	nematode - *Schistonchus*
Deg Imi 2	0,8	nematode - *Acrostichus*

3.3.3 Comparison of PCR product length using gel electrophoresis

Running samples on 2% gel for longer time confirmed that lengths of PCR products from 18S primers are variable, but did not separate similar lengths significantly enough so it could be used to reliably distinguish samples with amplified nematode DNA (Table 10, Figure 6).

Table 10: Comparison of PCR product lengths via gel electrophoresis.

lane	sample	sequence quality	BLAST results
1	Ladder	-	-
2	Num Ito 48	-	-
3	Num Ito 40	-	-
4	Kau Was 25	-	-
5	Kau Afa 10	-	-
6	Deg Imi 2	double peaks	nematode (Acrostichus)
7	Deg Imi 001	clear	fig wasp (Chalcidoidea)
8	Deg Imi 1	clear	fig wasp (Chalcidoidea)
9	Deg Afa 76	double peaks	nematode (Ficotylus)
10	Deg Was 23	double peaks	nematode (*Schistonchus*)
11	Deg Afa 60	double peaks	fig wasp (Chalcidoidea)
12	Deg Was 75	weak peaks	nematode (*Schistonchus*)
13	Deg Imi 6	clear	nematode (*Ficotylus*)
14	Deg Imi 009	double peaks	fig wasp (Chalcidoidea)

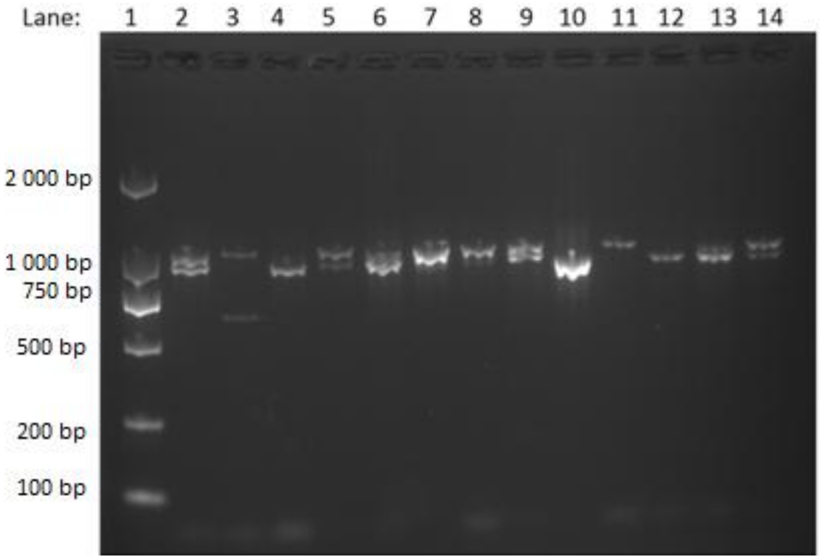

Figure 6: Comparison of PCR products length via gel electrophoresis. (The "double vision" effect is probably caused by combination of using deeper wells and longer time for the electrophoresis causing change of the angle of view.)

3.3.4 Temperature gradient during PCR primer annealing

Use of temperature gradient during annealing phase of PCR didn’t show any change in specificity of used primers (would be probably observed as change of product length or band strength, vanishing of band, etc.), but only decrease in the amount of amplified DNA with rising temperature (Table 11, Figure 7).

Table 11: Use of temperature gradient during primer annealing.

sample		sequence quality	BLAST results
1	Deg Imi 9	double peaks	fig wasp (Chalcidoidea)
2	Deg Imi 6	clear	nematode (*Ficotylus*)
3	Num Ito 40	not sequenced	-
4	Deg Was 75	double peaks	nematode (*Schistonchus*)
5	Deg Was 23	double peaks	nematode (*Schistonchus*)

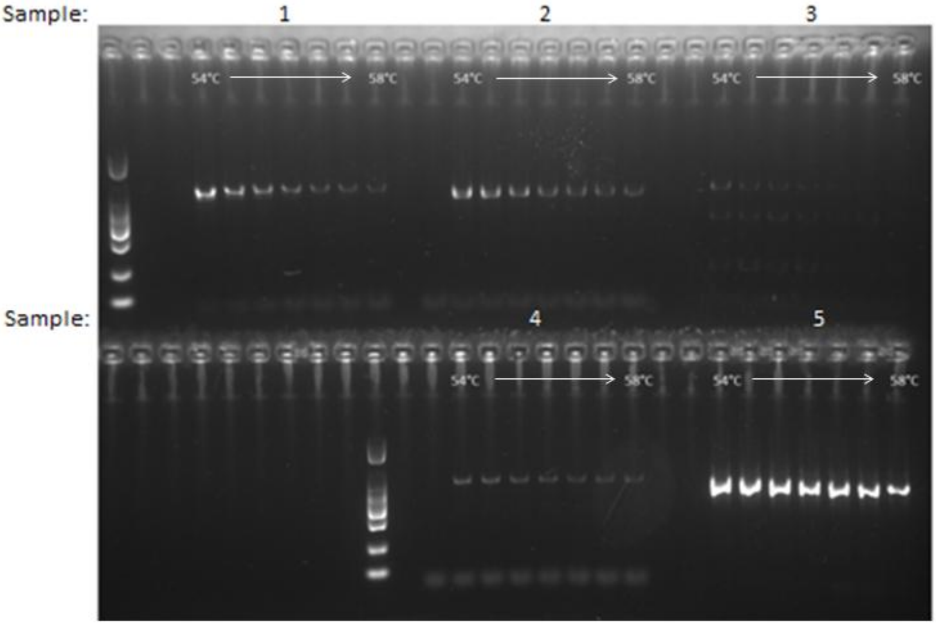

Figure 7: Use of temperature gradient during primer annealing.

3.3.5 Microdissections and optical microscopy

Wasp microdissections and light microscopy didn’t lead to finding of any nematode parasites.

3.4 Discussion

During the experimental part of my work, I had to face several complications. The main one issue was insufficient specificity of used primers which led to amplification of wasp DNA in most of the samples. Because of that I had to primarily focus on adjusting and modifications of used methods.

As the length of amplified 18S fragments was similar for both wasps and nematodes, using the gel electrophoresis did not help to significantly separate them and so clearly prove nematode presence in samples. Neither did the amending of annealing temperature lead to change of primer specificity.

Use of CO I primers revealed nematode presence in fig wasp samples, but didn’t provide their closer classification.

Using 18S primers I succeeded in the identification of nematodes belonging to different families in five samples, unfortunately the quality of most of the acquired sequences wasn’t high enough for relevant analysis. Only one clear nematode sequence was gained.

For insufficient number of nematode sequences, it was not possible to meet the third aim of the thesis which was to compare rate of nematode infection across the elevational gradient.

For future study I can see two main ways to follow:

The first one would be an improvement and use of other molecular methods for getting clear nematode DNA sequences. I would suggest finding or designing more specific primers targeting different genes. Next alternative might be use of blocking primers that would prevent fig wasp DNA from amplifying during PCR. Other but probably more expensive possibility would be to send all samples for next generation sequencing and so get complete information about the sample identity. Also using cloning comes to consideration in order to clearly separate wasp and nematode DNA in mixed samples.

The other option would be to focus on nematode detection at the very beginning of the study – during field collecting. Hand-picking of the living nematodes from fresh fig samples would provide sufficient amount of clear DNA material for further molecular analysis and moreover also possibility for morphological observations.

3.5 Conclusion

In order to detect nematodes in fig wasp samples, molecular methods were used. COI primers revealed nematode presence in fig wasp samples, but didn’t offer their closer identification. 18S primers showed to be not sufficiently specific to supply relevant results for further analysis. Unfortunately I wasn’t able to fulfil the aim of quantification of nematode infection rates across the elevational gradient due to insufficient amount of clear nematode sequences.

The used methods are still to be improved and tested and also some suggestions for future direction of the study were settled.

4. Summary

In my bachelor thesis I have been dealing with complex assemblage of interactions built on fig-fig wasp mutualism with focus on its nematode antagonists. I firstly provided general overview of current literature on nematodes associated with wasps.

Secondly, in the experimental part of my work, I attempted to screen fig wasp samples for nematode presence and identity using molecular markers (with aim to compare nematode infection rates across the elevational gradient). Further study of the issue and optimization of used methods are to be done.

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6. Attachments

Table 12: Results of comparing COI sequences with GenBank database (using BLAST) showing 10 closest hits for each sample.

Sample	**Organism**	**Identical Sites**	**Pairwise Identity**	**Accession**	**Bit-Score**	**E Value**	**Taxonomy (**Eukaryota; Metazoa; Ecdysozoa)
BruMic036	Pristionchus pacificus	93.2%	93.2%	YP_004300493	363,999	2,18E-121	Nematoda; Chromadorea; Diplogasterida; Neodiplogasteridae
	Oscheius chongmingensis	91.8%	91.8%	AJW75166	360,533	6,42E-120	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Nematodirus oiratianus	91.8%	91.8%	YP_009050223	360,147	7,72E-120	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Molineidae
	Phasmarhabditis sp.	95.7%	95.7%	ARX95143	355,525	2,81E-122	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Phasmarhabditis sp.	95.2%	95.2%	ART85725	354,369	5,38E-122	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Oswaldocruzia chambrieri	93.8%	93.8%	AMS36804	350,517	3,32E-120	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Molineidae
	Parelaphostrongylus andersoni	89.1%	89.1%	ABS89266	348,591	4,93E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Cyathostominae sp.	94.3%	94.3%	ANW09532	348,206	1,53E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; Cyathostominae
	Enochrus ater	94.7%	94.7%	SNU46046	347,436	3,37E-119	Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Holometabola; Coleoptera; Polyphaga; Staphyliniformia; Hydrophilidae; Hydrophilinae
	Ortleppascaris sinensis	92.4%	92.4%	AKP17095	346,665	6,89E-119	Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Ascarididae
BruMic038	Pristionchus pacificus	95.3%	95.3%	YP_004300493	358,992	2,66E-119	Nematoda; Chromadorea; Diplogasterida; Neodiplogasteridae
	Oscheius chongmingensis	93.8%	93.8%	AJW75166	355,91	4,21E-118	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Phasmarhabditis sp.	94.8%	94.8%	ARX95143	352,443	5,05E-121	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Parelaphostrongylus tenuis	89.0%	89.0%	ABR57316	346,665	1E-117	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Phasmarhabditis sp.	94.2%	94.2%	ART85725	345,51	1,62E-118	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Parelaphostrongylus andersoni	91.5%	91.5%	ABS89264	345,125	1,29E-117	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	91.5%	91.5%	ABS89266	345,125	1,08E-117	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Caenorhabditis brenneri	92.9%	92.9%	ACD61691	344,739	1,31E-117	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Peloderinae
	Oswaldocruzia chambrieri	92.9%	92.9%	AMS36804	344,739	6,03E-118	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Molineidae
	Heligmosomoides polygyrus	94.1%	94.1%	ABH10082	343,584	8,19E-118	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Heligmosomatidae

BruMic045	Pristionchus pacificus	96.7%	96.7%	YP_004300493	364,77	1,31E-121	Nematoda; Chromadorea; Diplogasterida; Neodiplogasteridae
	Oscheius chongmingensis	95.2%	95.2%	AJW75166	361,303	2,49E-120	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Nematodirus oiratianus	94.7%	94.7%	YP_009050223	360,918	3,69E-120	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Molineidae
	Phasmarhabditis sp.	96.6%	96.6%	ARX95143	351,673	1,01E-120	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Parelaphostrongylus andersoni	92.8%	92.8%	ABS89264	350,132	1,43E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	92.8%	92.8%	ABS89266	350,132	1,31E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Caenorhabditis brenneri	94.3%	94.3%	ACD61691	350,132	9,59E-120	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Peloderinae
	Parelaphostrongylus andersoni	92.8%	92.8%	ABS89258	349,747	2,26E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	92.8%	92.8%	ABS89261	349,747	2,41E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Toxocara canis	92.8%	92.8%	AGT99521	349,362	9,77E-120	Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Toxocaridae
DegWas023	Nematoda sp.	86.5%	86.5%	AGT20151	382,874	5,34E-133	Eukaryota; Metazoa; Ecdysozoa; Nematoda
	Ortleppascaris sp.	82.7%	82.7%	AFY06693	382,104	1,19E-132	Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Ascarididae
	Steinernema feltiae	85.6%	85.6%	AFD53225	381,333	1,68E-132	Nematoda; Chromadorea; Rhabditida; Panagrolaimoidea; Steinernematidae
	Steinernema feltiae	85.6%	85.6%	AFD53227	381,333	1,42E-132	Nematoda; Chromadorea; Rhabditida; Panagrolaimoidea; Steinernematidae
	Steinernema feltiae	85.6%	85.6%	AFD53229	381,333	1,57E-132	Nematoda; Chromadorea; Rhabditida; Panagrolaimoidea; Steinernematidae
	Steinernema feltiae	85.6%	85.6%	AFD53230	380,948	2,34E-132	Nematoda; Chromadorea; Rhabditida; Panagrolaimoidea; Steinernematidae
	Steinernema feltiae	85.5%	85.5%	AFD53245	379,407	8,09E-132	Nematoda; Chromadorea; Rhabditida; Panagrolaimoidea; Steinernematidae
	Steinernema sp.	85.1%	85.1%	AGN29995	379,407	7,54E-132	Nematoda; Chromadorea; Rhabditida; Panagrolaimoidea; Steinernematidae
	Ortleppascaris sinensis	85.4%	85.4%	AKP17095	378,637	1,33E-131	Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Ascarididae
	Monhystrella parvella	84.7%	84.7%	AIC32931	376,711	7,87E-131	Nematoda; Chromadorea; Monhysterida; Monhysteridae
DegWas075	Nematoda sp.	86.6%	86.6%	AGT20151	374,015	1,89E-129	Nematoda
	Ortleppascaris sp.	84.7%	84.7%	AFY06693	373,629	3,13E-129	Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Ascarididae
	Steinernema feltiae	85.6%	85.6%	AFD53225	372,859	5,12E-129	Nematoda; Chromadorea; Rhabditida; Panagrolaimoidea; Steinernematidae
	Steinernema feltiae	85.6%	85.6%	AFD53230	372,474	7,44E-129	Nematoda; Chromadorea; Rhabditida; Panagrolaimoidea; Steinernematidae
	Heterorhabditis bacteriophora	85.6%	85.6%	AEX97050	372,089	7,29E-129	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Heterorhabditidae
	Contracaecum sp.	84.7%	84.7%	AJC50694	372,089	4,93E-129	Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Anisakidae
	Monhystrella parvella	84.4%	84.4%	AIC32932	370,548	2,45E-128	Nematoda; Chromadorea; Monhysterida; Monhysteridae
	Contracaecum sp.	84.2%	84.2%	AJC50696	370,548	2,14E-128	Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Anisakidae
	Ortleppascaris sinensis	85.6%	85.6%	AKP17095	370,163	3,25E-128	Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Ascarididae
	Contracaecum sp.	84.2%	84.2%	AJC50691	369,777	4,71E-128	Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Anisakidae

NumArf006	Parelaphostrongylus andersoni	92.2%	92.2%	ABS89257	361,303	1,17E-123	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	92.2%	92.2%	ABS89263	361,303	1,44E-123	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	92.2%	92.2%	ABS89266	361,303	5,92E-124	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	92.2%	92.2%	ABS89258	360,918	9,04E-124	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	92.2%	92.2%	ABS89259	360,918	1,44E-123	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	92.2%	92.2%	ABS89261	360,918	1,1E-123	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	92.2%	92.2%	ABS89265	360,918	1,4E-123	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	92.2%	92.2%	ABS89269	360,918	1,37E-123	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	92.2%	92.2%	ABS89268	360,533	1,3E-123	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Phasmarhabditis sp.	93.9%	93.9%	ARX95143	359,762	7,02E-124	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
SnoMic046	Pristionchus pacificus	95.4%	95.4%	YP_004300493	373,244	5,35E-125	Nematoda; Chromadorea; Diplogasterida; Neodiplogasteridae
	Nematodirus oiratianus	94.0%	94.0%	YP_009050223	370,548	7,95E-124	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Molineidae
	Oscheius chongmingensis	94.0%	94.0%	AJW75166	370,163	9,56E-124	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Nematodirus spathiger	93.5%	93.5%	YP_009050211	368,622	4,02E-123	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Molineidae
	Phasmarhabditis sp.	95.8%	95.8%	ARX95143	362,844	3,9E-125	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Parelaphostrongylus andersoni	91.7%	91.7%	ABS89266	359,377	2,28E-123	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	91.7%	91.7%	ABS89261	358,992	5,59E-123	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	91.7%	91.7%	ABS89258	358,607	6,58E-123	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Parelaphostrongylus andersoni	91.7%	91.7%	ABS89268	358,607	7,12E-123	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Caenorhabditis brenneri	92.6%	92.6%	ACD61691	357,836	7,31E-123	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Peloderinae
SnoMic048	Pristionchus pacificus	94.9%	94.9%	YP_004300493	363,999	2,91E-121	Nematoda; Chromadorea; Diplogasterida; Neodiplogasteridae
	Oscheius chongmingensis	93.5%	93.5%	AJW75166	360,533	4,75E-120	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Phasmarhabditis sp.	94.4%	94.4%	ARX95143	356,681	9,63E-123	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Phasmarhabditis sp.	95.2%	95.2%	ART85725	355,14	2,1E-122	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Oswaldocruzia chambrieri	93.8%	93.8%	AMS36804	351,673	9,89E-121	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Molineidae
	Cyathostominae sp.	94.3%	94.3%	ANW09532	349,747	4,18E-120	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; Cyathostominae
	Enochrus ater	94.7%	94.7%	SNU46046	348,591	1,33E-119	Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Holometabola; Coleoptera; Polyphaga; Staphyliniformia; Hydrophilidae; Hydrophilinae
	Ortleppascaris sinensis	92.4%	92.4%	AKP17095	348,206	1,59E-119	Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Ascarididae
	Trichostrongylus axei	94.7%	94.7%	ADN53251	347,821	2,38E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Trichostrongylidae; Trichostrongylinae
	Murshidia longicaudata	93.8%	93.8%	AET95731	347,821	2,33E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; Cyathostominae

SnoMic049	Pristionchus pacificus	94.0%	94.0%	YP_004300493	364,385	1,96E-121	Nematoda; Chromadorea; Diplogasterida; Neodiplogasteridae
	Oscheius chongmingensis	92.6%	92.6%	AJW75166	361,303	3,31E-120	Eukaryota; Metazoa; Ecdysozoa; Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae; Oscheius
	Phasmarhabditis sp.	95.7%	95.7%	ARX95143	355,91	2,07E-122	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Phasmarhabditis sp.	95.2%	95.2%	ART85725	354,369	4,37E-122	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Oswaldocruzia chambrieri	93.8%	93.8%	AMS36804	349,747	5,32E-120	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Molineidae
	Cyathostominae sp.	94.3%	94.3%	ANW09532	349,747	4,64E-120	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; Cyathostominae
	Parelaphostrongylus andersoni	90.3%	90.3%	ABS89266	349,362	2,45E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Metastrongyloidea; Protostrongylidae; Elaphostrongylinae
	Enochrus ater	94.7%	94.7%	SNU46046	348,591	1,33E-119	Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Holometabola; Coleoptera; Polyphaga; Staphyliniformia; Hydrophilidae; Hydrophilinae
	Murshidia longicaudata	93.8%	93.8%	AET95731	347,821	2,68E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; Cyathostominae
	Trichostrongylus axei	94.7%	94.7%	ADN53251	347,436	3,44E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Trichostrongylidae; Trichostrongylinae
SnoMic050	Necator sp.	95.8%	95.8%	BAW02953	322,013	1,2E-107	Nematoda; Chromadorea; Rhabditida; Strongylida; Ancylostomatoidea; Ancylostomatidae; Bunostominae
	Phasmarhabditis sp.	96.8%	96.8%	ART85725	320,857	8,35E-109	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Phasmarhabditis sp.	97.4%	97.4%	ARX95143	320,857	1,54E-108	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Oswaldocruzia chambrieri	95.8%	95.8%	AMS36804	318,931	8,65E-108	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Molineidae
	Necator americanus	96.3%	96.3%	ANA52016	318,931	3,02E-108	Nematoda; Chromadorea; Rhabditida; Strongylida; Ancylostomatoidea; Ancylostomatidae; Bunostominae
	Heligmosomoides polygyrus	94.7%	94.7%	ABH10082	316,62	3,83E-107	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Heligmosomatidae
	Murshidia longicaudata	96.3%	96.3%	AET95731	316,62	4,42E-107	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; Cyathostominae
	Enochrus ater	96.8%	96.8%	SNU46046	316,235	7E-107	Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Holometabola; Coleoptera; Polyphaga; Staphyliniformia; Hydrophilidae; Hydrophilinae;
	Murshidia linstowi	95.7%	95.7%	AET95867	315,849	6,63E-107	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; Cyathostominae
	Murshidia longicaudata	95.7%	95.7%	AET95727	315,464	1,24E-106	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; Cyathostominae
SnoMic051	Pristionchus pacificus	95.3%	95.3%	YP_004300493	364,385	2E-121	Nematoda; Chromadorea; Diplogasterida; Neodiplogasteridae
	Oscheius chongmingensis	93.9%	93.9%	AJW75166	361,303	3,46E-120	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Phasmarhabditis sp.	94.9%	94.9%	ARX95143	357,451	4,7E-123	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Phasmarhabditis sp.	95.2%	95.2%	ART85725	355,525	1,52E-122	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Oswaldocruzia chambrieri	93.0%	93.0%	AMS36804	352,058	8,08E-121	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Molineidae
	Cyathostominae sp.	93.4%	93.4%	ANW09532	350,517	1,89E-120	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; Cyathostominae
	Enochrus ater	93.9%	93.9%	SNU46046	349,362	6,59E-120	Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Holometabola; Coleoptera; Polyphaga; Staphyliniformia; Hydrophilidae; Hydrophilinae
	Trichostrongylus axei	93.8%	93.8%	ADN53251	348,206	1,82E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Trichostrongylidae; Trichostrongylinae
	Murshidia longicaudata	92.9%	92.9%	AET95731	348,206	1,45E-119	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; Cyathostominae
	cf. Panagrolaimidae	92.5%	92.5%	AHK25084	348,206	1,93E-119	Nematoda; Chromadorea; Rhabditida; unclassified Rhabditida

SnoMic055	Pristionchus pacificus	95.7%	95.7%	YP_004300493	350,903	3,65E-116	Nematoda; Chromadorea; Diplogasterida; Neodiplogasteridae
	Phasmarhabditis sp.	96.1%	96.1%	ART85725	344,354	4,62E-118	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Phasmarhabditis sp.	96.6%	96.6%	ARX95143	344,354	7,86E-118	Nematoda; Chromadorea; Rhabditida; Rhabditoidea; Rhabditidae; Rhabditinae
	Oswaldocruzia chambrieri	94.6%	94.6%	AMS36804	338,961	1,26E-115	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Molineidae
	Cyathostominae sp.	95.0%	95.0%	ANW09532	337,035	4,89E-115	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; unclassified Cyathostominae
	Murshidia longicaudata	95.0%	95.0%	AET95731	336,265	7,75E-115	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; Cyathostominae
	Heligmosomoides polygyrus	94.1%	94.1%	ABH10082	335,88	8,23E-115	Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Heligmosomatidae
	Enochrus ater	95.5%	95.5%	SNU46046	335,88	1,24E-114	Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Holometabola; Coleoptera; Polyphaga; Staphyliniformia; Hydrophilidae; Hydrophilinae
	Ortleppascaris sinensis	93.1%	93.1%	AKP17095	335,495	1,89E-114	Nematoda; Chromadorea; Ascaridida; Ascaridoidea; Ascarididae
	Murshidia linstowi	94.5%	94.5%	AET95867	335,495	1,4E-114	Nematoda; Chromadorea; Rhabditida; Strongylida; Strongyloidea; Strongylidae; Cyathostominae

Table 13: Results of comparing 18S sequence (sample DegImi6) with GenBank database (using BLAST) showing 10 closest hits.

Sample	**Organism**	**Identical Sites**	**Pairwise Identity**	**Accession**	**Bit-Score**	**Taxonomy (**Eukaryota; Metazoa; Ecdysozoa)
DegImi6	**Ficotylus congestae**	99.0%	99.0%	EU018049	1038,94	Nematoda; Chromadorea; Tylenchida; Hexatylina; Sphaerularioidea; Neotylenchidae; Ficotylus
	Uncultured nematode	89.2%	89.2%	JN049686	725,007	Nematoda; environmental samples
	Ditylenchus ferepolitor	89.1%	89.1%	KJ636374	723,16	Nematoda; Chromadorea; Tylenchida; Tylenchina; Tylenchoidea; Anguinidae; Anguininae; Ditylenchus
	Tylenchidae sp.	89.0%	89.0%	JX291139	717,62	Nematoda; Chromadorea; Tylenchida; Tylenchina; Tylenchoidea; Tylenchidae; unclassified Tylenchidae
	Uncultured nematode	88.7%	88.7%	JN049687	708,387	Nematoda; environmental samples
	Ditylenchus sp.	88.5%	88.6%	KJ636302	704,694	Nematoda; Chromadorea; Tylenchida; Tylenchina; Tylenchoidea; Anguinidae; Anguininae; Ditylenchus
	Ditylenchus sp.	88.5%	88.5%	AY284637	702,847	Nematoda; Chromadorea; Tylenchida; Tylenchina; Tylenchoidea; Anguinidae; Anguininae; Ditylenchus
	Ditylenchus weischeri	88.5%	88.6%	MG383954	702,847	Ecdysozoa; Nematoda; Chromadorea; Tylenchida; Tylenchina; Tylenchoidea; Anguinidae; Anguininae; Ditylenchus
	Ditylenchus dipsaci	88.5%	88.5%	MG434348	701,001	Nematoda; Chromadorea; Tylenchida; Tylenchina; Tylenchoidea; Anguinidae; Anguininae; Ditylenchus
	Ditylenchus dipsaci	88.5%	88.5%	MG434349	701,001	Nematoda; Chromadorea; Tylenchida; Tylenchina; Tylenchoidea; Anguinidae; Anguininae; Ditylenchus