Characterization of small RNAs and differential chromatin amplification in the germline of the parasitic nematode family Strongyloididae

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Summary

The two nematode sister-genera *Strongyloides* and *Parastrongyloides* form the Strongyloididae family of nematodes. This nematode family is of particular interest because it contains important parasites of medical and veterinary relevance. Species of this family have a complex biphasic life-cycle, which has the advantage of alternating easily accessible, sexually reproducing free-living generations with parasitic generations. Therefore, the Strongyloididae offer unique opportunies to investigate not only the evolution and basic biology of these intestinal parasites, but also to investigate antihelminthic treatments. In this thesis I am presenting the results of my work on three Strongyloididae species: *S. ratti* (infecting rats), *S. papillosus* (infecting sheep) and *P. trichosuri* (infecting Australian possums). I worked on two main aspects of the Strongyloididae biology:

1.) Together with colleagues, I investigated differential chromatin amplification in the gonads and compared the chromosomal content of sperm of three Strongyloididae species. We could show that autosomes are present in higher copy numbers than X chromosomes in a population of giant nuclei with a very high DNA content, located in the distal parts of the gonads of free-living adults of all three species. Quantitative RNA sequencing showed that autosomal genes are higher expressed than X chromosomal ones in these cells, implying that differential chromatin amplification serves as a mechanism for the regulation of gene expression. Further we showed that *S. ratti*, but not *S. papillosus*, produces genetically male determining (nullo-X) sperm, although the free-living generations of both species do not produce any surviving male progeny.

2.) I sequenced and analyzed the small RNA classes of two different developmental stages of the three Strongyloididae. For comparison, I also re-sequenced the sRNAs of the two free-living model nematodes *Caenorhabditis elegans* and *Pristionchus pacificus*. Recent studies had shown considerable variation in the sRNAs among nematodes, but no Strongyloididae species or close relative had been included in these studies. Therefore, I established a sRNA sequencing protocol. I identified conserved and taxon specific micro RNAs of which many are differentially regulated between the stages. I found that Strongyloididae have lost the highly conserved class of piRNAs, but possess a novel class of ∼27 nucleotide long RNAs, starting with 5'G or A. These 27GA RNAs appear to have triphosphates at their 5' ends and are therefore presumably synthesized by RNA dependent RNA polymerases without 5' processing. The 27GA RNAs have the potential to target transposable elements and endogenous genes. Finally, I showed that no parasite-derived sRNAs circulate in the blood of *S. ratti*-infected rats at detectable levels.

Zusammenfassung

Die zwei Schwestergattungen *Strongyloides* und *Parastrongyloides* repräsentieren die Nematodenfamilie Strongyloididae. Diese Nemtodenfamilie ist von besonderem Interesse, da sie wichtige Parasiten von human- und tiermedizinischer Relevanz enthält. Spezies dieser Familie haben einen biphasischen Lebenszyklus, der den Vorteil hat, dass sich einfach zu handhabende, sexuell reproduzierende und freilebende Generationen mit eher schwierig zu handhabenden parasitischen Generationen abwechseln. Daher bieten die Strongyloididae Arten einzigartige Möglichkeiten, nicht nur zur Untersuchung der Evolution und Grundlagenbiologie dieser Darmparasiten, sondern sie ermöglichen auch die Erforschung von Antihelminthika. Meine Arbeit konzentriert sich auf zwei Aspekte der Biologie der Strongyloididae Arten *S. ratti* (infiziert Ratten), *S. papillosus* (infiziert Schafe) and *P. trichosuri* (infiziert australische Possums):

1.) Gemeinsam mit Kollegen habe ich die differentielle Amplifizierung von Chromatin in den Gonaden von drei Strongyloididae Arten untersucht und habe den chromosomalen Inhalt ihrer Spermien spezifiziert. Wir konnten zeigen, dass in der Keimbahn dieser Würmer, speziell den sogenannten "giant nuclei", Autosome in höherer Kopienzahl als X Chromosome vorliegen. Durch quantitative RNA Sequenzierung konnten wir zusätzlich zeigen, dass auotosmale Gene höher exprimiert sind als X-chromosomale Gene, was darauf hindeutet, dass die differentielle Chromatin Amplifikation dem Zweck dient, die Genexpression zu regulieren. Weiterhin haben wir gezeigt, dass *S. ratti*, aber nicht *S. papillosus*, männchenproduzierende (nullo-X) Spermien produziert, obwohl keine der beiden Spezies überlebende männlichen Nachkommen in der freilebenden Generation zeugt.

2.) Ich habe kleine RNA Klassen in zwei Entwicklungsstadien der Strongyloididae sequenziert und analysiert. Zum Vergleich habe ich die kleinen RNAs der freilebenden Modellnematoden, *C. elegans* und *P. pacificus*, resequenziert. Neueste Studien haben gezeigt, dass nicht-kodierende, kleine RNA Klassen innerhalb der Nematoden stark variieren, jedoch war keine Strongyloididae Art Teil dieser Studien. Daher habe ich ein Protokoll für die sRNA Sequenzierung etabliert und konservierte und taxonspezifische micro RNAs identifiziert, von denen viele zwischen den Stadien differentiell exprimiert sind. Ich habe herausgefunden, dass die ansonsten hochkonservierte Klasse der piRNAs nicht vorhanden ist, dafür aber eine neue Klasse an sRNAs, mit einer Länge von ∼27 Nukleotiden und 5'G oder A. Diese 27GA RNAs scheinen Triphosphate an ihren 5' Enden zu haben und werden daher mutmaßlich von RNA abhängigen RNA Polymerasen synthetisiert. Die 27GA RNAs haben das Potential Transposons und Gene zu regulieren und können damit möglicherweise den Verlust der piRNAs kompensieren. Abschließend konnte ich keine zirkulierenden parasitischen sRNAs im Blut von Wirten nachweisen.

List of publications

Teile der vorliegenden Arbeit wurden bereits veröffentlicht:

Differential chromatin amplification and chromosome complements in the germline of Strongyloididae (Nematoda).
Kulkarni A, Holz A, Rödelsperger C, Harbecke D, Streit A.
Chromosoma. 2016 Mar;125(1):125-36. doi: 10.1007/s00412-015-0532-y. Epub 2015 Jul 24.

Gain and loss of small RNA classes - characterization of small RNAs in the parasitic nematode family Strongyloididae.
Anja Holz and Adrian Streit.
Genome Biol Evol. 2017 Sep; doi: 10.1093/gbe/evx197.

Introduction

1.1 The phylum Nematoda

Representatives of the phylum Nematoda (roundworms) exhibit a wide variety of vastly different lifestyles. Many species of nematodes live as free-living species in terrestrial, marine or fresh-water environments. Those free-living species feed on bacteria, fungi and other nematodes that inhabit the same environmental niche. Other nematodes live as parasites, infecting all kinds of animals and plants through different mechanisms (Lee, 2002; Perry and Wharton, 2011). There are more than 23,000 nematode species that have been described so far and estimates suggest that over a million nematode species exist on earth (Lambshead and Boucher, 2003). Not only do the habitats differ remarkably, but also the reproductive modes across the nematode clades vary, with gonochoristic, hermaphroditic and parthenogenetically reproducing species (Pires-daSilva, 2007). The diversity of lifestyles and reproduction modes must have arisen from evolutionary adaptations in conserved and novel molecular, developmental and physiological processes and it is very interesting to study these processes by comparing them in nematodes of different taxa, in particular with respect to adaptations to parasitism.

The survival of adverse environmental conditions like food deprivation, temperature extremes, dehydration and osmotic stress is essential to ensure the survival of nematode populations. For example free-living nematodes have to cope with environmental fluctuations (e.g. drought, seasonal changes) and parasitic species have to survive outside of their hosts for a period of time, locating the next suitable host, where they face changes in pH and temperature and food deprivation. Nematodes have the ability to endure these adverse conditions by entering a persistent (dormant) stage, the so-called Dauer stage. All nematodes develop through four moulting larval stages (L1-L4) from embryos to adult worms. At the beginning

of their lives embryos reside inside an eggshell and depending on the species of nematode, the L1 or L2 larva hatches from the egg continuing their development through L3 and L4 stages into adult worms (Lee, 2002; Perry and Wharton, 2011).

The phylum Nematoda can be divided into five major clades (Figure 1.1) based on sequence similarity of the small subunit ribosomal DNA sequences (Blaxter et al., 1998). Relatively basal to the phylum nematoda are the clades I and II including vertebrate parasitic, insect parasitic, plant parasitic and free-living orders. Representatives of clade II differ from all other nematodes in their structure of the nervous system (Malakhov, 1994) and division patterns during early embryogenesis (Malakhov, 1994; Schierenberg, 2005; Voronov et al., 1998). Clade III groups vertebrate and arthropod-parasitic taxa from the orders Ascaridida (gut roundworms like *Ascaris* spp.), Spirurida (filarial nematodes like *Brugia* spp.), Oxyurida (pinworms like *Enterobius* spp.) and Rhigonematida (millipede gut parasites like *Rhigonema* spp.). Clade IV contains the free-living bacteriovore families Cephalobidae and Panagrolaimidae, the entomopathogenic genus Steinernema, the plant parasitic order of Tylenchida (*Globodera* spp. and *Meloidogyne* spp.) and animal parasitic species of the family Strongyloididae (*Strongyloides* spp.). In clade V the suborder Rhabditina, which includes the famous model nematode *Caenorhabditis elegans*, and the vertebrate-parasitic order Strongylida (including hookworms) group together with the entomopathogenic genus *Heterorhabditis* and the order Diplogasterida (including *Pristionchus pacificus*). Interestingly, the distal clades III-V have relatively high nucleotide substitution rates which is why nematode identification at species level is possible on basis of the SSU rDNA gene in these clades (Holterman et al., 2006). The acceleration of nucleotide substitutions could be caused by two factors: an exposure to free radicals due to increased metabolic rates and defense mechanisms of hosts or an accumulation of DNA replication errors due to shorter generation times (Gillooly et al., 2005). See Figure 1.1 for an overview of the five major clades of nematodes.

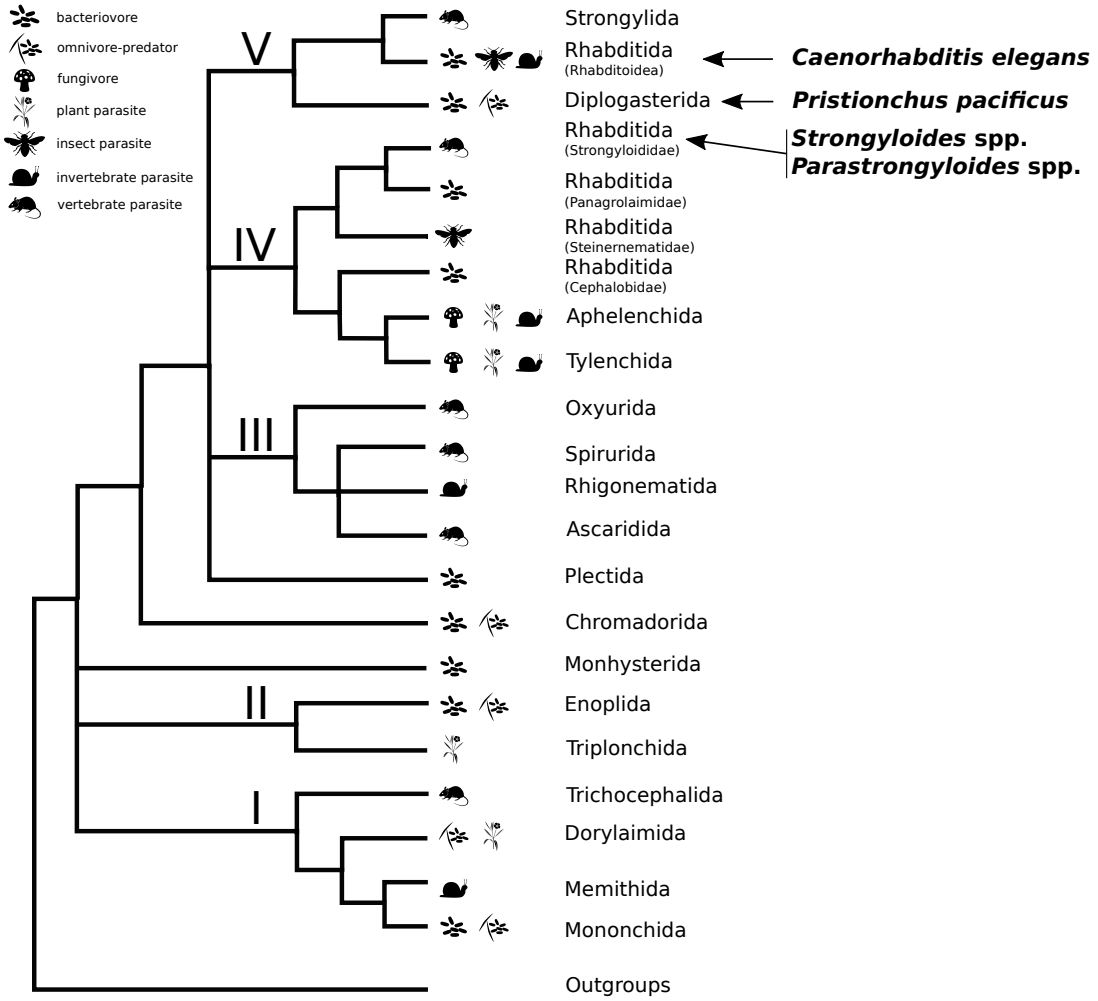

FIGURE 1.1: **Phylogeny of the phylum Nematoda**. Adapted from Blaxter et al. (1998). Dendrogram summarizing the results of the phylogenetic analysis of SSU sequences from 53 nematode species. The 5 clades are indicated as roman numerals and the trophic ecologies of the taxa are represented by symbols. The species/family of interest for this thesis are indicated with arrows. Note that the order of Rhabditida is found to be a paraphyletic.

1.2 Parasitic nematodes

Almost all animals and terrestrial plants can be infected with at least one species of parasitic nematodes, resulting in a great impact on crop yields, livestock production and of course impairment of human health (Lee, 2002; Perry and Wharton, 2011). Phylogenetic analyses of free-living and parasitic nematodes (Blaxter et al., 1998; Holterman et al., 2006) have shown that parasitism in the Nematoda must have evolved at least seven times independently (Blaxter, 2011; Blaxter et al.,

1998; Dorris et al., 1999). Others proposed up to 18 or 20 independent events of aquisition of parasitism in nematodes (Blaxter and Koutsovoulos, 2014).

For all parasitic nematodes, shifing to the next host is crucial for the survival of the population. The life-cycles can be cyclical, involving an intermediate host like arthropods, molluscs or other mammals, or involving only a single host species. Most parasitic nematodes have a special infective stage during their life-cycles (usually the L1 and/or L3 larval stage for animal parasites) that can be free-living or inside an intermediate host (Perry and Wharton, 2011). During this stage the larvae can endure a certain amount of time under adverse environmental conditions, waiting for the opportunity to infect the next definitive host.

Although it was originally postulated that parasitic species have smaller genomes than their free-living relatives, there is no general trend to genome size reduction and when changes in genome sizes are found, it usually comes with smaller intron sizes, shorter intergenic distances and less repeat regions (Blaxter and Koutsovoulos, 2014; Hunt et al., 2016). Many examples of horizontal gene transfers can be found in parasitic species, such as cellulolytic enzymes in plant parasitic nematodes, which enable the worms to degrade the cell-walls of their host plants. These enzymes originate from fungi and bacteria, which form part of the rhizosphere (Blaxter and Koutsovoulos, 2014). Similar enzymes have also been identified in the beetle associated *Pristionchus pacificus* (Mayer et al., 2011) and the free-living model organism *Caenorhabditis elegans* (Parkinson and Blaxter, 2003). Therefore horizontal gene transfer is not restricted to parasitic nematode species but also happens in free-living species. In animal parasitic species, horizontal gene transfer is not as abundant as in plant parasitic species but can still be found for example in species of the Onchocercidae, like *Brugia malayi*, as a copy of a bacterial ferrochelatase (involved in the synthesis of haem), suggesting an additional need of haem in these tissue and blood parasites (Elsworth et al., 2011).

Not only can horizontal gene transfer enable parasitic nematodes to acquire novel traits and abilities to interact with their hosts, it can also result in the acquisition of different symbiotic bacteria. For example the entomopathogenic genera *Heterorhabditis* (Baiet al.2013) and *Steinernema* (Goodrich-Blair,2007) share a strategy to kill newly invaded insect larvae by utilizing specific bacterial symbionts (*Photorhabdus* and *Xenorhabdus*). Another example is the symbiosis between *Wolbachia* symbionts and filarial nematodes of the family Onchocercidae.

It is hypothesized that *Wolbachia* assists the nematodes in evading the hosts' immune response and supporting the worms metabolically (Darby et al., 2012). By killing the *Wolbachia* with antibiotics, the nematodes are affected through loss of fecundity and high mortality (Bandi et al., 1999).

The understanding of the mechanisms by which parasitic nematodes can locate and invade their hosts and avoid host immunity and the comprehension of the evolutionary origins of plant and animal parasitism, are not only important for basic research, but also are of great medical, veterinary and agricultural interest.

1.2.1 Human-pathogenic nematodes

Diseases caused by parasitic nematodes are a great medical and economical burden throughout the world. Poor communities in the developing regions of the world have a high prevalence and are most badly affected. These diseases can cause severe disfigurement (lymphatic filariasis), blindness (onchocerciasis) and impairment of the nutritional status in particular in children (soil-transmitted helminthiasis) (Hotez et al., 2008).

It is a major goal of the World Health Organization (WHO) to combat and control Neglected Tropical Diseases (NTDs), which include several classes of diseases caused by parasitic nematodes. Guidelines and strategies have been developed in order to reduce this burden and in some cases even to eradicate the diseases (WHO, 2017b). Although there are cases like dracunculiasis, where eradication of the disease is almost achieved, it remains an important task to find new ways of preventing and treating the other parasitic diseases caused by nematodes.

Several antihelminthic drugs exist for the treatment of nematode infections, most importantly albendazole, mebendazole and ivermectin. Although these drugs are well established and still working fine, there are concerns that their small number will not be sufficient and resistances could develop, as it is already the case in veterinary medicine (Hotez et al., 2008). Therefore, improving the understanding of parasite biology is very important to develop novel targets and treatments.

Out of the 20 listed NTDs defined by the WHO, four are caused by parasitic nematodes (WHO, 2017e):
Dracunculiasis, also known as Guinea-worm disease, is caused by the species *Dracunculus medinensis*, the largest of skin tissue residing parasites of humans

(females can reach a length of 60-80cm). The infection occurs when people drink water that is contaminated with parasite-carrying copepods of the genus *Cyclops*. The L3 larvae are released in the stomach and migrate through the intestinal wall and develop into adult females and males. After mating the males die in the tissue and females migrate to the extremities (usually lower legs). The female worms emerge through the skin causing a burning sensation and affected people typically try to relieve the pain by immersing the area in water. The worm then releases thousands of L1 larvae into the water, where they are ingested by copepods again and develop into L3 larvae. Over the course of the last 20 years dracunculiasis has been almost eradicated and only 25 human cases were reported in 2016 (WHO, 2017c).

Lymphatic filariasis, also known as elephantiasis, is mainly caused by three species of filarial nematodes: *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. Lymphatic filariasis is endemic to tropical and subtropical regions of the world and an estimated number of 120 million people are infected worldwide (WHO, 2017d). Infective L3 larvae are transferred from human to human via the blood-feeding intermediate hosts, mosquito species of the family Culicidae (*Anopheles* spp., *Aedes* spp. and *Culex* spp.). The adult female and male worms accumulate in the lymphatic system, specifically the lymph nodes. After mating, embryonic L1 larvae (microfilariae) are released and migrate through the lymphatic vessels to the heart and lungs, where they enter the bloodstream. The circulating microfilariae can then be taken up by a mosquito, where they develop into infective L3 larvae. The majority of infected people are asymptomatic, but the infection can cause a variety of symptoms, including lymphoedema of the limbs and swelling of the scrotum or vulva, leaving the patients severely disfigured (Wenk and Renz, 2003). In communities at risk, elimination efforts are mainly based on deworming by mass drug administration with albendazole and ivermectin. This is supplemented with measures to reduce the density of mosquito vectors (Hotez et al., 2008; WHO, 2017d).

Onchocerciasis, also called river blindness, is caused by the filarial nematode *Onchocerca volvulus*. Onchocerciasis is mainly endemic in regions of West and Central Africa but also in northern parts of South America. In 2015 about 12 million individuals were treated against onchocerciasis worldwide (WHO, 2017f). *O. volvulus* larvae are transferred from one person to another by black flies of the family Simuliidae. Onchocerciasis is always linked to a proximity to rivers

because black flies only breed in fast-flowing rivers. After infection with L3 larvae during a bite from an infected fly, the larvae migrate subcutaneously, developing into adult females and males. The adult females mate with males and reside in subcutaneous fibrous nodules, thereby avoiding strong effects of the host's immune system. Embryonic L1 microfilariae exit the nodules and migrate through the skin and eyes, where they cause an inflammatory response, leading to depigmentation (so called leopard skin) and causing blindness through clouding of the cornea. Migrating microfilariae can enter a black fly during a blood meal and develop into L3 larvae in the flight muscle, completing the life-cycle (Wenk and Renz, 2003). From the 1970s until 2002 the Onchocerciasis Control Progamme (OCP) was in effect, slowly achieving a vector control with insecticides and since 1995 the African Programme for Onchocerciasis Control (APOC) is supplementing the success of the OCP by treating affected communities with ivermectin (WHO, 2017a).

Soil-transmitted helminthiasis is a disease class caused by several different species of intestinal nematodes. Soil-transmitted helminthiasis is the most prevalent disease caused by parasitic nematodes, affecting almost 25% of the world's population and 880 million children are in need of treatment, according to latest estimates (WHO, 2017g). Causing agents are mainly the parasitic nematode families Ancylostomatidae (*Necator americanus* and *Ancylostoma duodenale*), Ascaridae (*Ascaris lumbricoides*), Trichuridae (*Trichuris trichiura*) and Strongyloididae (*Strongyloides stercoralis*). All of these parasitic nematode families are intestinal parasites and infections don't require an intermediate host but can occur either passively through ingestion of eggs or actively through skin-penetration of L3 larvae in the soil. People with light infections usually have no symptoms but the infection causes nutritional impairment with an impact on growth and physical development especially in children. For control of soil-transmitted helminth infections, people living in endemic areas receive periodic treatments mainly with albendazole or mebendazole (WHO, 2017g) and the improvement of sanitary conditions is a key step in reducing infections and worm load.

1.2.2 Research on human-pathogenic nematodes

Studying the basic biology of human-pathogenic nematodes is quite troublesome and thorough research requires "alternative" hosts or model systems in order to be able to investigate the entire life-cycles and biology without having to infect

humans. There are a few examples of human-pathogenic nematodes that can be maintained under laboratory conditions in alternative hosts for their full life-cycle. Two examples are *Brugia malayi*, which can be maintained in mongolian gerbils and mosquitoes (Ash and Riley, 1970) and *Strongyloides stercoralis*, which can be maintained in dogs (Schad et al., 1989) or mongolian gerbils (Nolan et al., 1993). But for most of the human-pathogenic nematodes the maintenance under laboratory conditions for their entire life-cycles is impossible or not established yet. Single larval stages, or parts of the life-cycle can sometimes be maintained in vitro, but the removal of worms from their hosts and culturing them in vitro causes changes in transcriptional profiles (Ballesteros et al., 2016) and may have strong influences on their physiology and drug response. Another possibility is the usage of animal parasitic nematode species, Which are closely related to human-pathogenic nematodes, as model systems to study their basic biology and infection cycles.

1.3 The nematode family Strongyloididae

The 2 nematode sister-genera *Strongyloides* and *Parastrongyloides* form the Strongyloididae family which groups with the clade IV nematodes (Blaxter et al., 1998; Dorris et al., 2002). *Strongyloides* spp. are obligate intestinal parasites of vertebrates which can undergo facultative single non-parasitic generations with males and females between parthenogenetic parasitic generations (Viney and Lok, 2015). *Parastrongyloides* spp. are facultative intestinal parasites of various marsupials with their best-studied representative, *Parastrongyloides trichosuri*, parasitizing the Australian brushtail possum *Trichosurus vulpecula* (Grant et al., 2006b). The infective 3rd stage larvae (iL3) of both genera invade the host percutaneously and after a pulmonary passage (in *S. ratti* through the mucosa of the nose), the final development to adult worms occurs in the small intestine. The exact mechanism and route by which the iL3s reach the small intestine is not clear, although migration with the bloodstream and/or through the lymph vessels is most likely (Grove, 1996). For a detailed description of the general life-cycle and differences between the species important for this thesis, see Section 1.3.4 below.

In this thesis I am presenting the results of my work on 3 Strongyloididae species: *S. ratti*, *S. papillosus* and *P. trichosuri*. Their phylogenetic relationship is shown

in Figure 1.2 along with their natural hosts and their chromosomal complements in the 2 sexes.

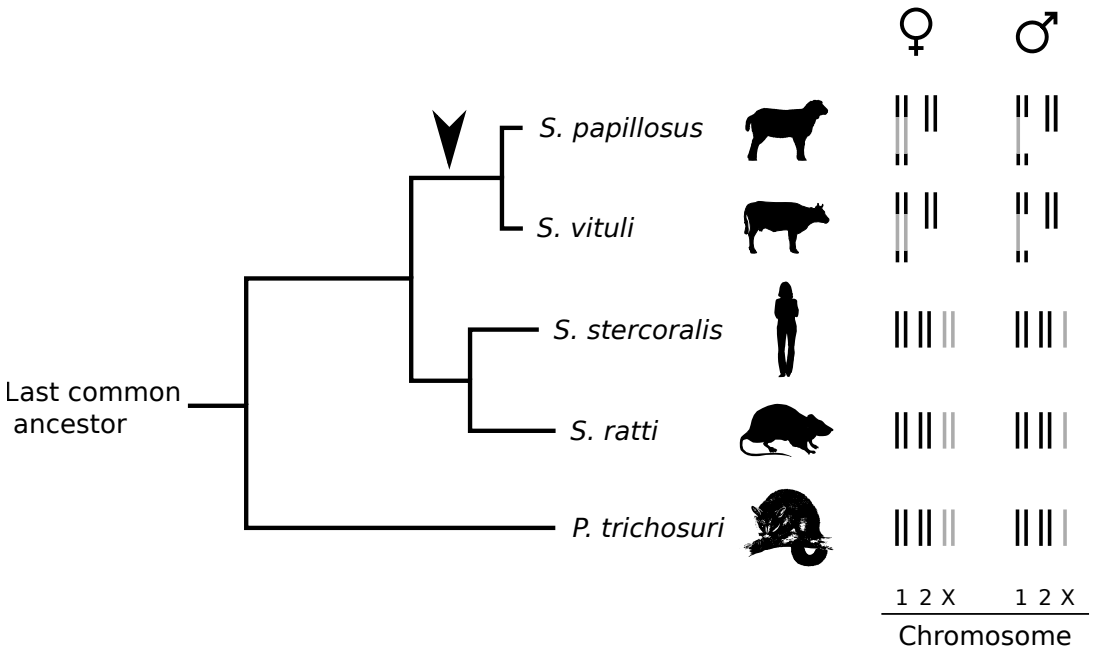

FIGURE 1.2: **Phylogeny of the family Strongyloididae**. Adapted from Kulkarni et al. (2013). Phylogenetic relationship, natural hosts and chromosome complements are shown for the 5 species indicated. Genomic regions present in two copies in both sexes (autosomal) are depicted in black, genomic regions present in two copies in females and in only one copy in males are depicted in grey (X). The arrowhead indicates the postulated X chromosome to chromosome 1 fusion, which led to the reduction of the haploid chromosome number from three to two in *S. papillosus* and *S. vituli*.

1.3.1 Historical remarks on Strongyloididae

Historically, *S. papillosus* was the first species of the Strongyloididae to be described by Wedl as *Trichosoma papillosum* in 1856 as a parasite of sheep (Wedl, 1856). However, it took more than 50 years until it was recognized that this species belongs to the genus of *Strongyloides* when *S. stercoralis* had already been established as type species (Ransom, 1911). In the meantime, *S. stercoralis* infective larvae had first been described by the french physician Louis Normand in 1876. He found the worms in the feces of soldiers returning from Vietnam and Arthur Bavay named them *Anguillula stercoralis* (Bavay, 1876; Grove, 1996). Due to the vastly different morphologies and lifestyles of the free-living and parasitic generations, the infective larvae and free-living adults in the feces were thought to belong

to another species than the intestinal parasitic worms (Grove, 1996). In 1883 the German parasitologist Rudolf Leuckart realized that these different worms were different stages of a single species (Leuckart, 1883) and in 1902 Stiles and Hassal proposed to rename the species to its final name *Strongyloides stercoralis* (Stiles and Hassal, 1902). By now more than 50 species of the genus *Strongyloides* are known to infect a wide variety of vertebrates (see Grove (1989, 1996) for a comprehensive list and references).

The genus *Parastrongyloides* was first described in 1928 by Morgan (1928) as parasites of moles and shrews and since then several species have been described, primarily as parasites of Australian marsupials (Mackerras, 1959).

1.3.2 *Strongyloides* spp. causing strongyloidiasis

The human-pathogenic *S. stercoralis*, and to a much lesser extent, *S. fuelleborni fuelleborni* and *S. fuelleborni kelleyi* cause strongyloidiasis (Nutman, 2017), an important chronic intestinal disease with an estimated prevalence of 30 - 100 million affected people worldwide, endemic in developing regions of Asia, Africa and Latin America (Hotez et al., 2008). It is reasonable to assume that the prevalence is underestimated due to biased sampling and screening methods of epidemiological studies (Bisoffi et al., 2013). Strongyloidiasis is included in the disease class of soil-transmitted helminthiasis, one of the NTDs defined by the WHO (see Section 1.2.1). Randomized clinical trials have been carried out, showing that ivermectin is best suited to treat strongyloidiasis and that a single dose of $200\frac{\mu g}{kg}$ for two days is highly effective and therefore the treatment of choice (Marti et al., 1996; Nutman, 2017; Suputtamongkol et al., 2011).

In contrast to other nematodes causing soil-transmitted helminthiasis, *S. stercoralis* is able to maintain an autoinfection cycle within a single host which can lead to decade long persistence in a single individual. *S. stercoralis* eggs already hatch in the intestine where they can develop into infective larvae and either directly penetrate the gut wall or the perianal skin (Grove, 1996). This is the reason why an infection can persist life-long, when no treatment occurs. Usually host-parasite interactions lead to a controlled infection that is asymptomatic in otherwise healthy patients. The mild symptoms of acute strongyloidiasis follow the route of infection with urticaria (rash) at the site of skin penetration, followed by dry cough and then gastrointestinal symptoms like diarrhoea, abdominal pain and constipation

(Keiser and Nutman, 2004). Chronic strongyloidiasis is mostly asymptomatic and can persist for decades, but patients may show subclinical symptoms like peripheral eosinophilia or elevated IgE levels (Rossi et al., 1993).

However, when the host's immune system is weakened, the asymptomatic chronic infection can develop into a hyperinfection syndrome, whereby the worms multiply and disseminate throughout many different tissues of the body, causing severe symptoms and death, if not properly treated. The occurrence of hyperinfection or disseminated strongyloidiasis is associated with administration of immunosuppressive drugs like corticosteroids in the context of organ transplantation, HTLV-1 infection (Human T-cell lymphotropic virus type 1) and HIV (Nutman, 2017). The medical relevance of strongyloidiasis had been underestimated for a long time but *S. stercoralis* is now increasingly acknowledged as an important pathogen in poor communities with unsatisfactory sanitary conditions.

1.3.3 Strongyloididae in wild and domestic animals

More than 50 species of *Strongyloides* have been described, infecting a wide variety of vertebrates (Grove, 1989, 1996). *Strongyloides* spp. can infect livestock such as cattle (*S. vituli*), swine (*S. ransomi*), horses (*S. westeri*) and sheep and goats (*S. papillosus*). Apart from domestic animals, *Strongyloides* spp. can also infect a wide variety of wild animals, from amphibians like frogs and toads to birds like herons and ducks, as well as wild mammals such as foxes and rats (Grove, 1989, 1996).

Parastrongyloides spp. parasitize various marsupials, with their best-studied representative, *Parastrongyloides trichosuri*, parasitizing Australian brushtail possums (Grant et al., 2006b). Several species have been described, primarily as parasites of Australian marsupials (Mackerras, 1959).

1.3.4 The biphasic life-cycle in the family Strongyloididae

All members of the family Strongyloididae share a complex life-cycle (Grove, 1996; Viney and Lok, 2015). The biphasic life-cycle alternates between free-living and parasitic cycles and is not common among parasitic nematodes (Figure 1.3). In all Strongyloididae, the parasitic worms reside in the small intestine and produce

eggs that are transported to the environment with the feces. To my knowledge, the only species among the Strongyloididae whose L1 larvae have been reported to hatch from embryonated eggs within the host's gut are *S. stercoralis* and *S. ratti*. For all other species investigated, the L1 larvae hatch outside of the host in the environment only. Therefore the autoinfection cycle and the potentially fatal disseminated strongyloidiasis described in Section 1.3.2 is a special characteristic of *S. stercoralis* infections in humans (Grove, 1996; Nutman, 2017; Viney and Lok, 2015) but has not been described for rats infected with *S. ratti*.

The progeny of the parasitic worms has two options for their further development: In the so-called homogonic cycle, the hatched larvae directly develop into infective iL3s and wait for the opportunity to invade their next host percutaneously. Alternatively, the larvae can go through the heterogonic cycle, where they develop into sexually reproducing males and females in the environment. In many *Strongyloides* species, the ratio of larvae, that directly develop into iL3s and larvae developing into free-living male and female adults is determined by environmental factors (reviewed in Streit, 2008).

Although the overall biphasic nature of the life-cycle is identical between *Strongyloides* spp. and *Parastrongyloides* spp., there are two striking and important differences: In the genus *Parastrongyloides*, the parasitic generation consists of male and female worms that reproduce sexually within the small intestine of their host (Grant et al., 2006b). On the contrary, in *Strongyloides* spp. the parasitic generation consists of females only that reproduce parthenogenetically (Grove, 1996; Nemetschke et al., 2010a; Streit, 2008). As mentioned above, the progeny of these parasitic females can develop either into free-living males and females or into exclusively female iL3s that will develop into parasitic females upon invasion of a suitable host. It has been shown, that the ratio of *S. ratti* male and female larvae is influenced by the immune status and the conditions inside the host (Crook and Viney, 2005; Streit, 2008). For *S. papillosus* the observation prevails, that less males are produced in the original hosts (sheep and goats) than in a permissive laboratory host like the rabbit (Matoff, 1936), implying an environmental influence on sex ratios.

The second important difference between the two genera is that *Parastrongyloides* spp. have the ability to go through an unlimited number of free-living generations, as long as the environmental conditions are favorable and no crowding occurs (Grant et al., 2006b; Kulkarni et al., 2013). The free-living generation of

Strongyloides spp., however, can only give rise to fertilized eggs, which are bound to develop into exclusively female iL3s. The life-cycles of the three Strongyloididae species, relevant for this thesis, and their peculiarities are shown in Figure 1.3.

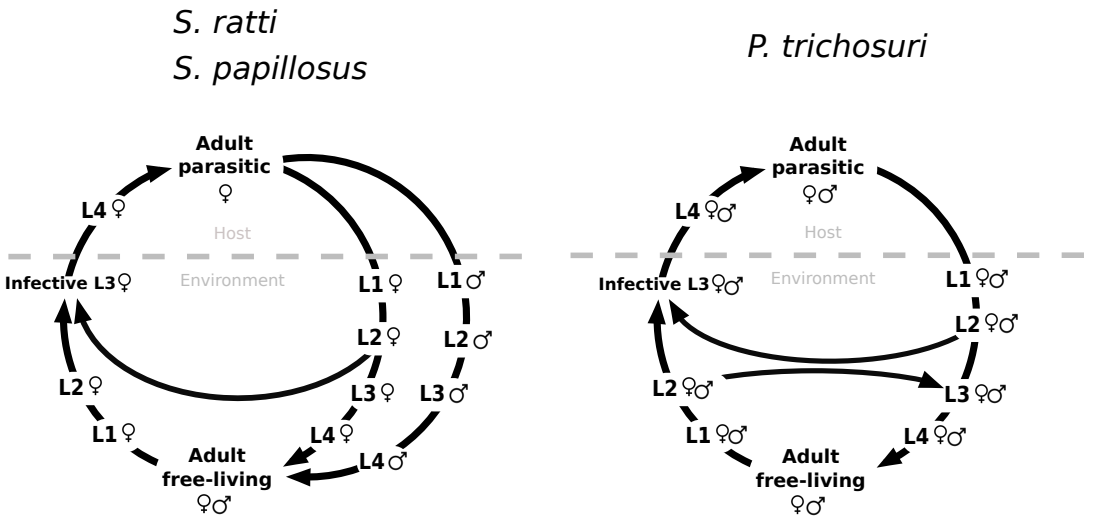

FIGURE 1.3: **Life-cycles of 3 Strongyloididae**. Schematic representation of the life-cycles of the nematode species under study: *S. ratti*, *S. papillosus* and *P. trichosuri*. All species go through the biphasic life-cycle, characteristic for the nematode family Strongyloididae. In contrast to the *Strongyloides* spp., *Parastrongyloides* reproduces sexually in both the parasitic and the free-living generation. Furthermore *Parastrongyloides* spp. can go through an unlimited number of free-living generations. L (larval stage).

1.3.5 Sex-determination and reproduction in the family Strongyloididae

Even though it was shown that environmental factors can influence sex determination in *Strongyloides* spp. (Crook and Viney, 2005; Streit, 2008), sex chromosomes have been shown to exist in *S. ratti* (Harvey and Viney, 2001; Nigon and E., 1952) and *S. stercoralis* (Hammond and Robinson, 1994a).

Interestingly, different chromosomal complements are found in different Strongyloididae species: *S. ratti*, *S. stercoralis* and *P. trichosuri* have three chromosome pairs and exhibit a XX/XO sex determining system where males and females have two pairs of autosomes and either one or two copies of the sex chromosome (X) respectively (Hammond and Robinson, 1994a; Harvey and Viney, 2001; Kulkarni et al., 2013). Contrary to those species, *S. papillosus*, *S. ransomi*, *S. vituli* and

S. venezuelensis females have only two chromosome pairs, one larger pair and one medium sized pair (2L2M karyotype). However, the males of *S. papillosus* and *S. vituli* have been shown to have a peculiar karyotype with one large, three medium sized and one small chromosomes (1L3M1S karyotype) (Albertson et al., 1979; Kulkarni et al., 2013; Nemetschke et al., 2010a). So far, the males of *S. ransomi* and *S. venezuelensis* have not been shown to have such a peculiar 1L3M1S karyotype (Hino et al., 2014; Triantaphyllou and Moncol, 1977). Instead, for *S. ransomi* males, the same 2L2M karyotype as in females had been described some 40 years ago (Triantaphyllou and Moncol, 1977), but in the same study they had also described a 2L2M karyotype for *S. papillosus* males, which has been proved to be wrong in the later work of Albertson et al. (1979) and Nemetschke et al. (2010a). The isolate of *S. venezuelensis*, that has been used by Hino et al. (2014), didn't produce any males and therefore it is not known what the karyotype of males is. It is not known if *S. venezuelensis* males can even be produced at all, as there seem to be only lab strains in use that produce only very few free-living females and no males. An overview of the karyotypes of five different species, together with their phylogeny is given in Figure 1.2.

Triantaphyllou and Moncol (1977) were the first to hypothesize that the L chromosome in *S. papillosus* and *S. ransomi* corresponds to a fusion between the ancestral X chromosome, which is still present in *S. ratti* and *S. stercoralis*, and one of the autosomes. This hypothesis has later been confirmed for *S. papillosus* (Nemetschke et al., 2010a) and some evidence on synteny suggests that the chromosomes in *S. venezuelensis* are derived from the same event (Hino et al., 2014; Hunt et al., 2016). In fact, *S. papillosus* males lose a part of one L chromosome through chromatin diminution, which represents a loss of the ancestral X chromosome and therefore a substitution of the ancestral XX/XO sex-determining system (see Figure 1.4 A) (Kulkarni et al., 2013; Nemetschke et al., 2010a). This special case of chromatin diminution in *S. papillosus* is discussed in the following section 1.3.5.1.

Strikingly, the free-living and sexually reproducing generation of all *Strongyloides* species produce only female offspring, which can only develop into iL3s (Figure 1.3). The cytological and molecular mechanisms underlying this avoidance of males in the offspring of the free-living worms is still not understood. Pseudogamy has been proposed, where eggs are activated by sperm, but sperm does not contribute genetically to the offspring (Grove, 1996; Hammond and Robinson,

1994a). Nevertheless, a male contribution of genetic material to the all-female offspring has later been demonstrated through genetic markers for *S. ratti* (Harvey and Viney, 2001; Nemetschke et al., 2010b).

For *S. papillosus* it has also been shown by Eberhardt et al. (2007) and Nemetschke et al. (2010a), that free-living females undergo standard meiosis, leading to equal representation of their alleles in the gametes, but in males only the intact L chromosome is incorporated into mature sperm, along with one of the M chromosomes. As a result, progeny of the free-living generation of *S. papillosus* consists entirely of females with the karyotype 2L2M. It remains to be clarified if the same mechanism is used by other *Strongyloides* spp., but some of our group's work (included in this thesis) has shown through sequencing of mature sperm DNA, that the free-living *S. ratti* males are able to produce a small amount of male (nullo-X) sperm, in contrast to *S. papillosus* (Kulkarni et al., 2016). We have some evidence from ongoing work in our group, that a significant number of *S. ratti* eggs don't develop and eventually die either within the gonad of the free-living females or once laid. It remains to be investigated if these dying embryos have a male karyotype.

The exact reproduction mechanism of the female-only parasitic generation of the *Strongyloides* genus is not known, but cytological and genetic evidence suggests that it is by mitotic parthenogenesis (reviewed in Streit, 2017). Even though this reproduction mode is clonal, not only female offspring but embryos of both sexes are produced. It remains a mystery how and through which mechanism one of the X chromosome copies (or sex-determining part of a chromosome) is eliminated and excluded from the male-destined progeny.

1.3.5.1 Chromatin diminution in *S. papillosus*

A specific loss of genetic material is not unique to this species of Strongyloididae, but can be found in a wide variety of phylogenetically distant taxa. The loss of genetic material can affect entire chromosomes (chromosome elimination), as in the fly *Sciara coprophila* (Goday and Esteban, 2001; Herrick and Seger, 1999), or parts of chromosomes (chromatin diminution), as happens during the formation of the macronucleus in protozoan ciliates like *Tetrahymena* spp. and *Paramecium* spp. (Chalker and Yao, 2011; Yao and Chao, 2005). Furthermore chromatin diminution has been described in copepods (Wyngaard and Gregory, 2001) and

several parasitic nematodes (Goday and Pimpinelli, 1993; Muller et al., 1996; Muller and Tobler, 2000; Nemetschke et al., 2010a).

The first observation and description of chromatin diminution was made by Theodor Boveri in 1887, who observed the development of *Parascaris univalens*, an intestinal parasite of horses. Today chromatin diminution in nematodes is best studied in *Ascaris suum* and *Parascaris univalens*, which are both clade III nematodes (Muller and Tobler, 2000).

Interestingly, the elimination process can serve different purposes in parasitic nematodes: In contrast to the family Ascarididae, where chromatin diminution is involved in the differentiation of germline and soma, *S. papillosus* chromatin diminution serves sex determination. In *S. papillosus*, chromatin diminution takes place during the mitotic parthenogenesis of parasitic females and allows the formation of male embryos (Albertson et al., 1979; Nemetschke et al., 2010a). By contrast, chromatin diminution in Ascarididae occurs early during the cleavage divisions of the developing zygote and is involved in the germline-soma differentiation (Tobler and Muller, 2001).

In *Ascaris* spp. internal parts of chromosomes, consisting of heterochromatin, are diminished selectively and telomeric repeats are added to the resulting chromosomal fragments, guaranteeing their integrity during further development (Muller et al., 1996; Muller and Tobler, 2000; Tobler and Muller, 2001). In *S. papillosus*, the different steps of chromatin diminution are not understood in detail, but the oocytes in the parasitic females, which give rise to a 1L3M1S karyotype, contain a pair of "beaded" L chromosomes during their maturation in the ovaries (see Figure 1.4 B) (Albertson et al., 1979; Nemetschke et al., 2010a). The mitotic maturation division of oocytes with beaded L chromosomes results in the fragmentation of one of the 2 L chromosomes. The "beads" remain in the cytoplasm and are eventually degraded (Albertson et al., 1979; Nemetschke et al., 2010a). It is not known how the decision is made, which of the 2 beaded L chromosomes is going to be diminished, and nothing is known about the molecular mechanism of selection, tagging and eventually degradation of the diminished chromosome parts.

Some small RNA classes are involved in the rearrangement of chromosomes in ciliates (Yao and Chao, 2005; Yao et al., 2003), but not in chromatin diminution in *Ascaris suum* (Wang et al., 2011). This implies that the mechanisms of chromatin diminution and chromosomal rearrangement are not widely conserved and have

evolved differently in different taxa. As the function of the chromatin diminution event is different in *Ascaris* and *Strongyloides*, it will be interesting to see if they also evolved different molecular mechanisms regulating the diminution, or if the molecular mechanism is still the same and only the function has changed and adapted differently.

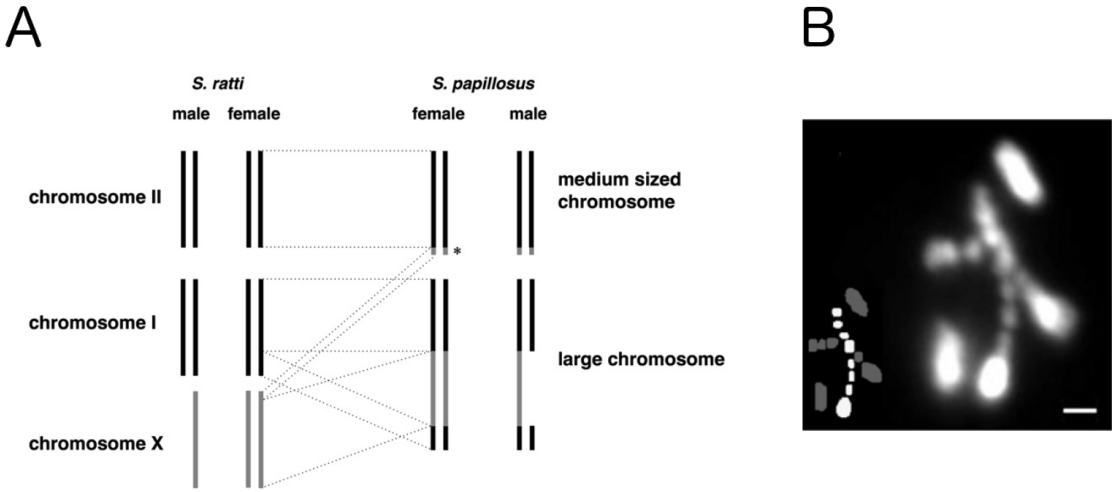

FIGURE 1.4: **Chromosomes in *S. papillosus***. Adapted from Nemetschke et al. (2010a). (A) Model for the evolutionary relationship between *S. papillosus* and *S. ratti* chromosomes. Autosomal regions are depicted in black, X chromosomal regions in grey. * Unpublished results suggest that the portion of the M chromosome related to the *S. ratti* X is located within the chromosome. (B) Beaded appearance of the two large (L) chromosomes in some oocytes inside the ovaries of *S. papillosus* parasitic females. A schematic representation with one of the L chromosomes in white is shown in the bottom left corner. The scale bar represents 1μm.

1.3.6 Model species and toolsets in Strongyloididae

Several species of the Strongyloididae are being developed as parasitic model organisms and they represent a unique possibility to investigate not only the evolution and basic biology of these intestinal parasites, but also possible mechanisms of antihelminthic treatments. The biphasic life-cycle has the advantage of combining an easily accessible, sexually reproducing free-living generation with the parasitic generation. That brings the unique possibility to do genetic analysis in the free-living generation by crossing and mutagenesis.

The general advantage of *P. trichosuri* over *Strongyloides* spp. is the ability to go through an unlimited number of generations in their free-living cycle (that's why

they are termed facultative parasites) (Grant et al., 2006b). Therefore, crosses, genetic analysis and general laboratory handling are easier for *Parastrongyloides*. Still, *S. ratti* is the best established laboratory model among the Strongyloididae and isofemale lines (clones) can be easily made by injecting rats with a single iL3 (Viney and Kikuchi, 2017; Viney et al., 1992) and a genetic map has been been constructed (Nemetschke et al., 2010b). *S. stercoralis* is inherently more difficult to handle because it bears the risk of human infections. Nonetheless, it can be studied in the laboratory by using alternative hosts like dogs (Schad et al., 1989) or mongolian gerbils (Nolan et al., 1993). For *S. papillosus*, maintenance in rabbits and a basic toolset have also been established (Eberhardt et al., 2008, 2007).

Protocols for producing transgenes are published for *S. ratti*, *S. stercoralis* and *P. trichosuri* (Grant et al., 2006a; Lok and Massey, 2002; Shao et al., 2012) but don't work very reliably in our experience. CRISPR/Cas9 induced site specific mutagenesis has been tried as a toolset in our lab for *S. ratti* and *P. trichosuri* without success and it has been attempted by several laboratories but until today no stable CRISPR/Cas9 induced mutant line of any species of *Strongyloides* has been reported.

Very importantly, the *S. ratti* genome is by now the second most contiguously assembled nematode genome after the *Caenorhabditis elegans* reference genome. With its size of ∼43 Mb, the *S. ratti* genome is less than half the size of the *C. elegans* genome and until now almost 12500 genes have been annotated, half as many as in *C. elegans* (Hunt et al., 2016). In total, the genomes of four *Strongyloides* and one *Parastrongyloides* species (*S. ratti*, *S. stercoralis*, *S. papillosus*, *S. venezuelensis* and *P. trichosuri*) have been published and represent a great toolset for the investigation of these important parasites (Hunt et al., 2016). The current *S. papillosus* genome is the biggest among the Strongyloididae with ∼60 Mb, but due to the poor quality of the assembly this might still change upon further improvements. General statistics and information on the genome assemblies that I used for this thesis are given in Table 1.

In comparison to the free-living *Rhabditophanes*, Strongyloididae have vastly expanded 2 gene families: astacin-like and SCP/TAPS (Hunt et al., 2016). Astacins are metallopeptidases playing a role in tissue migration of parasitic nematodes (Gomez Gallego et al., 2005; Lun et al., 2003; Williamson et al., 2006) and SCP/-TAPS genes have been previously associated with immunomodulatory effects, especially as inhibitors of neutrophil and platelet activity in hookworm infections

(Del Valle et al., 2003; Moyle et al., 1994). It has been shown, that genes of these expanded gene families are specifically upregulated in parasitic worms and it is likely that they play a key role in the parasitic lifestyle (Baskaran et al., 2017; Hunt et al., 2016).

Table 1: Properties of the genome assemblies

	S. ratti	*S. papillosus*	*P. trichosuri*	*P. pacificus*	*C. elegans*
Clade	IV	IV	IV	V	V
Number of chromosomes	3	2	3	6	6
Assembly version	PRJEB125[a]	PRJEB525[a]	PRJEB515[a]	Hybrid1[b]	PRJNA13758[a] (WS250)
Assembly size (Mb)	43,15	60,45	42,49	172,5	100,29
Number of scaffolds	135	4703	1810	18083	7
N50 of scaffolds (kb)	11694	86,36	836,94	1245	17494
N50 (number)	2	129	12	39	3
GC content (%)	21,4	25,6	28,25	42,82	35,44
Number of genes	12448	18456	15010	30228	23629

Genome statistics are based on all scaffolds, the analysis was done with BBTools; N50 length of scaffolds: 50% of the entire assembly is contained in scaffolds equal to or larger than this value. N50 (number): Number of scaffolds which contain 50% of the assembled genome.
[a] NCBI BioProject ID; [b] from http://www.pristionchus.org/ (last accessed 29.09.2017).

1.4 small RNA pathways in nematodes

Small non-coding RNAs (sRNAs) are 18-35 nucleotide long RNAs that are involved in regulating gene expression through translational inhibition, mRNA degradation, influencing mRNA storage and controlling epigenetic markers. sRNAs can target coding genes, pseudogenes, transposons and repeat regions and the different sRNA classes are defined by their interaction with distinct effector proteins of the Argonaute family (Billi et al., 2014; Buck and Blaxter, 2013; Sarkies and Miska, 2014). Some sRNA pathways are highly conserved across the animal kingdom, plants, fungi and even bacteria with the most conserved being the micro RNA (miRNA) pathway and the PIWI interacting RNA (piRNA) pathway (Czech and Hannon, 2011). *C. elegans* is one of the best-studied organisms with respect to sRNA families and their Argonaute partners. In fact, the very first miRNA was discovered in *C. elegans* in 1993 (Lee et al., 1993; Wightman et al., 1993) and since then small RNA classes and their associated effector proteins are being studied not only in *C. elegans* but all kinds of model organisms. Many new mechanisms of function and regulation have since been discovered and investigated (Billi et al., 2014). Known classes of sRNAs in *C. elegans* include miRNAs, piRNAs, primary

endogenous siRNAs and secondary endogenous siRNAs (see Figure 1.5). According to their differing biogenesis, these sRNA classes differ in their lengths, their 5'-nucleotide biases towards certain nucleotides and their 5'- and 3'-end modifications (Billi et al., 2014; Kawaji and Hayashizaki, 2008).

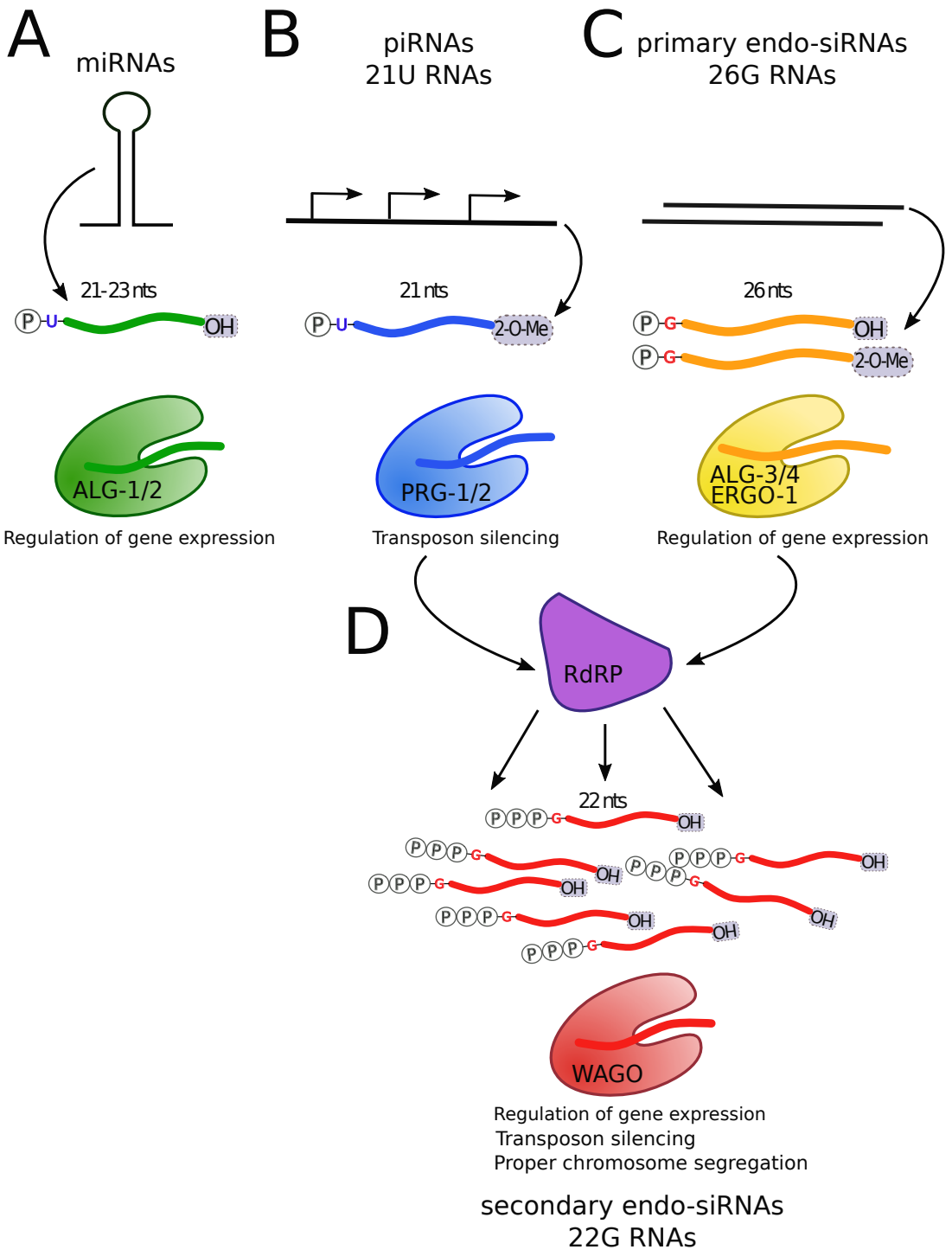

FIGURE 1.5: For description, see next page.

FIGURE 1.5: **Endogenous sRNA pathways in *C. elegans*.** Three main endogenous sRNA pathways exist in *C. elegans*: the (A) miRNA, (B) piRNA and the so-called (C) 26G primary endo-siRNA pathway. The piRNA and the 26G pathways can amplify their signal through the (D) 22G secondary endo-siRNA pathway. (A) In the miRNA pathway, mature miRNAs (21–23 nts and 5'-U) are derived from hairpin precursors cleavage (Drosha and Dicer). Mature miRNAs bind to the AGO proteins ALG-1 and ALG-2 to induce silencing of target genes. (B) piRNAs are transcribed as short (26–30 nts) precursors, which are processed to give rise to mature piRNAs with a length of 21 nts and a 5'-U, hence the name 21U RNAs. 21U RNAs bind to the Argonaute protein PRG-1 targeting and silencing transposable elements predominantly in the germline. (C) The 26G primary endo-siRNAs (26 nts and 5'-G) are derived from dsRNA that are processed by Dicer. The mature 26G RNAs are loaded into the AGO proteins ALG-3 and ALG-4, regulating gene expression. (D) The production of 22G secondary siRNAs (22 nts and 5'-G) is induced by both, the piRNA and 26G RNA pathways and can also be triggered by the exogenous siRNA pathway (not shown in this figure). They are produced by RdRPs, giving rise to triphosphorylated sRNAs. The 22G bind to different worm-specific Argonautes (WAGOs) which then function in the regulation of gene expression and transposon silencing. The 5'-end properties and nucleotides are depicted for each sRNA class. Monophosphorylated (P); Triphosphorylated (PPP); 5'-Uridine (U); 5'-Guanosine (G). The 3'-end properties are depicted as hydroxy (-OH) or as 2'-O-methyl group (2'-O-Me).

1.4.1 miRNAs

miRNAs play essential roles during development of many different organisms through gene regulation by translational repression or mediation of mRNA degradation. Many miRNAs from *C. elegans* are conserved even in humans, with many of them being involved in controlling important developmental transitions (Kaufman and Miska, 2010). Primary miRNAs (pri-miRNAs) are transcribed from miRNA genes by RNA polymerase II, resulting in 5'-capped and 3'-polyadenylated single-stranded RNAs, harboring from a single to several secondary hairpin structures (Lee et al., 2004). Within the nucleus the RNase III Drosha cleaves the pri-miRNAs into roughly 65nt long precursor miRNAs (pre-miRNAs), which have a characteristic stem-loop structure (Lee et al., 2003). The pre-miRNAs are transported into the cytoplasm (Lund et al., 2004), where they get cleaved into the 20-23nt long mature miRNAs by Dicer, another RNase III enzyme (Ketting et al., 2001) along with miRNA-specific Argonaute proteins (Bouasker and Simard, 2012).

C. elegans has two different proteins that belong to the miRNA-specific Ago-subfamily of Argonautes: ALG-1 and ALG-2 (Buck and Blaxter, 2013; Sarkies and Miska, 2014). Both of them initially bind double-stranded mature miRNAs but then release one strand, retaining the other and forming the so-called miRNA-induced-silencing complex (miRISC). Once the miRISC is associated with a mRNA, this target mRNA is post-transcriptionally silenced through the repression of translation and induction of deadenylation, thereby reducing the amount of translated proteins and promoting the degradation of the target mRNA (Djuranovic et al., 2012).

Generally, miRNAs are identified by sequencing sRNAs and subsequently using the read and genome information in specific bioinformatics pipelines that can predict the secondary hairpin structure in the genome and then check if reads from the sRNA sequencing match to those regions. One of those established miRNA prediction tools is miRDeep2 (Friedländer et al., 2012). miRNAs are classified into "seed families", according to their seed region (nucleotides 2-8), which is the primary determinant of the target RNA. All published miRNAs are stored in the miRNA-specific database miRBase (Kozomara and Griffiths-Jones, 2014). Interestingly, it has been hypothesized that mutations in miRNA genes can function as mediators of drug resistances in parasitic nematodes, rendering them an even more important field of study in parasitic nematode species (Devaney et al., 2010).

1.4.2 piRNAs

Among animals, the piRNA pathway is almost as conserved as the miRNA pathway with regard to their function, their 5'-U bias and the PIWI-subfamily Argonautes that bind them. Nonetheless, the sequences of piRNAs are not conserved and differ entirely even between very closely related species (de Wit et al., 2009). piRNAs are expressed in the germline and play an important role in the maintenance of genome stability and the trans-generational silencing of transposable elements (Ku and Lin, 2014; Siomi et al., 2011).

In mammals, mature piRNAs are reported to have a size range of 26-30 nucleotides, but in nematodes (specifically *C. elegans* and *P. pacificus*), piRNAs are also called 21U RNAs because they are precisely 21 nucleotides long and have a 5'-U. In *C. elegans*, each piRNA precursor is transcribed from an independent transcriptional

unit by the RNA polymerase II as a single-stranded, 26nt long and 5'-capped RNA. Many of the 21U RNA coding units contain a conserved eight nucleotides long upstream motif (Ruby motif) and most of them are located in one of two large clusters on chromosome IV (de Wit et al., 2009; Gu et al., 2012; Ruby et al., 2006; Weick et al., 2014). In *C. elegans*, the 26nt long piRNA precursors are bound by one of the two PIWI-related Argonautes, PRG-1 and PRG-2, and subsequently de-capped and two nucleotides are trimmed from the 5'-end (Gu et al., 2012). Afterwards, the 3'-ends are also trimmed and 2'-O-methylated by the highly conserved methyltransferase HENN-1, eventually giving rise to the mature 21nt long piRNA (Kamminga et al., 2012; Montgomery et al., 2012). The 2'-O-methyl group serves to increase the stability of the individual piRNAs over a longer timespan.

Once the piRNAs are bound to PRG-1 (the function of PRG-2 is not yet clear) and processed to their final length, they form the so-called PRG-1-piRNA-complex, targeting transcripts that are partially complementary (Bagijn et al., 2012). When the PRG-1-piRNA-complex is associated with a target transcript, the production of another class of sRNA, the secondary siRNAs or 22G RNAs, is triggered around the piRNA target site (Bagijn et al., 2012; Lee et al., 2012). This mechanism is dependent on RdRPs and serves to amplify the original signal and mediate trans-generational silencing via chromatin remodeling (Ashe et al., 2012; Bagijn et al., 2012; Lee et al., 2012; Shirayama et al., 2012). The 22G RNAs are bound by worm-specific Argonautes (WAGOs) and they will be discussed in more detail in section 1.4.4.

Given the wide conservation of piRNAs among animals, it is striking that piRNAs and PIWI proteins were only found in nematodes from clade V, which contains *C. elegans* and *P. pacificus*. This observation is based on the absence of recognizable Piwi genes in the genomes of several clade III (*A. suum*, *B. malayi*, both animal parasitic) and IV (free-living *Panagrellus redivivus* and plant parasitic *Globodera pallida*) nematodes (Sarkies et al., 2015). These authors proposed that the piRNA pathway has been lost several times independently during nematode evolution and that compensating mechanisms might have evolved in the form of more diverse endogenous siRNA classes. However, another possible explanation might be an early loss of the piRNA pathway in the Nematoda and a subsequent regain in clade V nematodes, possibly through horizontal gene transfer.

1.4.3 Primary endogenous siRNAs

A third class of sRNAs in *C. elegans* are the primary endogenous siRNAs. Because of their length of 26 nucleotides and their very strong bias towards a 5'-G they are also called 26G RNAs. They are derived from an endogenous double-stranded RNA trigger and their biogenesis is dependent on the RdRP RRF-3 and the RNase III Dicer (Billi et al., 2014; Han et al., 2009; Thivierge et al., 2011). 26G RNAs are enriched in the germline and two different subclasses can be defined according to their Argonaute partners and their sex-specificity: The 26G RNAs that are bound by the Argonautes ALG-3 and ALG-4 are required for sperm production and the ERGO-1 bound 26G RNAs play important roles in oocytes and embryos. Interestingly, in contrast to the ALG-3/4 26G RNAs, the ERGO-1 26G RNAs are methylated at their 3'-end through HENN-1, similarly to the piRNAs (Kamminga et al., 2012; Montgomery et al., 2012). In complex with their associated Argonaute, they target perfectly complementary transcripts (Han et al., 2009) and act as gene regulators via translational inhibition, enhancing transcript degradation and regulating chromatin modifications (Claycomb, 2012; van Wolfswinkel and Ketting, 2010). Upon the interaction with the target transcript, their action is amplified and enhanced through the local production of 22G secondary siRNAs, comparable to the piRNAs.

26G RNAs have also been described in *P. pacificus* (de Wit et al., 2009) and the clade III animal parasitic species *A. suum* (Wang et al., 2011), but no 26G peaks were detected in *B. malayi* (clade III) or the plant parasite *Globodera pallida* (clade IV) (Sarkies et al., 2015).

1.4.4 Secondary endogenous siRNAs

Secondary endogenous siRNAs in *C. elegans* are around 22nt long and have a 5'-G, hence their name 22G RNAs (Ashe et al., 2012; Gu et al., 2009; Lee et al., 2012; Pak and Fire, 2007; Shirayama et al., 2012; Vasale et al., 2010). As already mentioned before, both primary endogenous siRNAs (26G RNAs) and piRNAs (21U RNAs) can trigger the production of 22G RNAs. Additionally, exogenous dsRNAs can also induce the production of 22G RNAs, after they have been processed into exogenous primary siRNAs of 21-22nt through the action of Dicer (Sarkies and Miska, 2014). 22G RNAs result from unprimed RNA synthesis by

the RdRPs RRF-1 and EGO-1, are not processed afterwards and are therefore 5'-triphosphorylated and have a very strong 5'-G bias (Sijen et al., 2007). They can roughly be separated into two different subsets according to their Argonaute partners and their function: WAGO-bound 22G RNAs and CSR-1-bound 22G RNAs. WAGO-bound 22G RNAs are involved in the repression of transposons, erroneous transcripts and certain genes and are perfectly complementary to their targets (Gu et al., 2009). On the other hand CSR-1-bound 22G RNAs have been shown to be essential for proper chromosome separation during mitosis and meiosis via targeting germline-specific genes and serve as protection against piRNA mediated degradation of bona fide genes in the germline (Wedeles et al., 2013).

In the study from Sarkies et al. (2015), the comparison of sRNAs of many different nematodes has shown, that the presumably secondary endogenous siRNAs in other species than *C. elegans* can vary in length, abundance and 5'-nucleotide bias. In some of the studied species, a comparable class of secondary endogenous siRNAs has not been detected, indicating a potential loss and a highly flexible sRNA adaptation during evolution in nematodes.

1.4.5 Extracellular sRNAs from parasitic nematodes

For some parasitic nematodes, it has been recently described that parasite-derived sRNAs are found to be circulating in the blood and other tissues of their animal hosts. Circulating parasite miRNAs were found in the blood of vertebrate hosts infected with the filarial nematodes *Dirofilaria immitis* and *Onchocerca volvulus* (Tritten et al., 2014) and in hosts infected with trematodes of the genus *Schistosoma* (Cai et al., 2015). It has been shown for the intestinal parasitic nematode *Heligmosomoides polygyrus* (infecting rodents), that it can secrete vesicles that contain miRNAs as well as an Argonaute protein (Buck et al., 2014). So far, the function of these parasite-derived sRNAs has not been investigated in greater detail, but a potential influence on the host's immune system and gene expression has been postulated.

1.4.6 Comparison of sRNAs from different nematodes

The comparative study of sRNAs of different nematode species is a great opportunity to investigate the evolution and diversification of sRNA mediated functions

and gene regulations. It has been found that sRNAs and their biological functions are surprisingly diverse among nematodes (de Wit et al., 2009; Sarkies et al., 2015; Shi et al., 2013; Wang et al., 2011). For example, piRNAs have been lost in multiple independent nematode lineages outside of clade V, and they appear to have evolved different compensating sRNA pathways, all involving RNA-dependent RNA Polymerases (RdRPs) during their biogenesis (Sarkies et al., 2015; Wang et al., 2011). In order to investigate the functions of sRNA classes in parasitic nematodes from clade IV and their possible importance for the establishment and maintenance of infections, it is necessary to characterize the basic sRNA classes and properties in these parasites. To my knowledge there is only a single study in *S. ratti* that has described sRNAs (focusing on miRNAs) in a Strongyloididae species so far (Ahmed et al., 2013). However, this study used methodology that included only sRNAs with 5'-monophosphates and would have missed potential 5'-triphosphorylated sRNAs, which have been reported to form a large portion of the sRNAs in other nematodes (Sarkies et al., 2015). That is why I conducted comparative sRNA sequencing for three Strongyloididae species, namely *S. ratti*, *S. papillosus* and *P. trichosuri*, and compared their profiles to the sRNA profiles of the well-studied *C. elegans* and *P. pacificus*.

1.5 Aims of this thesis

The aim of this thesis was to address two main topics of the biology of the three Strongyloididae species *S. ratti*, *S. papillosus* and *P. trichosuri*:

1.) Clarification of the DNA content of Strongyloididae germ cells.
A) A population of "ginat nuclei" with a DNA content of up to several hundred times more than normal diploid cells exists in the distal region of the gonads of both sexes of Strongyloididae species. The aim of this work was to use quantitative DNA and RNA sequencing to determine if different parts of the the genome are amplified to different degrees and, if yes, if this is also reflected in gene expression.
B) Free-living adults of *Strongyloides* spp. produce no male progeny. The aim of this work was to use quantitative DNA sequencing to determine if genetically male determining sperm (i.e. sperm with no X chromosome) are produced at all by males in these species.

2.) Characterization of all small RNAs in the three Strongyloididae and comparison to other nematodes.
The aim was to provide a comprehensive description of all sRNAs in the three Strongyloididae species by performing sRNA sequencing experiments on two different developmental stages with three biological replicates each: free-living adults and infective larvae. For comparison the sRNAs of the corresponding two developmental stages of the model nematodes *C. elegans* and *P. pacificus* were re-sequenced and analysed with the exact same methods. In particular I addressed the following questions:
A) Do the Strongyloididae produce triphosphorylated secondary siRNAs, as described in *C. elegans*, and if yes, what are their potential targets?
B) Do the Strongyloididae possess piRNAs?
C) Are there conserved miRNAs that are differentially expressed between two developmental stages?
D) Are there parasite-derived sRNAs circulating in the blood of hosts?
In a few cases of other parasitic nematodes it has been shown that parasite-derived sRNAs circulate in the blood of the host. The aim was to test if this is also the case for *S. ratti* by sequencing the sRNAs isolated from blood of *S. ratti*-infected rats and uninfected controls.

Results and Discussion

2.1 Differential chromatin amplification and chromosome complements in the germline of Strongyloididae (Nematoda).

Kulkarni A, Holz A, Rödelsperger C, Harbecke D, Streit A.
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2.1.1 Synopsis

Nematodes of the family Strongyloididae are intestinal parasites that can infect a wide variety of vertebrates, including humans and livestock. In comparison to the famous free-living nematode model organism *Caenorhabditis elegans*, they differ in many aspects of their reproductive biology and in their gonad organization. The distal parts of the gonads of *Strongyloides* males and females contain less than 50 so-called giant nuclei with a much higher DNA content than one would expect from a diploid nucleus (Hammond and Robinson, 1994b). Another peculiarity of the *Strongyloides* gonad is a band of up to 150 small, compact nuclei in the loop region of young adult worms. In order to learn if different portions of the genome are differentially amplified in the giant nuclei, we used DNA sequencing on the distal parts of the gonads of free-living male and female worms. To gain a broader perspective, we used and compared three closely related Strongyloididae species, namely *S. ratti* a parasite of rats, *S. papillosus* a parasite of sheep and goats and *Parastrongyloides trichosuri* a facultative parasite of Australian possums. In addition we conducted RNA sequencing on the same regions of the gonads of the

three species mentioned, to assess a potential correlation of gene expression and amplification of certain parts of the genome. Furthermore, we also quantitatively sequenced the DNA of mature sperm of the three species, in order to see if nullo-X sperm is produced in free-living males of the two *Strongyloides* species. In contrast to *P. trichosuri*, *S. ratti* and *S. papillosus* do not produce male offspring in the free-living generation. There are two main findings of this study: Firstly, autosomes are present in higher copy numbers than X chromosomes in the distal parts of the germlines of both sexes in all three Strongyloididae under study. Consistent with this, RNA sequencing showed that genes on autosomes are higher expressed than X chromosomal ones, implying that differential chromatin amplification can serve as a mechanism for the regulation of gene expression in the germline. Secondly, as expected, male-determining sperm is present in *P. trichosuri*, but absent in *S. papillosus*. Surprisingly, sperm that would be expected to lead to male progeny (i.e. sperm without an X chromosome) appears to be present in *S. ratti*, even though this species does not produce male progeny. These findings suggest that closely related *Strongyloides* spp. use different mechanisms for controlling sex ratios in the offspring of the free-living generation.

2.1.2 Own contributions

Together with AK I dissected the distal parts of the gonads and isolated the mature sperm samples with support from DH. I was responsible for RNA isolation and low input RNA library preparation and assisted AK with the preparation of the DNA libraries. I consider my contribution to this study to be approximately 30% of total work.

2.2 Gain and loss of small RNA classes - characterization of small RNAs in the parasitic nematode family Strongyloididae.

Anja Holz and Adrian Streit.
Genome Biol Evol. 2017 Sep; doi: 10.1093/gbe/evx197.

2.2.1 Synopsis

The nematode family Strongyloididae contains important intestinal parasites of medical and veterinary relevance, occupying an interesting phylogenetic position within the nematodes. All species of Strongyloididae go through the biphasic life-cycle, which is characteristic for this nematode family. The life-cycle is complex and alternates between free-living and intestinal-dwelling, parasitic generations. The small non-coding RNA pathways in nematodes show some peculiarities and differ quite substantially from other taxa. Recent comparative studies have shown that even within the nematodes the sRNA pathways and their effector protein partners, the Argonautes, vary considerably. In order to shed light on the sRNA pathways that are present in the Strongyloididae, I sequenced the sRNAs of three biological replicates of each of two developmental stages of the three different Strongyloididae, *Strongyloides ratti*, *Strongyloides papillosus* and *Parastrongyloides trichosuri*, and compared them with the well-studied free-living nematodes *Caenorhabditis elegans* and *Pristionchus pacificus*. To ensure that not only monophosphorylated sRNAs are included into the libraries, I prepared them with and without prior treatment with tobacco acid pyrophosphatase, representing either sRNAs with 5'-mono-, 5'-di- or 5'-triphosphates and potential 5'-caps or sRNAs with exclusively 5'-monosphosphates, respectively. With the acquired datasets I was able to describe the micro RNAs of *S. papillosus* and *P. trichosuri* for the first time and I found that the Strongyloididae have conserved and taxon specific miRNAs. Many of those miRNAs have differential expression patterns in the two developmental stages. Furthermore, I was able to identify a new class of ∼27 nucleotide long RNAs that start with 5'-G or -A, therefore named 27GA RNAs. A substantial fraction of those 27GA RNAs have the potential to target transposable elements and endogenous genes. Most likely, these sRNAs have

triphosphates at their 5'-ends and therefore their biogenesis is presumably dependent on RNA dependent RNA polymerases. Similarly to some other nematode taxa, but in strong contrast to *C. elegans* and *P. pacificus*, the Stronglyoididae seem to have lost the highly conserved piRNA pathway, lacking a characteristic 21U RNA peak. Additionally, I was not able to identify any Argonaute proteins of the Piwi family, the effector proteins of the piRNA pathway, in any of their genomes. Finally, I could not find any circulating parasite-derived sRNAs in the blood of infected hosts.

The supplementary data for this paper is mostly not printable on A4 paper, hence only the main manuscript is included in the version attached below. The supplementary data can be accessed through the following link:
https://academic.oup.com/gbe/article/doi/10.1093/gbe/evx197/4237399/Gain-and-loss-of-small-RNA-classes

2.2.2 Own contributions

I designed and performed all experiments with input from AS. All samples were collected and the sRNA libraries constructed by myself. I performed the analysis pipeline and interpreted the data with help from AS. All figures and tables were prepared by me with input from AS. The manuscript was mainly written by me with contributions from AS. I consider my contribution to this study to be approximately 90% of total work.

2.3 Unpublished results

2.3.1 tRNA-derived fragments in nematodes

2.3.1.1 Known tRNA fragments

transfer RNAs (tRNAs) are the adapter molecules that link a mRNA codon to an amino acid and are therefore a fundamental part of the translation machinery within cells. tRNAs are conserved 70-90 nt long non-coding RNAs that have a cloverleaf secondary structure and a L-shaped tertiary structure with many post-transcriptional modifications (Söll and RajBhandary, 1995). They are one of the most abundant RNA classes in cells, and their functions in translation as well as their biogenesis are very well known in a wide variety of bacteria and eukaryotes. Precursor tRNAs are transcribed by RNA polymerase III and subsequently RNase P and RNase Z remove the 5'-leader and 3'-trailer sequences, before finally the three nucleotides CCA are added to the 3'-end by a nucleotidyl transferase (Söll and RajBhandary, 1995). Additionally, a multitude of different base modifications can be found on tRNAs, executed by a wide variety of modification enzymes (Söll and RajBhandary, 1995).

Commonly, one of the first steps during the analysis of RNA and sRNA sequencing experiments is to filter out any "uninteresting" sequences, i.e. rRNA or tRNA matching reads. Those reads are then ignored as allegedly non-functional degradation products during the rest of the analyses. However, several experimental and bioinformatic approaches have shown that tRNA-derived RNAs can indeed be functional molecules with roles in cancer, viral infections, apoptosis, regulation of translation, sperm maturation and even as epigenetic factors (Anderson and Ivanov, 2014; Cole et al., 2009; Sharma et al., 2016; Soares and Santos, 2017; Sobala and Hutvagner, 2011). Usually, these functional tRNA fragments (tRFs) are classified according to their origin on the full-length tRNA. When the mature tRNA is cleaved at the anticodon site by the RNase angiogenin in mammalian cells, (Saikia et al., 2014; Yamasaki et al., 2009), the resulting products are called 5' and 3' tRNA halves (Soares and Santos, 2017; Sobala and Hutvagner, 2011). The cleavage of mature tRNAs into tRNA halves was first described in ciliates upon amino acid starvation (Lee and Collins, 2005), and later the same has been desribed in mammalian cell lines upon nutritional stress, heat shock and other

stresses (Fu et al., 2009; Yamasaki et al., 2009). Experiments in cell culture have shown that transfection with 5' tRNA halves inhibits protein synthesis via inhibiting cap-dependent translation, and contribute to the stress response by inducing the formation of stress granules (Emara et al., 2010). tRNA halves have also been found in mature sperm of mice with an important role in regulation of gene expression (Sharma et al., 2016). Also, 20-30 nt long 5'- and 3'-tRFs have been desribed of which some are produced by Dicer and found loaded into Argonaute proteins, similar to miRNAs (Maute et al., 2013). Recently another subclass of tRFs has been described as internal parts of the mature tRNA (i-tRFs) with variable lengths, including the intact anticodon (Telonis et al., 2015).

2.3.1.2 All described tRNA fragment classes can be found in nematodes

In my experiments, between 4-21% of all sRNA reads mapped to tRNAs and I could not find any general differences between Adults and Dauers/Infectives or 5'-monophosphate (untreated) or 5'-all-phosphate (TAP-treated) libraries (see Figure 2.1 A). There are no striking differences between clade IV and clade V nematodes with respect to the number of tRNA-derived reads and none of the five species shows a clear enrichment of tRNA-derived reads upon TAP treatment. When looking at the 5' nucleotides and length profiles of the tRNA-derived reads no striking differences between Adults and Dauers/Infectives can be seen within each species (see Figure 2.1 B). In clade V nematodes, the 5' nucleotides are biased towards U due to highly expressed tRNA halves, specifically in *C. elegans*, and most of the reads are longer than 30 nts. In the Strongyloididae species, the 5' nucleotides are rather biased to 5' G, although in Adults 5'-monophosphate libraries this trend is not clear. Some sequences seem to be enriched by TAP-treatment, specifically 5'U reads that are longer than 30 nts in *C. elegans* and 5'G reads shorter than 30 nts in the Strongyloididae. These trends are not always consistent across replicates or closely related species and are therefore difficult to interpret.

I could find all the different kinds of tRNA fragments that have been described in literature, specifically 5'- and 3' halves and shorter 5'- and 3' tRFs. Figure 2.2 shows examples of tRNA fragments that I found consistently in all datasets and that even seem to be conserved across all 5 nematode species. The 5' halves

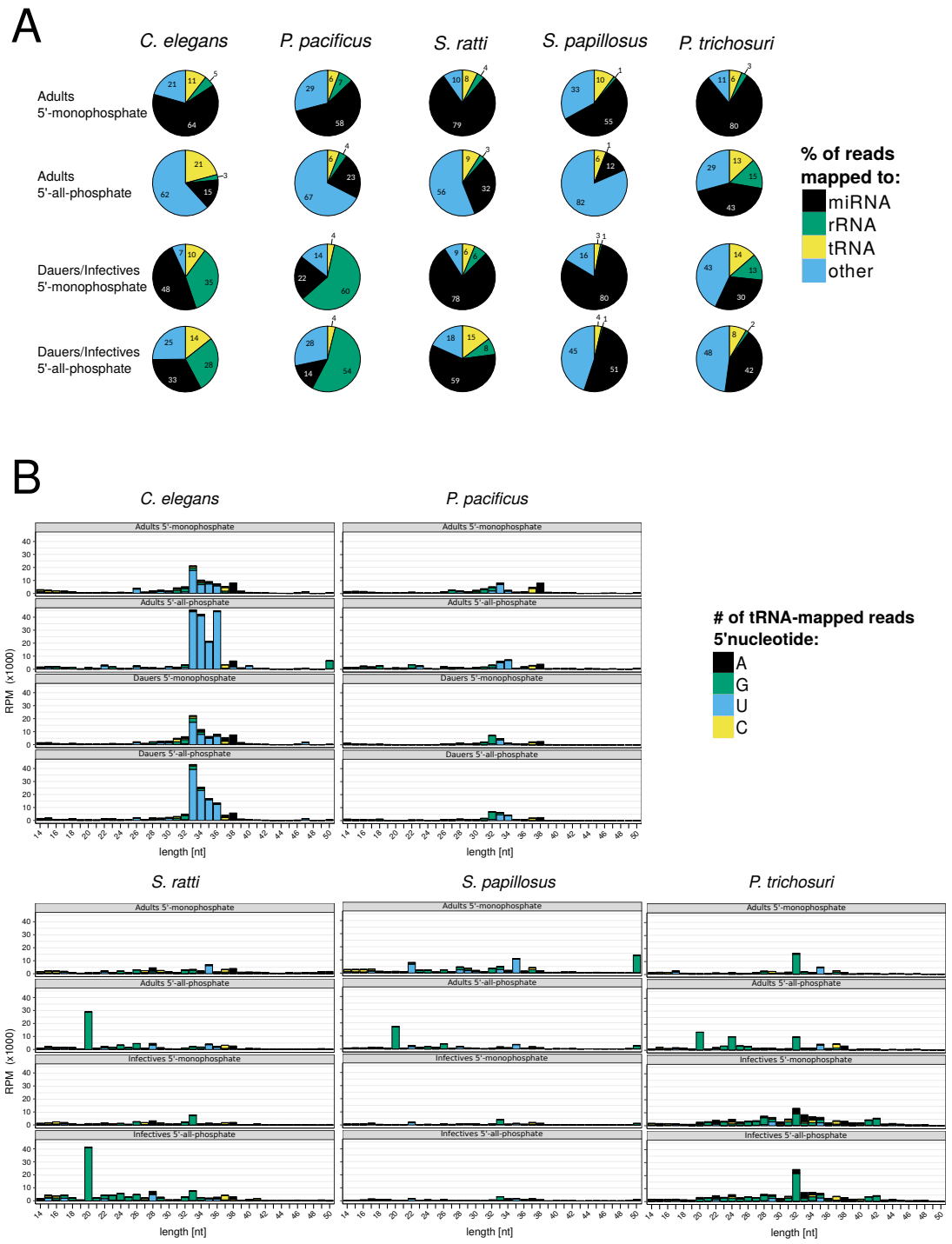

FIGURE 2.1: **5' nucleotides of tRNA-derived sRNAs**. (A) Piecharts representing the percentage of all mapped reads classified as miRNAs (black), rRNA-derived (green), tRNA-derived (yellow) or other (blue). (B) Barplots of the length distribution and 5' nucleotide of sRNAs classified as tRNA-derived. Reads starting with A, G, U or C are represented in black, green, blue and yellow, respectively. In all cases the average of the three biological replicates is given. 5'-monophosphate RNA not treated with TAP before library construction; 5'-all-phosphate RNA treated with TAP before library construction; RPM: reads per million of total mapped reads; length[nt]: read length in nucleotides.

of Gly^{GCC} tRNAs can be found in all samples and species, although in different abundances (see Figure 2.2 A). The cleavage site lies exactly within the anticodon and seems to be highly conserved, as previously reported in literature. The 3' halves of $Thr^{AGT/CGT}$ tRNAs start just after the anticodon, which is consistent across all species under study (see Figure 2.2 B). I could also find shorter (∼20nts) 5' and 3' tRFs on conserved tRNAs, $Leu^{TAA/AAG}$ and Phe^{GAA}, respectively (see Figure 2.2 C and D). Also i-tRFs are present in the libraries, but the patterns are not as clear as for the other fragments.

For now I can't assign a possible function to any of these fragments, but given the fact that they are well conserved across all five nematodes, and generally among many eukaryotes, it is reasonable to assume that they have a conserved function. The fact, that the same tRNA types show the specific patterns, regardless of the nematode species, is another very interesting fact that hints to a highly conserved feature of specific tRNAs. I can only hypothesize about the function of these tRNA fragments in nematodes, but it seems likely that they may have a similar function during stress response, to those described in ciliates and mammalian cells. It will be interesting to investigate the function of tRNA fragments further, especially their potential role in Dauer entry and exit, and their a link to starvation and downregulation of protein synthesis in nematodes.

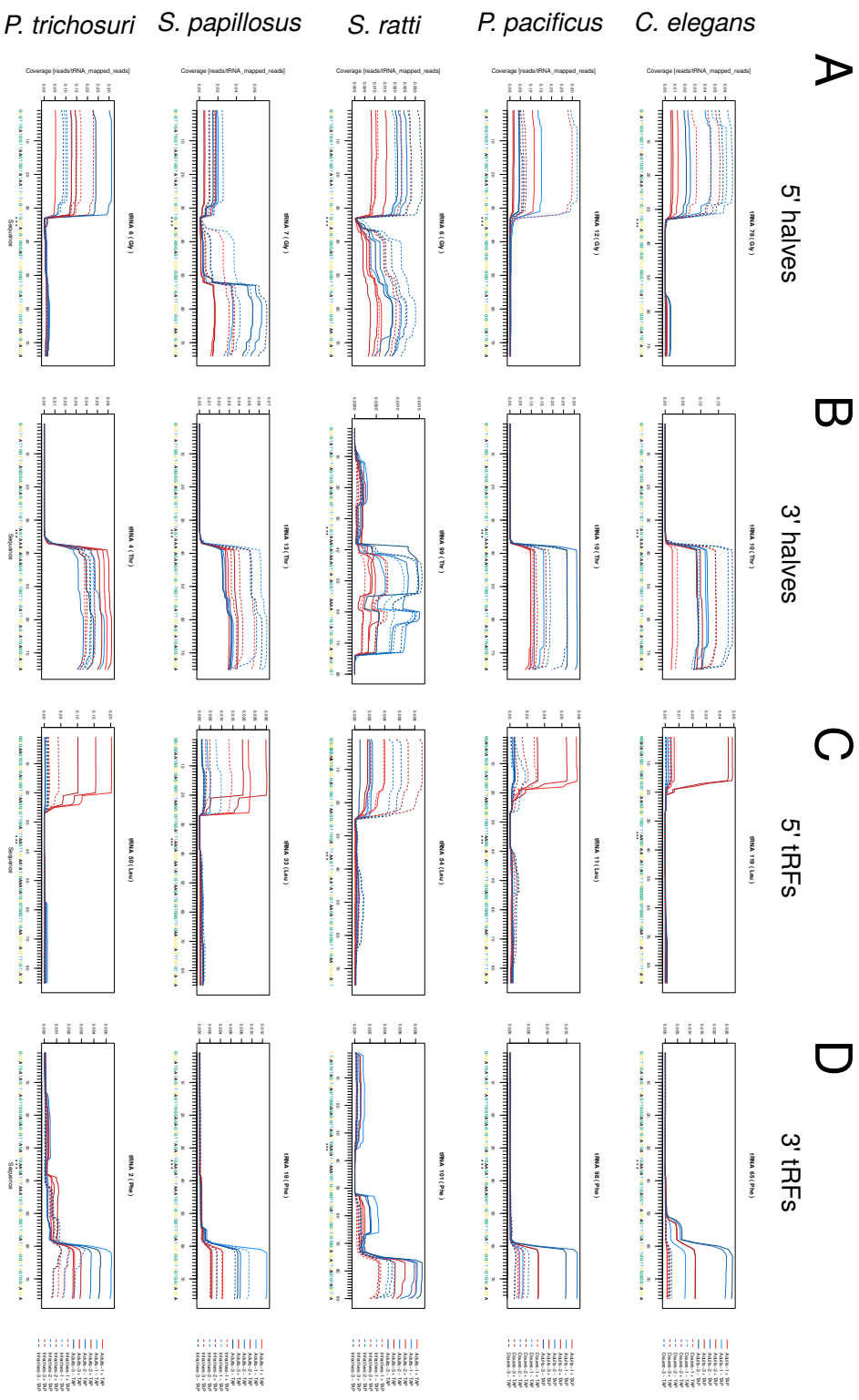

FIGURE 2.2: **tRNA fragment profiles**. Plots representing the coverage of tRNA-derived sRNA reads along predicted tRNAs for all 5 nematode species. (A) 5' halves of Gly^{GCC}; (B) 3' halves of $Thr^{AGT/CGT}$; (C) short 5' tRFs of $Leu^{TAA/AAG}$; (D) short 3' tRFs of Phe^{GAA}. The different biological replicates for adults are represented in solid lines, for Dauers/Infectives in dashed lines. TAP-treated samples are represented in red, untreated samples with blue lines. Anticodons are indicated with star symbols. Coverage is shown as ratio of read count and number of reads mapped to all predicted tRNAs.

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ORIGINAL ARTICLE

Differential chromatin amplification and chromosome complements in the germline of Strongyloididae (Nematoda)

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Abstract Nematodes of the genus *Strongyloides* are intestinal parasites of vertebrates including man. Currently, *Strongyloides* and its sister genus *Parastrongyloides* are being developed as models for translational and basic biological research. *Strongyloides* spp. alternate between parthenogenetic parasitic and single free-living sexual generations, with the latter giving rise to all female parasitic progeny. *Parastrongyloides trichosuri* always reproduces sexually and may form many consecutive free-living generations. Although the free-living adults of both these species share a superficial similarity in overall appearance when compared to *Caenorhabditis elegans*, there are dramatic differences between them, in particular with respect to the organization of the germline. Here we address two such differences, which have puzzled investigators for several generations. First, we characterize a population of non-dividing giant nuclei in the distal gonad, the region that in *C. elegans* is populated by mitotically dividing germline stem cells and early meiotic cells. We show that in these nuclei, autosomes are present in higher copy numbers than X chromosomes. Consistently, autosomal genes are expressed at higher levels than X chromosomal ones, suggesting that these worms use differential chromatin amplification for controlling gene expression. Second, we address the lack of males in the progeny of free-living *Strongyloides* spp. We find that male-determining (nullo-X) sperm are present in *P. trichosuri*, a species known to produce male progeny, and absent in *Strongyloides papillosus*, which is consistent for a species that does not. Surprisingly, nullo-X sperm appears to be present in *Strongyloides ratti*, even though this species does not produce male progeny. This suggests that different species of *Strongyloides* employ various strategies to prevent the formation of males in the all-parasitic progeny of the free-living generation.

Introduction

The nematode *Strongyloides stercoralis* is one of the most prevalent parasitic round worms in humans. Strongyloidiasis is considered a neglected tropical disease (Olsen et al. 2009). The rat parasite *Strongyloides ratti* and the sheep parasite *Strongyloides papillosus* are two other, more experimentally accessible members of the nematode genus *Strongyloides*, which consists of small-intestinal parasites of numerous vertebrates (Viney and Lok 2007; Dorris et al. 2002; Speare 1989). *S. ratti* can be maintained in the laboratory in their natural host while *S. papillosus* can be reared in rabbits. The easy access to the free-living adults (see below) and a number of recently developed resources for working with *Strongyloides* spp. and their relative *Parastrongyloides trichosuri*, a facultative parasite of Australian possums (Grant et al. 2006a; 2006b; Shao et al. 2012; Eberhardt et al. 2007; Nemetschke et al. 2010b; Viney et al. 2002), render this group of parasites highly attractive not only for parasitological research of medical and veterinary interest but also for the study of basic biological questions like host parasite interactions (Bleay et al. 2007; Crook and Viney 2005; Viney et al. 2006) and evolution (Fenton et al. 2004; Gemmill et al. 2000; Streit 2014).

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The life cycles of *S. ratti and S. papillosus* (Fig. 1a) have been reviewed in several places (Streit 2008; Viney and Lok 2007). In brief, the parasitic worms are all female and live in the small intestines of their respective hosts. They give rise to both female and male offspring by mitotic parthenogenesis. The young females either develop directly into infective third-stage larvae (L3i) and search for a new host (termed homogonic or direct development) or, together with all the males, give rise to a facultative free-living generation that reproduces sexually (termed heterogonic or indirect development). In the genus *Strongyloides*, offspring of free-living adults are all female and bound to develop into L3i, with very few known exceptions (Streit 2008; Yamada et al. 1991). *Parastrongyloides* with its best-studied representative *P. trichosuri* is a genus closely related to *Strongyloides* (Dorris et al. 2002). *Parastrongyloides* spp., like *Strongyloides* spp., also form parasitic and free-living generations of reproducing adults. However, the life history (Fig. 1b) and reproductive modes of this genus differ in interesting ways from those of *Strongyloides* spp. (Grant et al. 2006b). The distinguishing feature that led to the installation of the new genus *Parastrongyloides* was the presence of males in the parasitic generation (Mackerras 1959). Linked to this, a major difference from *Strongyloides* spp. is that free-living *Parastrongyloides* spp. produce progeny of both sexes. For *P. trichosuri*, it was confirmed genetically that reproduction in both generations is indeed sexual (Grant et al. 2006b; Kulkarni et al. 2013). In addition, *P. trichosuri* can undergo an apparently unlimited number of consecutive free-living generations (Grant et al. 2006b). It is therefore a facultative parasite.

P. trichosuri employs XX/XO chromosomal sex determination with $2n=6$ in females and $2n=5$ in males. There is no indication of an environmental influence on sex determination in this species (Grant et al. 2006b; Kulkarni et al. 2013). However, in *S. ratti* and in *S. papillosus*, as in all species of *Strongyloides* investigated thus far, the sex ratio in the progeny of parthenogenetic parasitic females is influenced by the immune status of the host (reviewed in Streit 2008) such that an increasing immune response of the host against the worms leads to a higher proportion of males. Nevertheless, in *S. ratti* and in *S. papillosus*, males and females differ in their chromosomal complement. In *S. ratti*, females have a pair of X chromosomes along with two pairs of autosomes while males have only one X chromosome. Hence, they employ an environmentally controlled XX/XO sex determination with $2n=6$ in females and $2n=5$ in males (Harvey and Viney 2001; Nigon and Roman 1952). In *S. papillosus*, the genetic material homologous to autosome I and to the X chromosome of *S. ratti* is combined into one chromosome (Nemetschke et al. 2010a). Additionally for this species, oocytes that give rise to males undergo a sex-specific chromatin diminution event that creates a hemizygous region corresponding in sequence to the X chromosome in *S. ratti*, presumably functionally restoring the ancestral XX/XO sex-determining system (Albertson et al. 1979; Nemetschke et al. 2010a; Kulkarni et al. 2013). In this process, an internal portion of one chromosome is eliminated and both ends are retained as separate chromosomes. This leads to karyotypes of $2n=5$ in males and $2n=4$ in females. Given that *Strongyloides* males are heterogametic, it is puzzling that they sire only female (homogametic) progeny. There are multiple, not mutually exclusive, hypothetical explanations for this: (i) male-determining mature sperm (nullo-X sperm in the case of *S. ratti* and sperm lacking the region undergoing chromatin diminution in *S. papillosus*, for simplicity referred to from here on as nullo-X sperm) may be rare or never formed at all; (ii) nullo-X sperm may be incapable or highly inefficient at fertilizing oocytes; or (iii) genetically male zygotes may be unviable. It is currently unknown as to which of these explanations hold true in *Strongyloides* spp. For *S. papillosus*, absence of nullo-X sperm was postulated based on genetic experiments (Nemetschke et al. 2010a), while for *S. stercoralis*, measurements of DNA content of sperm based on DNA binding dyes indicated that nullo-X sperm may be present (Hammond and Robinson 1994).

In the model nematode *Caenorhabditis elegans*, as in many other nematodes (Rudel et al. 2005), the gonads in both sexes are essentially tubes. In the hermaphrodites, the gonad has two arms, one extending anteriorly and one posteriorly but both terminating in a central vulva. The male gonad has just one arm with a posterior opening. These arms contain a germ cell production and differentiation line (Hubbard and Greenstein 2005). Although the overall morphology of the gonad arms is very similar to that of *C. elegans*, the organization and appearance of the germ cells is very different in *Strongyloides* and *Parastrongyloides* spp. (Hammond and Robinson 1994; Triantaphyllou and Moncol 1977). In these genera, the distal region contains giant nuclei that take on various shapes (Fig. 1c). No nuclear divisions have ever been reported in this region of the gonad in adult free-living stages. These nuclei have a DNA content of up to several hundred C, where one C is the DNA content of a haploid set of chromosomes (Hammond and Robinson 1994). Interestingly, these authors noted that the DNA contents they observed in different nuclei and among individuals were not full multiples of the entire genome, suggesting that different portions of the genome are amplified to various extents. The region with these giant nuclei is followed proximally by a band of very small, compact nuclei. At the end of this band, nuclei with condensed and presumably meiotic chromosomes can be observed. Further down in the gonad, depending on sex, differentiated oocytes or sperm are present, very similar to *C. elegans*.

Here, we elaborate on the chromatin and chromosome complements in germ cells of Strongyloididae. Based on quantitative sequencing approaches, we show that the X chromosome (or the X-derived region in the case of *S. papillosus*) is underrepresented in comparison to the autosomes in the giant germline nuclei of both sexes in two species of *Strongyloides*

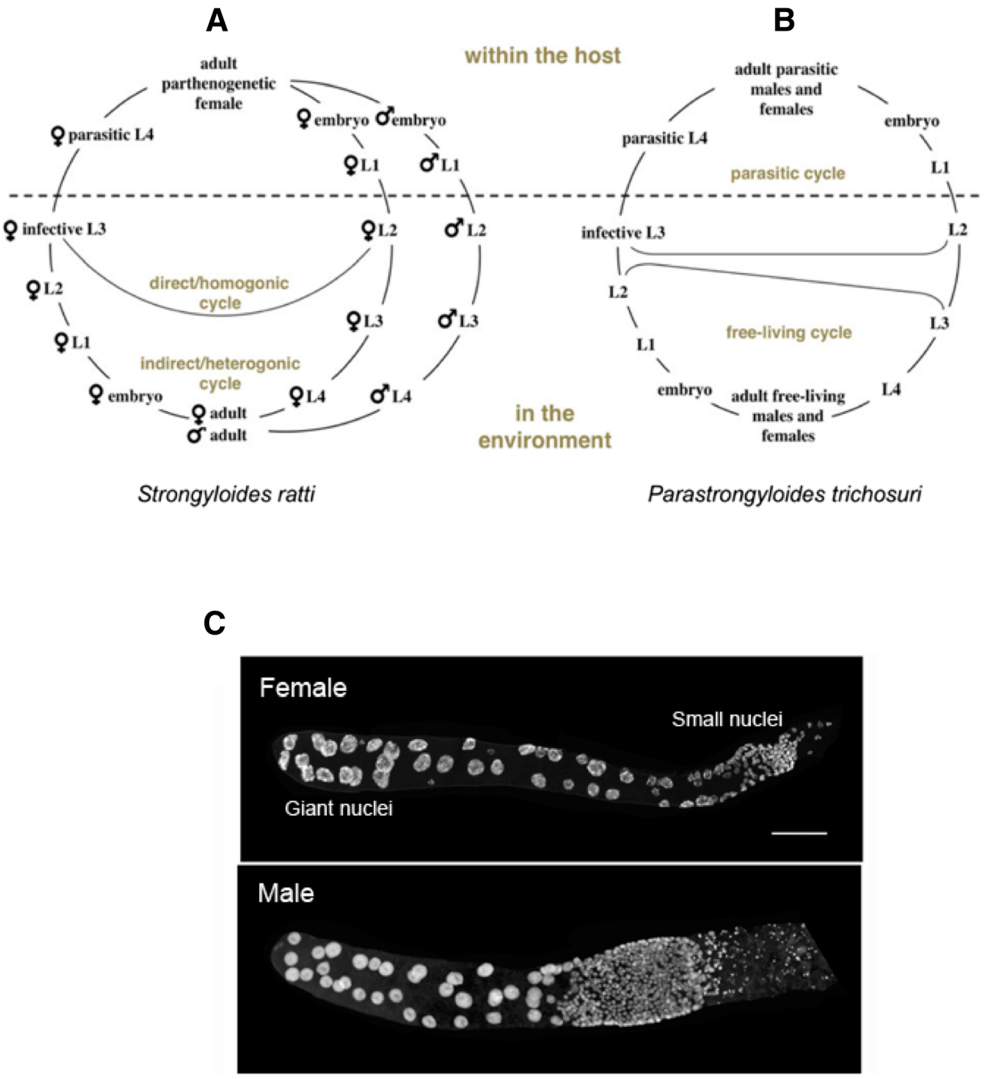


Fig. 1 Life cycle and introduction to the *Strongyloides* gonad **a** The generalized life cycle of *Strongyloides* species. **b** The life cycle of *Parastrongyloides trichosuri*. **c** Representative examples of dissected DAPI-stained gonads from *Strongyloides* spp. females (*top*) and males (*bottom*) showing the gonadal organization with giant nuclei occupying the entire distal arm, followed by a region of small compact nuclei. *Scale bar* 20 μm

and in *P. trichosuri*. Differential chromatin amplification likely serves as a way of controlling gene expression since X-encoded genes are, on average, expressed at a much lower level than autosomal genes in the distal gonad of *S. ratti* females. Additionally, based on quantitative sequencing of isolated mature sperm, we confirm the absence of nullo-X sperm in *S. papillosus* but, surprisingly, its presence in *S. ratti*. For this species, we found evidence for the presence of nullo-X sperm and unviable early embryos, suggesting that the two species of *Strongyloides* employ different strategies to avoid the formation of males in the progeny of the free-living generation.

Materials and methods

Culturing and manipulating nematodes

S. ratti ED321 and *S. papillosus* isolate LIN were maintained as described (Eberhardt et al. 2007; Nemetschke et al. 2010b; Viney et al. 1992). All animal experimentation was done according to national and international guidelines. The required permits were granted by the local authorities. *P. trichosuri* was cultured in continuous free-living cycles (Grant et al. 2006b) at 20 °C on NGM plates seeded with *E. coli* OP 50 bacteria (Stiernagle 1999) supplemented with a piece of autoclaved rabbit feces.

DAPI staining

Adult worms (of the desired age) were fixed with ice cold 100 % methanol and directly mounted (without a rehydration series) on polylysine-coated glass slides in 10 μL of Vectashield containing 1 μg mL^{-1} 4′,6-diamidino-2-phenylindole (DAPI).

DNA extractions from dissected gonads for Illumina sequencing

About 500 distal arms of the gonads per biological replicate were manually dissected from adult worms (both sexes) of all

three species. Samples were frozen in liquid nitrogen and stored at −20 °C for DNA extraction using the Illumina Epicenter Masterpure™ DNA purification kit. The samples were measured for their DNA content using the Qubit High Sensitivity DNA measuring kit and then used for making libraries according to the Illumina platform. Libraries were made using the NEXTflix™ ChIP-Seq10ng kit (Bioo Scientific) and then sequenced.

RNA extraction from dissected gonads

For each of the four independent replicates, approximately 100 distal gonad arms were dissected from adult free-living female worms of *S. ratti* and samples were immediately put on dry ice. Five hundred microliters of TRIzol was added to the samples, and they were then frozen in liquid nitrogen and stored at −80 °C until RNA extraction. RNA was extracted using the RNA micro kit (Invitrogen) and quantified using the Qubit High Sensitivity RNA measuring kit. cDNA was prepared using SMARTer Ultra Low Input RNA for Illumina Sequencing (Clontech Laboratories). Libraries were made using the Low Input Library Prep Kit (Clontech Laboratories) and then Illumina sequenced.

DNA extraction from sperm

Adult males of all three species were incubated in a solution of 0.5 μg/L (0.2 M) levamisole at room temperature for 45 min as described for *S. papillosus* (Nemetschke et al. 2010a). This causes muscle contraction and as a consequence the release of sperm. Then the ejaculated mature sperm were collected using a mouth pipette and transferred into sterile Eppendorf tubes. DNA extraction was done using the Illumina Epicenter Masterpure™ DNA purification kit. Libraries were made according to the Low Input Library Prep Kit (Clontech) and Illumina sequenced.

Analysis of sequencing data

Draft genome assemblies including chromosome information and gene annotations for *S. ratti*, *S. papillosus*, and *P. trichosuri* were provided by the Sanger Institute. We used version 0.5.9-r16 of BWA software (Li and Durbin 2009) to align raw reads of all three species to their respective genome assemblies. Read counts per 2-kb window were calculated from the resulting alignment files using custom Perl scripts. To ensure comparability across samples, read counts were normalized to one million aligned reads.

For transcriptome analysis of the *S. ratti* samples, we employed version 2.0.3 of TopHat aligner to map RNA-seq reads to the *S. ratti* genome and used version 2.0.1 of Cufflinks software to quantify expression levels (Trapnell et al. 2012). Tests for higher expression levels on autosomes with respect to the X chromosomes were done using a Wilcoxon ranksum test, as implemented in R.

Results

Appearance and number of giant nuclei

We observed that in the species studied, the giant nuclei in the distal gonad arms can take on various shapes depending on age. In general, they went from being relatively small and round or oval in young virgins (Fig. 2a) to becoming highly elongated, irregular, and large (>15 μm) in older females that are becoming infertile (Fig. 2b). This change in size and shape appeared to be more extreme in *P. trichosuri* than in the two species of *Strongyloides* (Fig. 2d). In these nuclei, frequently the nucleoli were clearly visible by DIC microscopy (Fig. 2e), as expected for nuclei in interphase. In all three species, the number of giant nuclei per gonad arm decreased significantly with age at least in females (Fig. 2f) but individual giant nuclei appeared to incorporate more DAPI in older worms than in younger ones, raising the question if their DNA content is higher. The number of giant nuclei per gonad arm ranged from around 20 to 50. The two arms within a single female tended to have similar but rarely equal numbers of giant nuclei. For example, among 33 *S. papillosus* females, only three had the same number in both arms while the maximum difference observed was 14. However, 25 (75 %) of the females fell within a range of 2–7 giant nuclei more in one arm than in the other. In 17 worms, the anterior arm had the higher number; in 13, this was the posterior arm.

In males, a band of small presumably early spermatogonic nuclei (Triantaphyllou and Moncol 1977) migrates anteriorly with age, thereby increasing the volume filled with mature sperm and decreasing the volume containing giant nuclei (Fig 2c).

Differential DNA amplification in the giant nuclei

The giant nuclei in the distal arm have been postulated to be a result of repeated replication of the chromosomes in the absence of intervening cell divisions (Hammond and Robinson 1994). Although these authors had already noted that it was unlikely that the DNA content of the giant nuclei was the product of uniform full genome amplifications, no information about what sequences are amplified was available. We isolated distal arms of gonads containing giant nuclei from free-living adults of *S. ratti*, *S. papillosus*, and *P. trichosuri* and subjected them to quantitative sequencing (Figs. 3 and 4). In all species, it appeared that the portion of the genome that is present in only one copy in males (the X chromosome and the region undergoing chromatin diminution, respectively) was underrepresented in both sexes. More precisely, in XX/XO-

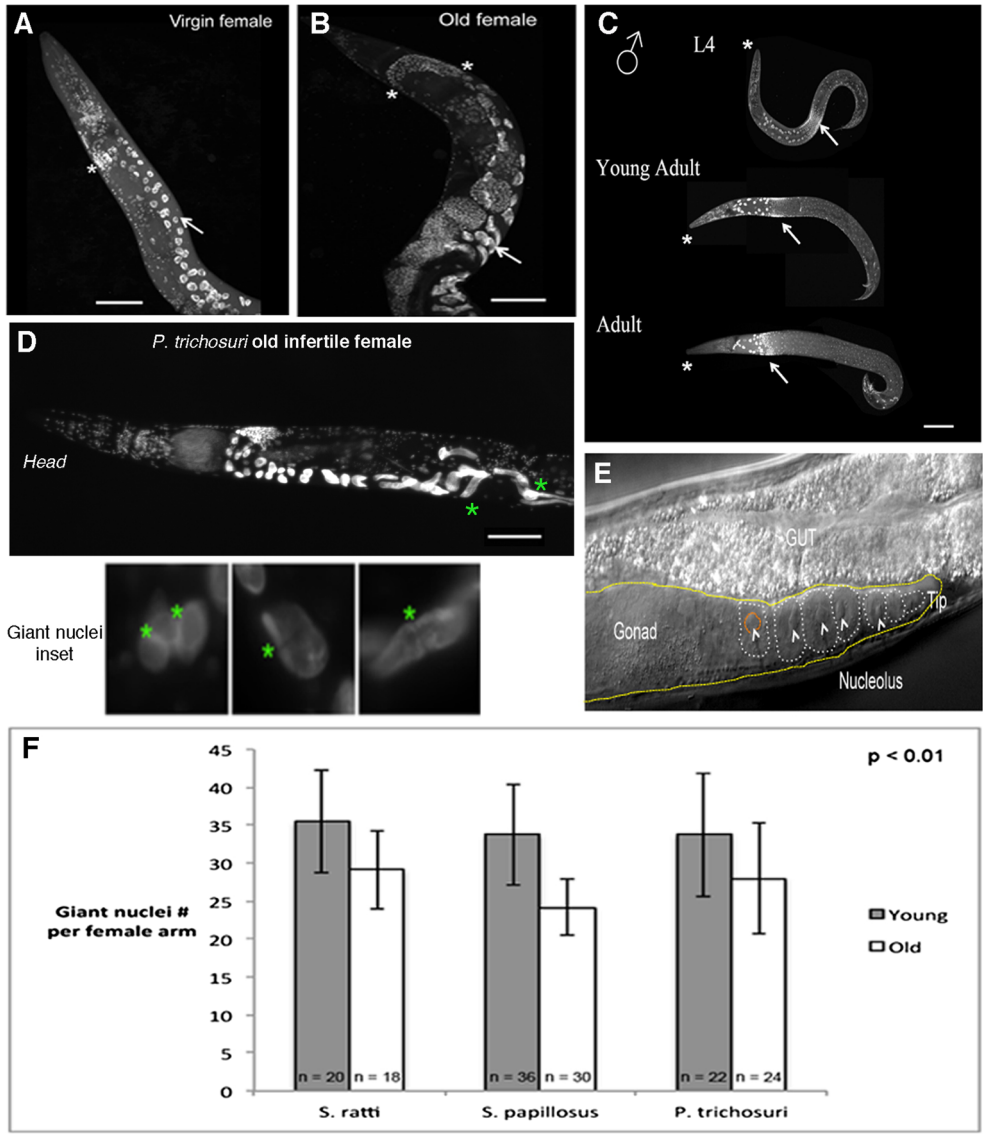


Fig. 2 Morphology and number of germline nuclei. DAPI-stained virgin (**a**) and old *S. papillosus* female (**b**), respectively. *Arrows* point to giant nuclei in the distal gonad arm. Note the change in size, number, and morphology of giant nuclei. The *asterisks* mark the position of the band of small nuclei. Note the change in position accompanied by an increase in number of small nuclei from virgin to old females. **c** DAPI-stained free-living *S. papillosus* male worms. *Arrows* point to the band of small nuclei. Note the change in position in males from L4 to adult. The band moves anteriorly over time reducing the space occupied by the giant nuclei in the gonad. *Asterisks* mark the anterior end of the worms. **d** DAPI-stained female *P. trichosuri* showing the extreme irregular giant nuclei (*asterisk*) in older infertile females. Giant nuclei *insets* show that often multiple giant nuclei are seen clumping together, and sometimes a thin line is seen within a nucleus (*asterisks*, See Suppl Movies 1 and 2). **e** DIC image showing the giant nuclei (outlined in *white*) in the distal gonad arm (outlined in *yellow*) showing a clearly visible nucleolus (outlined in *orange*, *arrow heads*). **f** The changes in the numbers of giant nuclei over age in females of all three species. For each species, there is a significant reduction in giant nuclei number over time

based sex determination, one would expect a two times higher coverage of autosomes as compared to the X chromosome and the region undergoing chromatin diminution respectively in males, but equal coverage in females. Instead, we find a four- to sixfold increase in median coverage for autosomal regions in both sexes of *S. ratti* (Fig. 3a) and *P. trichosuri* (Fig. 4a) with no obvious difference between the sexes. Although it is difficult to analyze this type of data statistically, it appears that in *S. papillosus* (Fig. 4b), which does not have a free X chromosome, the difference is smaller (only about two- to threefold) and the underrepresentation might be slightly more pronounced in males than in females.

The high quality of the draft genome sequence available for *S. ratti* (*Strongyloides* sequencing consortium, submitted for publication) allowed us to analyze the chromatin amplification along the individual chromosomes (Fig 3b, Suppl Fig. 1a). While we cannot exclude slight differences, we found no clear indication of differential DNA amplification among different

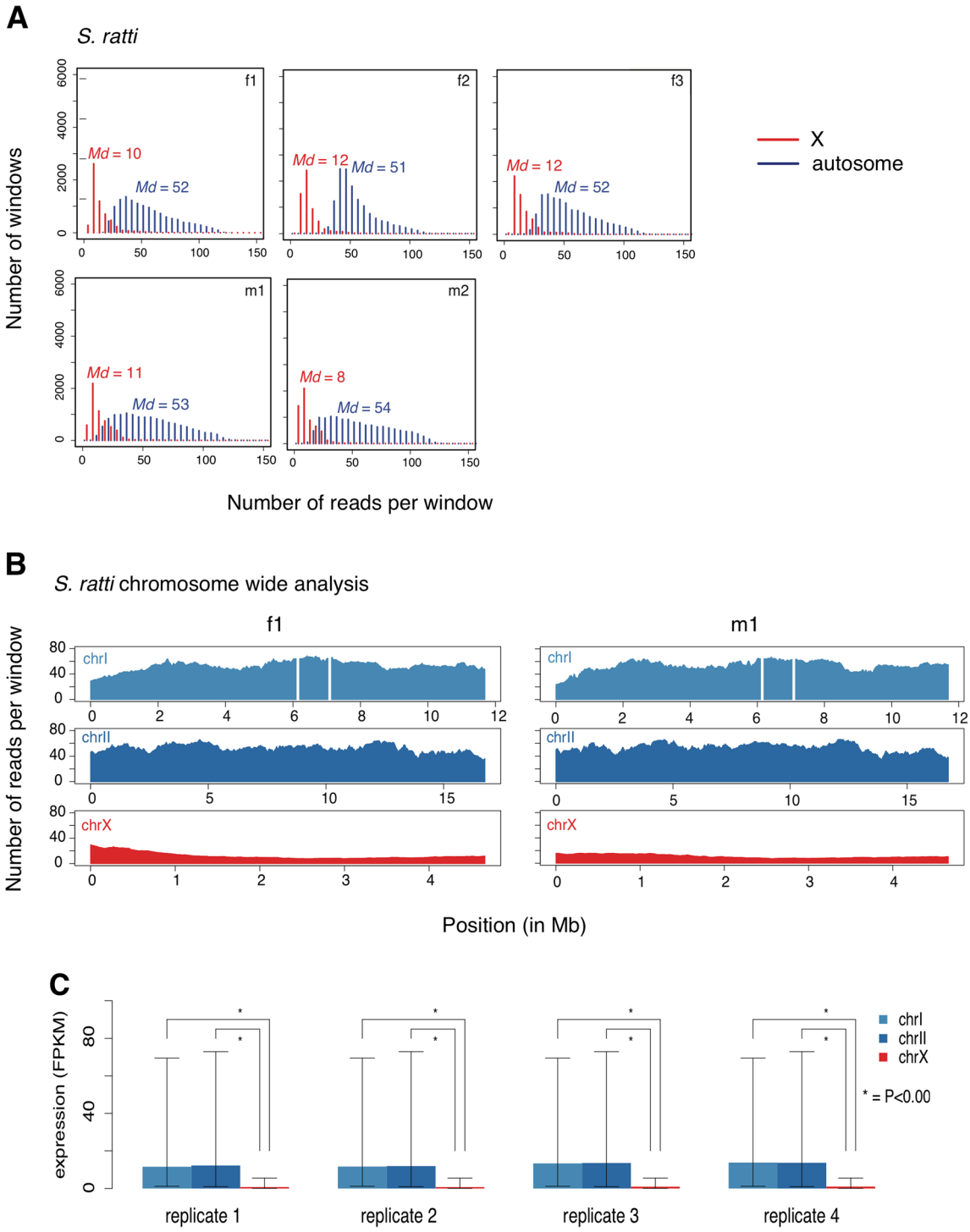


Fig. 3 DNA and RNA sequencing of *S. ratti* giant nuclei **a** Graphs indicate the genome-wide distribution of coverage (non-overlapping 2-kb windows) obtained from sequencing giant nuclei in *S. ratti* females and males, respectively (*f1*, *f2*, and *f3* indicate female biological replicates while *m1* and *m2* are male biological replicates). The higher coverage peak corresponds to the autosomes (*blue*) and the lower coverage peak is that of the X chromosome (*red*). *Md* indicates median values. **b** Chromosome-wide analysis of one female (*left*) and one male (*right*) replicate showing a relatively uniform amplification of DNA across the length of a chromosome (See Suppl Fig. 1a for the other replicates). The slight increase in coverage towards the left end of the X may or may not be real (see text). **c** Quantitative RNA sequencing from the giant nuclei in *S. ratti* female replicates shows that autosomal genes show a strong trend towards higher expression ($p<0.001$, Wilcoxon ranksum test), consistent with the underrepresentation of the X chromosomal genes compared with the autosomal ones

regions of chromosomes. In Fig. 3b, it appears as if sequences at the left end of chromosome X are present in somewhat higher copy number than the rest of the chromosome. However, the quality of the assembly of the X chromosome is not

as good as for autosomes, and for the moment, we cannot tell if the left end is indeed amplified more than the rest of the X or if this slight increase is artificially caused due to genome assembly problems. To determine whether the lower copy number of the X chromosomes is also reflected in the levels of gene products, we also isolated and sequenced the RNA of the distal portion of the gonads from *S. ratti* females. Indeed, X-derived mRNAs are on average much less abundant than transcripts encoded on autosomes (Fig. 3c, Suppl Fig. 1b) while there is no difference between the two autosomes.

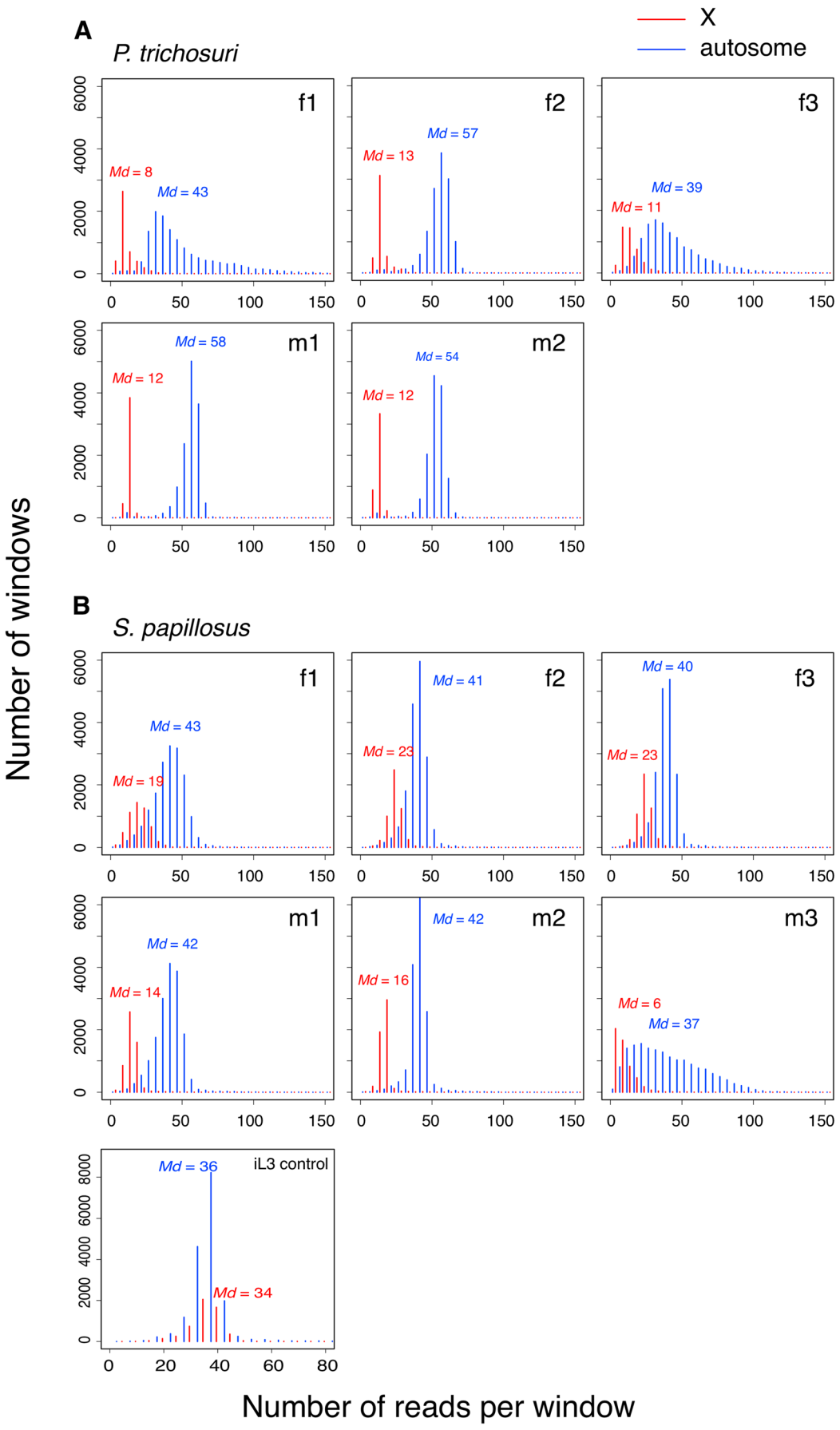
Presence or absence of male determining sperm

As explained in the "Introduction," reproduction in the free-living generation of the two species of *Strongyloides* in question is sexual but, unlike in *P. trichosuri*, produces only females. In order to address if genetically male-determining (nullo-X) sperm exists, we isolated mature sperm from free-living males of both species of *Strongyloides* and from *P. trichosuri* and we quantitatively sequenced the sperm genomes (Fig. 5a). As expected, in *P. trichosuri*, which produces both sexes, X chromosomal sequences are covered by only about half as many reads as autosomal regions indicating that X-bearing and nullo-X sperm are present in about equal numbers (Fig. 5a, top left panel). In *S. papillosus*, on the other hand, we did not observe an underrepresentation of the X-derived sequences, confirming the genetic findings (Fig. 5a, top right panel). Surprisingly, X chromosomal sequences were underrepresented in mature sperm in *S. ratti*, indicating that nullo-X sperm are present (Fig. 5a, bottom panels). It must, however, be noted that the two independent experiments, shown in Fig. 5a bottom panels, differed rather strongly. While in one experiment (Fig. 5a, bottom right) the under representation of the X chromosome was very clear and the difference was very close to the expected 50 %, in the second one, the difference was less than expected (Fig. 5a, bottom left). This might indicate that the proportion of nullo-X sperm formed might be variable over time or cultures. Alternatively, it may be a consequence of stochastic fluctuations, which have to be taken into account given the very small amount of starting material available. However, there is additional evidence for the existence of nullo-X sperm in *S. ratti*. In contrast to *S. papillosus* (Nemetschke et al. 2010a), we observed two different karyotypes among the very young embryos of *S. ratti*, namely with five or with six chromosomes, which correspond to the diploid number of chromosomes for males and females, respectively (Fig. 5b, Suppl Movies 3, 4, and 5). In addition, within the uteri of *S. ratti*, but not *S. papillosus* females, we observed dying embryos among normally developing ones (Fig. 5c). Dying embryos were observed at a frequency of 13 % (n=116). Although we do not know when exactly the embryos die and this number is therefore an underestimate, it is considerably less than the 50 % that would be expected if half of the sperm were nullo-X leading to non-viable embryos (see "Discussion"). For comparison with *S. papillosus*, 0 out of 55 embryos were scored to be developing abnormally.

Discussion

Giant non-dividing nuclei had been noticed and described in the distal gonads of *Strongyloides* spp. by multiple authors over the years (see, for example, Basir 1950; Hammond and Robinson 1994; Triantaphyllou and Moncol 1977). For *S. stercoralis*, it had also been proposed that the DNA content of these nuclei is as high as several hundred C and that the exact DNA amount per nucleus could not have resulted from a succession of consecutive full genome duplications (Hammond and Robinson 1994). This suggests that different regions of the genome are amplified to variable extents. All these earlier studies were based on cytological observations using DNA binding dyes, and no information about the genomic regions amplified was available. Here we show that in *S. ratti*, *S. papillosus* and *P. trichosuri* X chromosomal regions (in *S. papillosus* the evolutionarily X chromosome-derived portion of the larger chromosome) are present in lower copy numbers than autosomal regions. Interestingly, for *S. papillosus*, in which the X chromosome is fused with an autosome (Kulkarni et al. 2013; Nemetschke et al. 2010a), the difference is smaller. While in males this difference can be partially explained by the lower dose of the X due to XX/XO sex determination, in females, it must be caused solely by differential amplification. Quantitative RNA sequencing consistently confirmed that X-linked genes are expressed at lower levels on average than autosomal genes (shown for *S. ratti* females), suggesting that the differential DNA amplification contributes to the control of gene expression in the germline. Underexpression of X chromosomal sequences compared with autosomal ones also occurs in the gonads of the model nematode *C. elegans*, and this phenomenon appears to be widespread among nematodes (Kelly et al. 2002). In *C. elegans*, the expression differential appears to be due to differential chromatin modifications and consequentially differential transcription from equal copy numbers. By contrast, in *Strongyloides* spp., an increase in autosomal copies might be an important determinant for the higher expression of autosomal genes. However, the about five to six times lower copy number of X chromosomal genes, compared with autosomal ones, does not fully explain the more than ten times lower median expression of X-linked genes. There must be additional gene-specific and/or chromosome-wide control mechanisms at work.

In general, endoreplication in the germline seems to be less common than in other tissues, with the exception of amplification of ribosomal RNA genes, which can be construed as an adaptation for rapid oogenesis. Incidentally, the giant nuclei

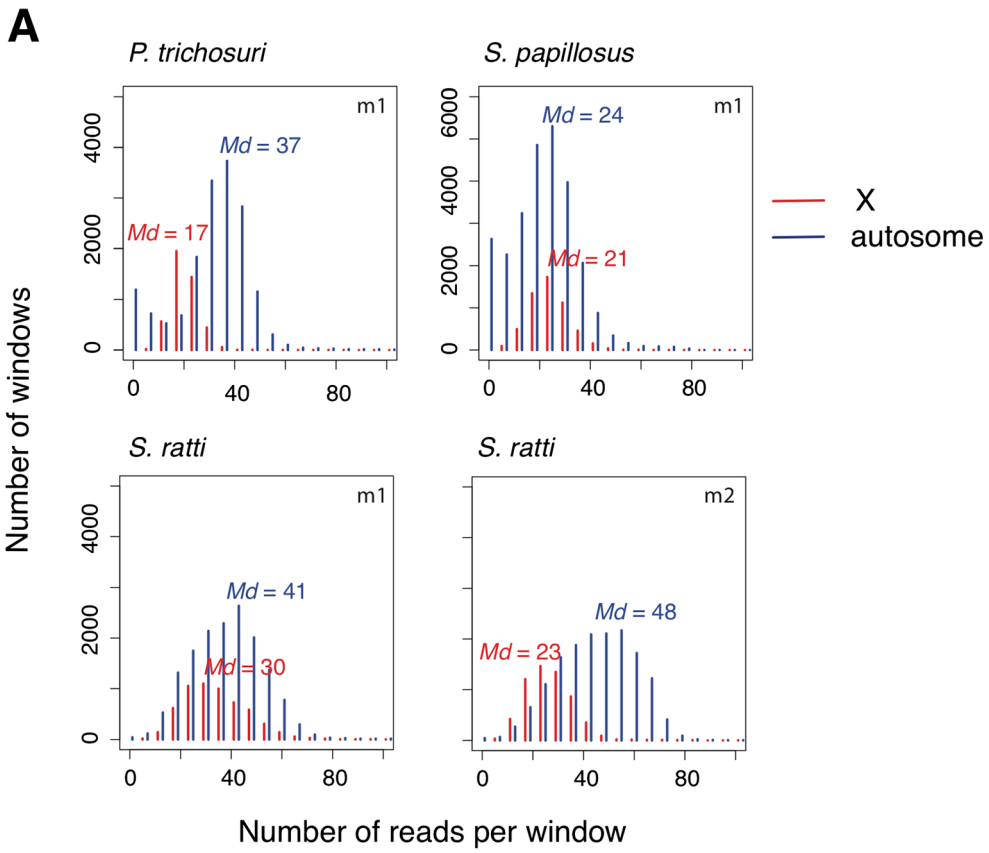

A
P. trichosuri
X
autosome
f1
Md = 8
Md = 43
f2
Md = 57
Md = 13
f3
Md = 39
Md = 11
m1
Md = 58
Md = 12
m2
Md = 54
Md = 12
B
S. papillosus
f1
Md = 43
Md = 19
f2
Md = 41
Md = 23
f3
Md = 40
Md = 23
m1
Md = 42
Md = 14
m2
Md = 42
Md = 16
m3
Md = 6
Md = 37
iL3 control
Md = 36
Md = 34
Number of windows
Number of reads per window

◀ **Fig. 4** DNA sequencing from *P. trichosuri* and *S. papillosus* giant nuclei. **a**, **b** Graphs plotting the genome-wide distribution of coverage (non-overlapping 2-kb windows) obtained from giant nuclei sequencing in *P. trichosuri* and *S. papillosus*, respectively (*f1*, *f2*, and *f3* indicate female biological replicates while *m1*, *m2*, and *m3* are male biological replicates). The higher coverage peak corresponds to the autosomes (*blue*) and the lower coverage peak is that of the X chromosome (*red*). The panel iL3 (*bottom left*) shows the equal coverage of autosomal and X chromosomal sequences obtained from sequencing infective larvae of *S. papillosus*, which are all female and lack giant nuclei in their gonads. This experiment serves as a control demonstrating that underexpression of the X chromosomes compared to the autosomes in the panels above is not a consequence of some feature of the X chromosome rendering it inefficient for sequencing. *Md* indicates median values

have previously been proposed to act as nurse cells supporting the development of oocytes (Hammond and Robinson 1994), a role that in *C. elegans* is assumed by early meiotic cells in pachytene (Hubbard and Greenstein 2005). In this regard, it is interesting to note that one of the best-characterized nurse cells, those found in the egg chambers of the fruit fly, also amplify their genome (Bastock and St Johnston 2008; Lee et al. 2009). On closer inspection of our RNA data, we observed high expression of mRNAs encoding ribosomal components, chaperones, and proteasome components, among others. These signs of high gene expression activity further support that the giant nuclei of *Strongyloides* and *Parastrongyloides* act as nurse cells. An interesting though somewhat puzzling observation is the reduction in number of the giant nuclei in females with age. One possible explanation would be that some of them are broken up into small, diploid nuclei, thereby replenishing the population of germ cells available for oogenesis. Such a process has been proposed for certain snakes (Becak et al. 2003), and it was shown that some endopolyploid tumor cells use a meiosis-related mechanism to revert to normal diploidy (Erenpreisa et al. 2009; Ianzini et al. 2009). In fact, we noticed a significant ($p<0.01$ in a t test) increase in small nuclei number from around 78 (±24.4 [standard deviation]) per arm in young *S. papillosus* females ($n=26$ arms) to about 97 (±17.2) at peak reproductive age ($n=36$ arms). The corresponding numbers for *S. ratti* were 56 (±16.0) in young ($n=18$) and 83 (±19.1) in older ($n=20$) worms. Nevertheless, we failed to observe any mitotic figures or condensed mitotic chromosomes in this region, a somewhat puzzling observation, which has however been reported before (Triantaphyllou and Moncol 1977). Alternatively, some giant nuclei may undergo apoptosis or fuse with each other, as suggested by the elongated shape of these nuclei (Fig. 2d giant nuclei insets, Suppl Movies 1 and 2). However, for the moment, these hypothetical explanations remain speculative and the actual dynamics of the giant nuclei in *Strongyloides* spp. will need to be investigated in live worms. To this end, GFP-tagged histone proteins expressed from transgenes, as has been established in *C. elegans* (Praitis et al. 2001), will be advantageous. For the moment, although transgenic techniques for *Strongyloides* spp. have been developed (Lok 2013), no germline-expressed promoters are available.

Most species of *Strongyloides* do not produce males in the progeny of the free-living generations (Streit 2008). Based on genetic arguments for *S. papillosus* (Nemetschke et al. 2010a), it was proposed that *Strongyloides* spp. males do not produce male-determining (nullo-X) sperm. However, DNA quantification using DNA binding dyes in *S. stercoralis* (Hammond and Robinson 1994) provided evidence that some species of *Strongyloides* might produce such sperm. While our quantitative sequencing of mature sperm confirmed absence of male-determining sperm for *S. papillosus*, we did find evidence for the presence of nullo-X sperm in *S. ratti*. Additionally, we also found early embryos with a male karyotype in this species. This might suggest that these two species use different strategies to prevent males among the infective larvae, either by avoidance of male-determining sperm as in *S. papillosus* or by inviability of male embryos as in *S. ratti*. In addition, nullo-X sperm might be less successful in fertilizing oocytes, reducing the number of "wasted" non-viable embryos. However, the difference might also be only quantitative and it might depend as much on the isolate as on the species. A very small proportion of nullo-X sperm (more precisely, sperm not containing a copy of the region undergoing chromatin diminution) in *S. papillosus* would probably have gone unnoticed in the earlier studies as our observations indicate that the number of nullo-X sperm in *S. ratti* might vary among experiments. A mechanism for producing an (variable) excess of X-bearing sperm has been described for the nematode *Rhabditis* sp. SB347 (Shakes et al. 2011). Therefore, it is also possible that both mechanisms are at work in both species but to variable extents.

The results presented here, on one hand, enhance our understanding of the reproductive biology of a fascinating group of parasitic nematodes. On the other hand, they also illustrate the usefulness of the *Strongyloides*/*Parastrongyloides* system for comparative evolutionary studies over very different phylogenetic distances. It will be highly revealing to further study the interesting differences (representing evolutionary changes) within the group and in comparison to other nematode model systems in evolutionary biology, in particular *Caenorhabditis* spp. and *Pristionchus* spp. (Sommer and Bumbarger 2012).


A
P. trichosuri
S. papillosus
S. ratti
S. ratti
m1
m1
m1
m2
Md = 37
Md = 17
Md = 24
Md = 21
Md = 41
Md = 30
Md = 48
Md = 23
X
autosome
Number of windows
Number of reads per window
B
S. ratti male karyotype
(2n = 5)
S. ratti female karyotype
(2n = 6)
C
S. ratti
S. papillosus

◀ **Fig. 5** X chromosomes in sperm of *P. trichosuri*, *S. papillosus*, and *S. ratti*. **a** DNA sequencing of mature sperm showing genome-wide distribution of coverage (non-overlapping 2-kb windows) of the autosomes (*blue*) compared with the X chromosome (*red*) of *P. trichosuri* (*m1*, *top left*), *S. papillosus* (*m1*, *top right*), and *S. ratti* (*bottom panels m1* and *m2*), respectively. The X chromosome is underrepresented in *P. trichosuri* and *S. ratti* indicating presence of nullo-X sperm in these species. *S. ratti* (*bottom panels*, *m1* and *m2*) shows variability in the amount of nullo-X sperm between replicates. *Md* indicates median values. **b** The two distinct karyotypes seen in early embryos of free-living *S. ratti* females, with $2n=5$ the expected male karyotype (*top*) and $2n=6$ the female karyotype (*bottom*). Also see Suppl Movies 3, 4, and 5. **c** Dying or abnormal embryos as seen in DAPI-stained free-living *S. ratti* females (*top*), marked with green *asterisks*, amid normally developing ones. In contrast no such abnormal embryos are observed in *S. papillosus* (*bottom*)

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Compliance with ethical standards

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Animal experiments All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Gain and Loss of Small RNA Classes—Characterization of Small RNAs in the Parasitic Nematode Family Strongyloididae

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Abstract

The nematode family Strongyloididae is of particular interest because it contains important parasites of medical and veterinary relevance. In addition, species of this family can form parasitic and free-living generations and it also occupies an interesting phylogenetic position within the nematodes. Nematodes differ in several ways from other taxa with respect to their small noncoding RNAs. Recent comparative studies revealed that there is also considerable variability within the nematodes. However, no Strongyloididae species or close relative was included in these studies. We characterized the small RNAs of two developmental stages of three different Strongyloididae species and compared them with the well-studied free-living nematodes *Caenorhabditis elegans* and *Pristionchus pacificus*. Strongyloididae have conserved and taxon-specific microRNAs, many of which are differentially regulated between the two developmental stages. We identified a novel class of around 27-nucleotide-long RNAs starting with 5′G or A, of which a large fraction have the potential to target transposable elements. These RNAs most likely have triphosphates at their 5′ ends and are therefore presumably synthesized by RNA-dependent RNA polymerases. In contrast to *C. elegans* but similarly to some other nematode taxa, Strongyloididae have no Piwi-interacting RNAs, nor do their genomes encode Argonaute proteins of the Piwi family. Finally, we attempted but failed to detect circulating parasite small RNAs in the blood of hosts.

Key words: nematodes, parasites, piRNAs, miRNAs, secondary siRNAs, TAP-treatment.

Introduction

Nematodes exhibit a wide variety of lifestyles ranging from soil-dwelling or marine free-living species feeding on bacteria, fungi, and/or other nematodes, to parasitic species infecting all kinds of animals and plants through different mechanisms (Lee 2002; Perry and Wharton 2011). Reproductive modes vary across the nematode clades, with gonochoristic, hermaphroditic, and parthenogenetically reproducing species found amongst the more than 23,000 nematode species that have been described so far. Phylogenetic analysis of free-living and parasitic nematodes (Blaxter et al. 1998; Holterman et al. 2006) led to the conclusion that parasitism must have evolved independently several times within this phylum (Blaxter et al. 1998; Dorris et al. 1999; Blaxter 2011). This diversity of lifestyles must have arisen from evolutionary adaptations in conserved and novel molecular, developmental and physiological processes, all of which are associated with changes in the genome organization and in gene expression. Small noncoding RNAs (sRNAs), produced through multiple regulatory pathways, play important roles in the regulation of these processes, in particular the fine-tuning of the expression of a wide variety of genes (Ghildiyal and Zamore 2009). They are therefore likely targets for evolution to act on during the adaptation to the different lifestyles. Indeed, some interesting differences in the sRNA complements between nematodes and other phyla, and between different nematode taxa have been described (Czech and Hannon 2011; Wang et al. 2011; Sarkies and Miska 2014; Sarkies et al. 2015). Within the animal kingdom, three major conserved classes of sRNAs (18–35 nucleotides [nt]) have been described: microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), and small-interfering RNAs (siRNAs) (Czech and Hannon 2011). These different sRNA classes are defined by their biogenesis and their interaction with different proteins of the Argonaute family. They play important roles in transcriptional and posttranscriptional gene regulation and genome maintenance (Carthew and Sontheimer 2009; Ghildiyal and Zamore 2009; Moazed 2009; Sabin et al. 2013). The

nematode *Caenorhabditis elegans* is one of the best-studied organisms with respect to sRNA families and their Argonaute partners (Buck and Blaxter 2013; Billi et al. 2014; Sarkies and Miska 2014). Known classes of small RNAs in *C. elegans* include the conserved miRNAs (20–23 nt), which are transcribed as single-stranded precursor RNAs with a secondary hairpin structure. This precursor is then processed by Drosha and Dicer resulting in the mature miRNA, which binds to the Ago subfamily of Argonautes (Sarkies and Miska 2014). Many miRNAs from *C. elegans* are conserved even in humans. They repress translation or promote degradation of mRNAs, with many of them involved in controlling important developmental transitions (Kaufman and Miska 2010). Another conserved sRNA class in *C. elegans* is the piRNAs, also called 21 U RNAs because they are 21 nt long and start with a 5′U. They target and silence transposable elements in the germline (Siomi et al. 2011; Ku and Lin 2014). 21 U RNAs are transcribed as short (26–30 nt) precursors, which are processed to give rise to mature 21 U RNAs (Ruby et al. 2006; de Wit et al. 2009; Gu et al. 2012; Weick et al. 2014). Each 21 U RNA is transcribed from its own transcription unit and many of them contain a conserved ~8 nt upstream motif (Ruby motif). Most 21 U RNA coding units are located in one of two large clusters on chromosome IV.

A third class of sRNAs in *C. elegans* is the primary siRNAs. Endogenous primary siRNAs are around 26 nt in length and have a strong bias toward a 5′ G (hence 26 G RNAs) and their biogenesis is dependent on an RNA-dependent RNA polymerase (RdRP) and Dicer (Han et al. 2009; Thivierge et al. 2011; Billi et al. 2014). 26 G RNAs are enriched in the gonad (Han et al. 2009; Thivierge et al. 2011; Billi et al. 2014). Exogenous primary siRNAs of 21–22 nt in length are formed upon challenge with exogenous double-stranded RNA through the action of Dicer (Sarkies and Miska 2014).

Both the primary siRNAs (exogenous and endogenous) and the piRNAs (21 U RNAs) can trigger the production of secondary siRNAs, thereby amplifying and enhancing their action. Secondary siRNAs in *C. elegans* are around 22 nt long and have a 5′ G (22 G RNAs) (Pak and Fire 2007; Sijen et al. 2007; Gu et al. 2009; Vasale et al. 2010; Ashe et al. 2012; Lee et al. 2012; Shirayama et al. 2012;). According to their differing biogenesis, the different sRNA classes vary in their lengths, their 5′-nucleotide biases toward certain nucleotides and in their 5′- and 3′-end modifications. miRNAs, 21 U and 26 G RNAs have 5′-monophosphates, due to the fact that their mature 5′ ends are generated through endonucleolytic cleavage. In contrast, the secondary siRNAs (22 G RNAs) are produced by nematode-specific RdRPs without 5′ endonucleolytic processing, resulting in 5′-triphosphates (Pak and Fire 2007; Sijen et al. 2007; Billi et al. 2014).

Although no other nematode has been studied as well as *C. elegans*, it has recently emerged that both nematodes small RNAs and their resulting biological functions are surprisingly diverse. For example, PIWI proteins and piRNAs have apparently been lost independently in multiple branches of the nematode phylogenetic tree, and 5′-triphosphorylated small RNAs were found in nematodes of clades III–V but not clade I (Wang et al. 2011; Sarkies et al. 2015).

The phylogenetic clade IV of nematodes (according to Blaxter et al. 1998) is of particular interest because it contains free-living, entomopathogenic, plant parasitic, and animal parasitic species. The animal parasitic clade IV family Strongyloididae consists of the two fairly closely related genera: *Strongyloides* and *Parastrongyloides*. *Strongyloides* spp. are obligate small intestinal parasites of vertebrates which can undergo facultative single nonparasitic generations with males and females between parthenogenetic parasitic generations (Viney and Lok 2015). *Parastrongyloides* spp. are facultative parasites of various marsupials with their best-studied representative, *Parastrongyloides trichosuri*, parasitizing Australian Brush-tailed Possums (Grant et al. 2006). The Strongyloididae have enjoyed a dramatic increase in scientific attention over the last few years, mainly because of two reasons. Firstly, this family contains *Strongyloides stercoralis*, one of the most prevalent nematode parasites of humans. Although its medical relevance had been underestimated for a long time, it is now increasingly appreciated as an important pathogen in poor communities with unsatisfactory sanitary conditions, and as an important complication in the context of immunosuppressive treatments for cancer and organ transplantation patients (Bisoffi et al. 2013; Nutman 2017). Secondly, with its interesting life cycle (supplementary fig. 1A–C, Supplementary Material online) at the interface of free-living and parasitic lifestyles, this family represents a highly interesting set of model species for basic biological and translational research (Lok 2007; Viney 2017; Baskaran et al. 2017). Recently, high-quality draft genomes of *Strongyloides ratti*, *Strongyloides venezuelensis* (which are both parasites of rats), *S. stercoralis*, *Strongyloides papillosus* (parasite of sheep), and *P. trichosuri* have been published (Hunt et al. 2016). The authors of this article also analyzed and compared the gene expression between different developmental stages and different species. However, this analysis is limited to mRNAs and protein coding genes and does not include data for sRNAs. To our knowledge, only a single analysis of the small RNAs of *S. ratti* has been published (Ahmed et al. 2013). However, this study focused on miRNAs and used methodology that included only small RNAs with 5′ monophosphates and therefore would have missed 5′ triphosphorylated sRNAs, which in other nematodes form a large portion of the sRNAs. In a recent extensive analysis of all known classes of small RNAs of numerous nematodes neither representatives of the family Strongyloididae nor of any other animal parasitic species of clade IV were included (Sarkies et al. 2015).

In order to compare the sRNA complement of the Strongyloididae, which are not only important parasites but also occupy an interesting phylogenetic position, with the sRNAs of other nematodes, we characterized the small RNA

contents of two developmental stages (free-living adults and infective larvae) of three different species of Strongyloididae (*S. ratti*, *S. papillosus*, and *P. trichosuri*). For comparison, we also reanalyzed the sRNAs of adult hermaphrodites and dauer larvae of the two well-studied free-living nematodes *C. elegans* and *Pristionchus pacificus*. Dauer larvae in *C. elegans* and *P. pacificus* and infective larvae in Strongyloididae are believed to be evolutionary equivalents (Ogawa et al. 2009; Wang et al. 2009; Crook 2014). Like *C. elegans* and *P. pacificus*, Strongyloididae have a prominent class of small RNAs with 5′ triphosphates. However, different from *C. elegans*, *P. pacificus*, and any other nematode analyzed so far, these RNAs are longer and do not show the strong bias toward a G nucleotide at their 5′-end. Further, Strongyloididae possess no class of small RNAs that show the typical features of piRNAs and, consistently, there are no recognizable orthologs of PIWI protein genes present in their genomes.

Materials and Methods

Nematode Strains, Culture Conditions, and Sample Collection

Wild-type strains of five different nematode species were used for this study: *C. elegans* N2 (received from the *C. elegans* Genetics Center at the University of Minnesota), *P. pacificus* RS2333 (received from Ralf J. Sommer), *S. ratti* ED321 (Viney 1996), *S. papillosus* LIN (Eberhardt et al. 2007), *P. trichosuri* (received from Warwick N. Grant, for description of isolate, see Kulkarni et al. (2013)).

Caenorhabditis elegans and *P. pacificus* were maintained on 6 cm plates with Nematode Growth Medium (NGM) agar on a lawn of *Escherichia coli* OP50 at 20 °C as previously described (Brenner 1974; Stiernagle 2006). For mixed-stage *P. pacificus* cultures, three adults per plate were picked onto 6 cm NGM plates and grown at 20 °C for 7 days. For mixed-stage *C. elegans* cultures, ~50 L1/L2 larvae were picked onto 6 cm NGM plates and grown at 20 °C for 3 days. To obtain adult hermaphrodites, mixed-stage worms from twenty 6 cm plates were washed off with 0.1 M NaCl into a 50 ml Falcon tube, and gravid adult worms were allowed to sink to the bottom of the tube for 3–5 min. The supernatant (with young larvae) was removed and the remaining worm pellet was resuspended in 50 ml 0.1 M NaCl. The washing and settling was repeated three times. After the washing, the supernatant was removed and the pellet of worms transferred into a 1.5 ml Eppendorf tube and filled with 0.1 M NaCl. The adult worms were finally pelleted by centrifuging for 30 s and as much supernatant removed as possible. *Caenorhabditis elegans* and *P. pacificus* dauer larvae were obtained from liquid culture (Lewis and Fleming 1995): Mixed-stage worms from thirty 6 cm NGM plates were inoculated into 150 ml S-medium without Cholesterol containing 1% (w/v) OP50, 50 μg/ml Streptomycin and 50 μg/ml Nystatin and grown at 22 °C and 180 rpm for 8 days. For the first replicate of dauer cultures, clean dauer larvae were obtained by washing with 0.1 M NaCl as mentioned before. For the second and third replicate of dauer cultures, dauer larvae were purified by sucrose flotation and Ficoll precipitation (http://diamond.tuebingen.mpg.de/wiki/index.php/Dauer_purification_by_Sucrose_Floatation_and_Ficoll_precipitation (last accessed October 2, 2017)).

Strongyloides ratti and *S. papillosus* were maintained under laboratory conditions in female Wistar rats or female New Zealand White rabbits, respectively, as previously described (Viney et al. 1992; Eberhardt et al. 2007). All relevant national and international animal welfare regulations and guidelines were followed. The experiments were approved by the Regierungspräsidium Tübingen (AZ: 35/9185.82-5/15.07.2015). *Strongyloides ratti* and *S. papillosus* adults (males and females) were obtained from 48 h fecal cultures at 19 °C for *S. ratti* and 25 °C for *S. papillosus*. Adult free-living worms were isolated from fecal cultures using the Baermann technique (2–3 h) (Lok 2007) and washed with tap water. Young larvae were removed by letting worms sink down for 3–5 min in 50 ml Falcon tubes filled with tap water. The supernatant (with young larvae) was removed and the remaining worm pellet was resuspended in 50 ml of tap water. The washing and settling was repeated three times. After the washing the supernatant was removed and the pellet of worms transferred into a 1.5 ml Eppendorf tube and filled with water. The adult worms were finally pelleted by centrifuging for 30 s and as much supernatant removed as possible. *Strongyloides ratti* infective larvae were obtained from 7-day fecal cultures using the Baermann technique (overnight) and washed three times with tap water. *Strongyloides papillosus* infective larvae were obtained from 7-day cultures as described (Eberhardt et al. 2008). In brief, 48 h fecal culture petri dishes were placed in a larger petri dishes with tap water. The infective larvae that accumulated in the water were collected using the Baermann technique (overnight) and washed three times with tap water. *Parastrongyloides trichosuri* was maintained in continuous free-living cycles on 6 cm NGM plates with a piece of autoclaved rabbit feces on a lawn of *E. coli* OP50 at 20 °C (Grant et al. 2006; Kulkarni et al. 2013). For mixed-stage *P. trichosuri* cultures, ~100–200 adults were picked onto NGM plates and grown at 20 °C for 8 days. As the worms tend to burrow into the agar, the agar of 5–10 plates was chopped into smaller pieces and the worms isolated using the Baermann technique (2 h) with M9 buffer. The worms were transferred to 50 ml Falcon tubes and adult worms were allowed to sink to the bottom of the tube for 3–5 min. The supernatant (with young larvae) was removed and the remaining worm pellet was resuspended in 50 ml M9 buffer. The washing and settling was repeated three times and the final worm pellets obtained as in *C. elegans*. *Parastrongyloides trichosuri* infective larvae were obtained by picking ~100–200 adults onto NGM plates and grown at 20 °C for 15 days. For each replicate, 10–20

plates were used and infective larvae isolated and washed like the adults.

Collection of Whole Blood from Rats Infected and Uninfected with *S. ratti*

Three 4-week-old female Wistar rats (Charles River) were infected with ~500 infective larvae from the *S. ratti* strain ED231 in 200 µl PBS as previously described (Viney et al. 1992). At the same time, two 4-week-old rats were mock-infected with 200 µl PBS. On the 8th day postinfection, the rats were first sedated with CO_2 for ~3 min and then killed by cervical dislocation. The thoracic cavity of the rats was opened, the heart was cut with a clean scalpel blade and whole blood obtained from the blood puddles forming in the cavity. The blood was collected in RNAprotect Animal Blood Tubes (500 µl Qiagen) and incubated for 5 h at room temperature. All relevant national and international animal welfare regulations and guidelines were followed. The experiments were approved by the Regierungspräsidium Tübingen (AZ: 35/9185.82-5/15.07.2015, Anzeige vom 13.10.2016).

RNA Extraction

For the nematodes, 1 ml TRIzol (Thermo Fisher Scientific) was added to each worm pellet and briefly grinded with a pestle. The mixture was then frozen in liquid nitrogen and thawed at room temperature three times. RNA of adult worms was extracted with the standard TRIzol protocol followed by a separation of large and small RNAs with the PureLink miRNA Isolation Kit (Thermo Fisher Scientific). RNA of dauer/infective larvae was extracted the same as adult RNA but the small RNA fraction contained a lot of large RNAs and was reseparated into large and small RNA fractions using the RNA Clean & Concentrator kit (Zymo Research). For the mouse blood, the total RNA (including short RNAs) from whole blood of infected and uninfected rats was extracted with the RNeasy Protect Animal Blood kit (Qiagen) according to the manufacturer's protocol. Subsequently, the large RNA and small RNA fractions were obtained with the RNA Clean & Concentrator kit (Zymo Research). The quality and quantity of the extracted RNA fractions was assessed using 1% Agarose gels, 2100 Bioanalyzer RNA 6000 Nano chips (Agilent Technologies), and Qubit RNA BR Assays (ThermoFisher Scientific) according to the manufacturer's protocol. The RNA was stored at –80 °C until library preparation.

Library Preparation

Following RNA isolation, each small RNA replicate was divided, and one part was treated with 25 U tobacco acid pyrophosphatase (Epicenter) or Cap-Clip acid pyrophosphatase (Biozym) in 50 µl volume for 2 h at 37 °C to remove 5′-triphosphates and allow 5′-independent library preparation. The other part was mock treated under the same conditions (5′-dependent library preparation). From those treated or untreated small RNA fractions, libraries were prepared with the TruSeq Small RNA Library Preparation Kit (Illumina) according to the manufacturer's protocol. cDNA constructs containing both adapters were cut from a gel by hand in the range of ~140–160 base pairs (bp) corresponding to sRNAs with lengths in the range of ~17–37 nt. Every library was validated with 2100 Bioanalyzer DNA 1000 chips.

Small RNA Sequencing and Read Preprocessing

Forty-eight samples were sequenced on the same flowcell on a HiSeq 3000 (Illumina; 150 bp single-end). Eighteen samples were sequenced in four batches on a MiSeq (Illumina; 50 bp single-end). See supplementary table 1, Supplementary Material online for more detailed information on the nematode data set and supplementary table 2, Supplementary Material online for information on the whole blood samples from infected and uninfected rats. Samples were demultiplexed, adapter sequences were removed with flexbar (v2.5; parameters '-u 100 -m 14 -at 1 -n 8 -ag -7 -ao 3') (Dodt et al. 2012) and only reads with 14 nt or longer retained. Fastq files were converted to fasta files using FASTX Toolkit (v0.0.14). All read data will be made publically available through appropriate databases upon acceptance of the manuscript.

miRNA Prediction and Quantification

miRDeep2 (v0.0.7) (Friedlander et al. 2012) was used for novel miRNA prediction and quantification of known miRNAs. The full initial list of predicted novel miRNAs was filtered against a custom database containing tRNAs, rRNAs, snoRNAs, snRNAs, 21 U RNAs, and repeats. All sequences with a miRDeep2 score <2 and/or matching the database and/or showing a ratio of 5′-all-phosphate to 5′-monophosphate read counts ≥ 1 were excluded. miRBase release 21 was used for reference precursors and mature miRNAs. A 10 nt genomic sequence on the 5′ and 3′ end of all precursors was added to ensure complete capture of miRNA-related reads. Mature miRNAs arms were defined by calculating the ratio of read counts matching to the 5p- or 3p-arm (all sample counts combined, 1 pseudocount was added to avoid division by zero). For ratios >1, the 5p-arm was considered to be the mature miRNA; for ratios <1, the 3p-arm (supplementary table 3, Supplementary Material online).

miRNA Classification into Seed-Families and Inference of Phylogenetic Trees

The known and novel miRNAs of all species were grouped into families based on the identity of their seed sequences (positions 2–8 of the mature miRNA; see supplementary table 4, Supplementary Material online). For the conserved miRNA seed families, seed-constrained multiple sequence alignments

were constructed using LocARNA (Will et al. 2007, 2012), which takes the predicted secondary structure of the miRNAs into account. For each miRNA group, the similarity matrix (LocARNA scores) was transformed into a distance matrix as described in Will et al. (2007), and used to infer phylogenetic trees with the Unweighted Pair Group Method with Arithmetic Mean hierachical clustering method (upgma, R package phangorn v1.99-7 (Schliep 2011)). The resulting phylogenetic trees were combined with our miRNA expression fold changes between dauers/infectives and adults, and the 5p/3p ratio information of miRNA arm usage, into plots with R.

sRNA Classification

All sRNA reads were classified into four classes: tRNA-, rRNA-, miRNA-derived, or other. For this classification, all reads were aligned to defined tRNA, rRNA, miRNA precursors, and finally to the respective genome assemblies (see supplementary table 5, Supplementary Material online). tRNAs were predicted for all species genomes using tRNAscan-SE (v1.3.1; default parameters) (Lowe and Eddy 1997). For the alignments, bowtie was used with no allowance for mismatches (v1.0.0; parameters '-v 0 –k 1 -a –best –strata') (Langmead et al. 2009).

Length Distribution and 5′ Nucleotides

Custom Shell and Perl scripts were used to get the length and first nucleotide of all reads. The output was read into R to calculate means and generate barplots.

Differential Expression Analysis

MicroRNAs

The miRDeep2 quantifier module was used to quantify reads from untreated (5′-monophosphate) libraries representing either dauers/infectives or free-living stages in triplicates for each of the five species. The sum of read-counts on the 3p-arm and 5p-arm of each miRNA was calculated for each sample. For the differential expression analysis of miRNAs, the R package edgeR (v 3.14.0) was used (Robinson et al. 2010). For this analysis, only miRNAs that have at least 1 read per million (miRNA mapping reads) in at least three samples were kept. The data were TMM (trimmed mean of M values) normalized (Robinson and Oshlack 2010), and differential expression determined using the generalized linear model (GLM) quasi-likelihood (QL) *F*-test (Lund et al. 2012). Up- and downregulated miRNAs are defined as significantly differentially expressed if they have a log2 fold change of ≥ 1 and ≤ -1, respectively and a false discovery rate (FDR) $<5\%$ (supplementary table 3, Supplementary Material online).

Other-Mapped Reads

All other-mapped reads from all samples were filtered, and only reads with a single unambiguous match in the respective genomes were kept. Other-mapped reads falling on annotated mRNAs were then counted with htseq-count from the HTSeq framework (v0.6.0; parameters '-s no -m union') (Anders et al. 2015). For the differential expression analysis of the other-mapped reads, R package edgeR (v 3.14.0) (Robinson et al. 2010) was used. For the analysis, only those genes that have at least 1 read per million in at least three samples were kept and 5′-all-phosphate and 5′-monophosphate samples were compared. The data were TMM normalized (Robinson and Oshlack 2010), and differential expression determined using the GLM QL *F*-test (Lund et al. 2012). Up- and downregulated target genes are defined as significantly differentially expressed if they have a log2 fold change of ≥ 1 and ≤ -1, respectively and an FDR $<5\%$. In order to investigate if tobacco acid pyrophosphatase (TAP)-treatment enriches sRNAs specifically matching to certain protein families containing specific protein domains (PFAM v.29), an hmmsearch of annotated proteins (supplementary table 5, Supplementary Material online) against the PFAM v.29 HMM library was performed. Gene set enrichment analysis was done in R with camera (Competitive Gene Set Test Accounting for Intergene Correlation (Wu and Smyth 2012)) from the package limma (v. 3.28.14). Significantly (FDR $< 5\%$), enriched protein families that are up- or downregulated between 5′-all-phosphate and 5′-monophosphate samples were compared bewteen all five nematode species.

Investigation of Other-Mapped Reads and Their Abundance on Annotated Genes

All other-mapped reads from all samples were filtered, and only reads with a single unambiguous match in the respective genomes were kept. Other-mapped reads mapping sense or antisense on annotated mRNAs (in case of *C. elegans* also transposons) were counted using htseq-count from the HTSeq framework (v0.6.0; parameters '-s yes/reverse -m union') (Anders et al. 2015). The percentage of alignments being sense, antisense, or not aligned to an annotated gene was then calculated.

Comparison of Our miRNA Expression Changes to Published Data from Illumina and SOLiD Sequencing

miRNA expression changes was compared with published data obtained by Illumina and SOLiD sequencing of dauers/infectives and mixed-stages of *C. elegans*, *P. pacificus*, and *S. ratti* (Ahmed et al. 2013). For all miRNA genes that were shared and highly enough expressed in both data sets (at least three reads per million in three samples), the expression changes were calculated into up-, downregulated, and unaffected. The miRNA genes were then grouped into a 3×3 contigency table. A χ^2 test was then performed in R to assess statistical independence of the two data sets.

Calculation of Genome Coverage of Potential piRNAs

All other-mapped reads from all samples were filtered and only reads with a single unambiguous match in the respective genomes, a 5′U and 21 (*C. elegans*) or 20–24 nt (*S. ratti* and *S. papillosus*) length were considered. samtools (v. 0.1.19 (Li et al. 2009)) and bedtools (v. 2.17.0 (Quinlan and Hall 2010)) were used to calculate the number of reads matching 100 kb bins of the genomes (considering only scaffolds with >100 kb length). The number of reads was normalized to reads per million in relation to all mapped reads (tRNA-, rRNA-, miRNA-, and other-mapped). The coverage was plotted with R.

piRNA Motif Detection

All other-mapped reads from all samples were filtered and only unique sequences with a single unambiguous match in the respective genomes, a 5′U and 21 (*C. elegans* and *P. pacificus*) or 20–24 nt (Strongyloididae) length were kept. The filtered sequences from adults were merged, and the 60 nt upstream and the first 2 nt of each location of these sequences were taken. meme (v. 4.11.1 (Bailey and Elkan 1994; Bailey et al. 2015), -dna -oc -maxsize 3000000 -mod zoops -nmotifs 1 -minw 4 -maxw 62 -p 64) was run on a random subset of 5,000 upstream sequences for each species to discover a common motif. fimo (v. 4.11.1 (Bailey et al. 2015; Grant et al. 2011), –oc –verbosity 1 –thresh 1.0E-4 –norc) was used to detect the predicted motifs in the entire sets of upstream sequences, and those which have at least one hit with a *P*-value of $\leq 1 \times 10^{-4}$ were counted.

Phylogenetic Analysis of Argonautes and RdRPs

To obtain a list of RdRPs and Argonaute proteins in *C. elegans*, *P. pacificus*, *S. ratti*, *S. papillosus*, and *P. trichosuri*, a jackhmmer (HMMER 3.1b1 (Eddy 2011)) search with five iterations (default options) against the accessions in supplementary table 5, Supplementary Material online using the protein sequences of *C. elegans* rrf-1 (Wormbase ID CE27141) and alg-1 (Wormbase ID CE31525) was performed. For Argonautes, the same search was also performed in *Homo sapiens*, *Mus musculus*, and *Drosophila melanogaster*. This resulted in RDRP orthologs with *E*-values $<2 \times 10^{-46}$, and Argonaute orthologs with $<2 \times 10^{-8}$. Multiple sequence alignments were then performed using MUSCLE with default settings (Edgar 2004). As outgroups for RdRPs, *Arabidopsis thaliana* (RDR1-6; GenBank accessions OAP18817.1, AEE82976.1, O82190.2, O82189.2, O82188.2, AEE78550.1) and *Saccharomyces pombe* (RDP1, GenBank accession CAB11093.1) RdRPs were used. As outgroups for the PIWI Argonautes, *H. sapiens* (PIWIL1 and PIWIL4; GenBank accessions Q96J94.1 and AAH31060.1), *M. musculus* (PIWIL1-2; GenBank accessions AAI29859.1 and AAK31965.1), and *D. melanogaster* (PIWI and AUBERGINE; GenBank accessions AGL81535.1 and CAA64320.1) PIWIs and the Argonaute protein from the Archaea *Pyrococcus furiosus* (PDB: 1Z25) were used. Unrooted trees were constructed with the outputs of MUSCLE, using the R package phangorn (v1.99-7 neighbour joining, maximum likelihood optim.pml, LG model, 100 bootstraps (Schliep 2011)).

Results

We isolated and sequenced sRNAs from three biological replicates for two developmental stages (free-living males and females/hermaphrodites, and infective/dauer larvae) of three species of Strongyloididae (*S. ratti, S. papillosus*, and *P. trichosuri*), and for comparison, of the two well-characterized clade V free-living nematodes *C. elegans* and *P. pacificus*. In order to obtain additional information about the chemical nature of the 5′ ends of the different sRNAs, from each RNA preparation, we prepared sequencing libraries with (5′-all-phosphate) and without (5′-monophosphate) prior treatment with TAP. 5′-Monophosphate libraries only include sRNAs with a 5′-monophosphate, whereas 5′-all-phosphate libraries also represent sRNAs that have 5′-di-, -triphosphates or cap-structures in vivo. After preprocessing and filtering (see Materials and Methods), the sRNA reads were classified into four groups: miRNAs (aligning to identified miRNA precursors), tRNA-derived (aligning to tRNA genes), rRNA-derived (aligning to ribosomal RNA genes), or "other" (aligning to any other part of the respective genome). See supplementary figure 1, Supplementary Material online for a graphical overview over the samples, treatments, and the analysis pipeline, and supplementary table 1, Supplementary Material online for detailed information on all samples and treatments.

Improvement of the miRNA Annotations

In our attempt to classify the sRNA reads, we had to consider the fact that the quality and the quantity of the miRNA precursor annotations varied dramatically between the five species under consideration. While *C. elegans* has been analyzed extensively and miRBase (release 21) contains a close to complete list of miRNA precursors for this species; for *S. papillosus* and *P. trichosuri*, no miRNAs have been annotated yet. Therefore, we used miRDeep2 (Friedlander et al. 2012) to predict novel miRNAs based on our 5′-monophosphate sequencing reads and the available genome information (for details see Material and Methods). We then compared our predictions with the entries in miRBase release 21 (table 1).

We found evidence for expression of the vast majority of the listed miRNAs in all three species where annotations were available. We identified only seven novel miRNAs in *C. elegans*, the species where the most extensive prior analysis has been conducted. Furthermore, 37 novel miRNA predictions for *P. pacificus*, 33 for *S. ratti*, 140 for *S. papillosus*, and 163 for *P. trichosuri* fulfilled our criteria for inclusion in the analysis

Table 1
Number of Expressed miRNA Precursors

	C. elegans	*P. pacificus*	*S. ratti*	*S. papillosus*	*P. trichosuri*
miRBase listed miRNA precursors[a]	250	354	106	—	—
expressed miRBase listed miRNAs[b]	218	344	106	—	—
newly predicted miRNA precursors[c]	7	37	33	140	163
Total expressed miRNAs	225	381	139	140	163

[a]miRNA precursors listed in miRBase (http://www.mirbase.org; last accessed October, 2 2017) release 21 for this particular species.
[b]Number of miRBase listed miRNA precursors for which we found mature miRNAs in any of our samples from the particular species.
[c]miRNA precursors not previously described for this particular species (new predictions were only made if the corresponding miRNA was detected in our samples).

(see Materials and Methods and supplementary table 6, Supplementary Material online). In total, we found 225 (*C. elegans*), 381 (*P. pacificus*), 139 (*S. ratti*), 140 (*S. papillous*), and 163 (*P. trichosuri*) expressed miRNA precursors and used those as basis for further analysis. The differences in the numbers of miRNA precursors may reflect the differences in the genome sizes as was proposed by Ahmed et al. (2013). For each known or novel miRNA precursor, the mature miRNA arm was predicted by calculating the ratio of read counts matching to the 5p- or 3p-arm. For ratios >1, the 5p-arm was considered to be the mature miRNA, and for ratios <1, the 3p-arm (see supplementary table 3, Supplementary Material online). Although there were cases with ratios relatively close to 1, in particular for weakly expressed miRNAs, in the vast majority of the cases the preference for one arm was strong. Next, all miRNAs were grouped into families based on their seed sequences (perfect match at positions 2–8 of the mature miRNA, see supplementary table 4, Supplementary Material online). This procedure is common (Wheeler et al. 2009) because the seed sequence is the main determinant of the target specificity of miRNAs. In total, we found 541 different seeds of which only 26 are shared among all five species (fig. 1*A*). However, members of these 26 seed families amount to a considerable fraction of the total miRNAs, namely 62/224 (28%) in *C. elegans*, 86/381 (23%) in *P. pacificus*, 48/139 (35%) in *S. ratti*, 44/140 (31%) in *S. papillosus*, and 42/163 (26%) in *P. trichosuri*. Further we identified 20 seed families that are shared only between Strongyloididae and 12 seeds that are shared only between the two clade V nematodes.

Results in *C. elegans* and *P. pacificus* and Comparison with Previously Published Literature

In *C. elegans* and *P. pacificus* adult hermaphrodites, miRNAs were the most abundant class in 5′-monophosphate samples, representing on average 64% and 58% of all mapped reads with a size distribution around 22 nt length and either a 5′U or 5′A start (fig. 2*A* and *B* and supplementary fig. 2, Supplementary Material online). The second most abundant class of sRNAs in the 5′-monophosphate libraries were other-mapped reads, representing on average 21% and 29% of all mapped reads in *C. elegans* and *P. pacificus* adults. Most of these other-mapped reads included in the 5′-monophosphate libraries have a distinct length of 21 nt and a 5′U representing 21 U RNAs (fig. 2*A*–*C*). These 21 U RNAs are known to be the piRNAs (Ruby et al. 2006; de Wit et al. 2009; Gu et al. 2012; Weick et al. 2014). Additionally there is a small other-mapped peak in both species at 26 nt with 5′Gs representing the primary (endo-)siRNA class of 26 G RNAs (fig. 2*B* and *C*) (Han et al. 2009; Thivierge et al. 2011; Billi et al. 2014). In the 5′-all-phosphate adult samples, miRNA reads amounted to only 15% and 23% of all mapped reads in *C. elegans* and *P. pacificus*, respectively, whereas other-mapped reads accounted for the majority of all mapped reads (62% and 67%, respectively). The TAP-enriched other-mapped reads have a size distribution around 22 nt of length and a very strong 5′G bias (22 G RNAs) (fig. 2*A*–*C*). In the dauer samples, the same trend as in adults can be observed, but a larger proportion (25–60%) of the reads are rRNA derived. Because most of them are in sense orientation, they probably represent degradation products (fig. 2*A*). The 22 G RNAs have been described to be secondary siRNAs, which are produced by the RdRPs RRF-1 and EGO-1 (Gu et al. 2009) without 5′ processing and therefore have 5′-triphosphates (Pak and Fire 2007; Sijen et al. 2007; Gu et al. 2009; Vasale et al. 2010; Ashe et al. 2012; Lee et al. 2012; Shirayama et al. 2012;). For the length distribution and 5′ nucleotides of dauer samples and other sRNA classes, see supplementary figure 2, Supplementary Material online. In order to also have a quantitative comparison with published data, we reclassified the sRNA reads from the previously published *C. elegans* and *P. pacificus* data sets from Weick et al. (2014) in the same way as our own data set and compared them with our data (supplementary table 7, Supplementary Material online). We found that in the published data roughly the same percentage of reads from 5′-monophosphate samples were miRNAs (69% and 54% in *C. elegans* and *P. pacificus*, respectively, compared with 64% and 58% in our sample) or other-mapped (12% and 33% compared with 21% and 29% in our sample). Also in the published data set, the proportion of miRNA reads was lower after TAP-treatment (21% and 19% compared with 15% and 23% in our sample) and the majority of reads fell in the class of other-mapped reads (72% and 75% compared with 62% and 67% in our sample). Overall, our

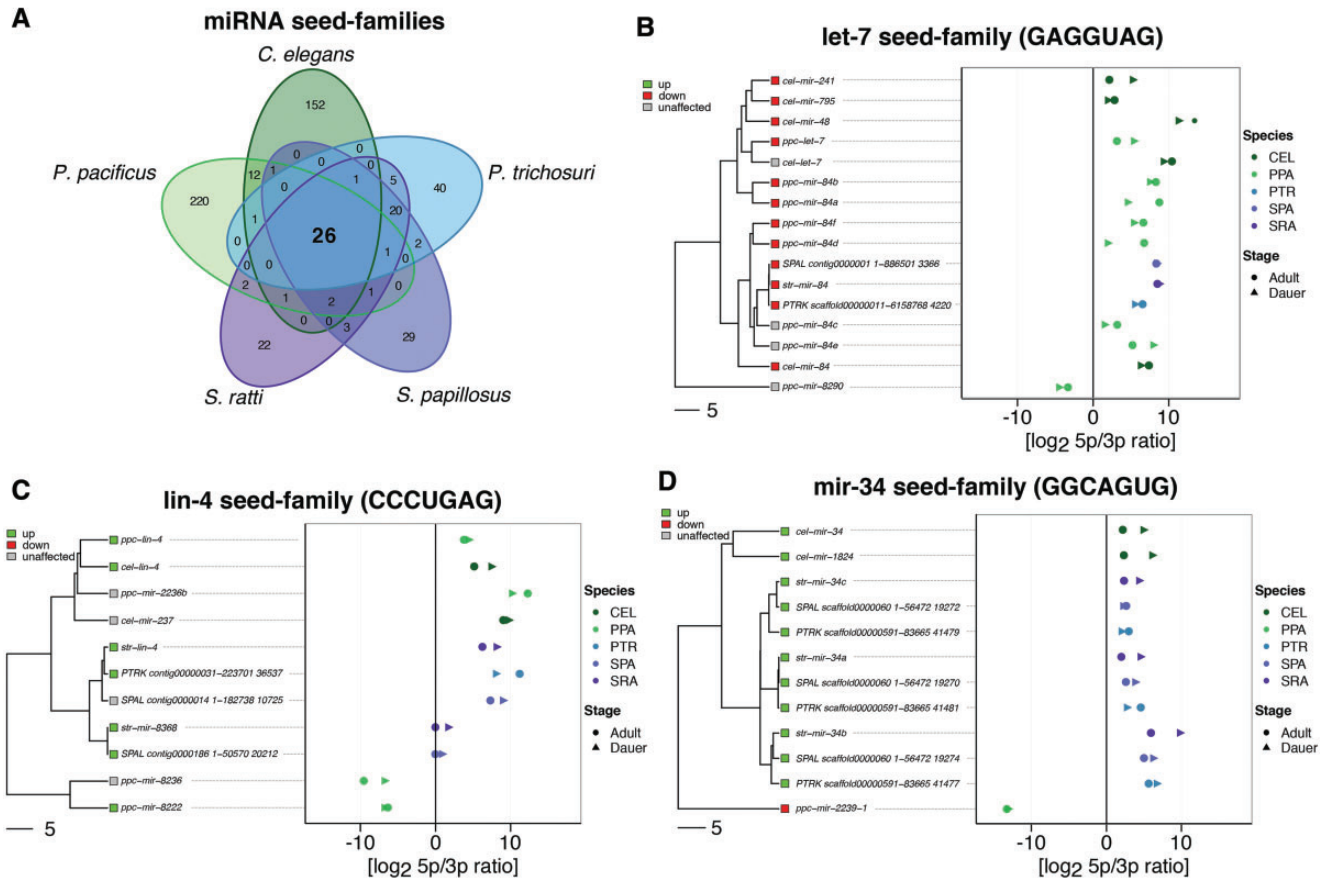

Fig. 1.—Comparative analysis of miRNA seed-families. (*A*) Venn diagram showing the number of miRNA seed families and their overlaps between the five nematodes species under study. (*B–D*) Phylogenetic trees based on multiple sequence alignments of all members of three miRNA seed-families (left) and arm preference (right) represented as ratio of reads derived from the 5p arm and the 3p arm of the miRNA precursor. The species are color coded, filled circles represent reads from libraries derived from adults, filled arrow heads represent reads from dauer/infective larvae derived libraries. The seed sequence is given in parentheses. (*B*) One example of a conserved seed family (present in all five species) that is consistently downregulated in dauer/infective larvae compared with adults (red squares). (*C*) One example for a conserved seed family that is consistently upregulated in dauer/infective larvae compared with adults (green squares). For the other conserved differentially expressed miRNAs see supplementary figure 7, Supplementary Material online. (*D*) The mir-34 seed family. Notice that *P. pacificus* mir-34 is differentially expressed in the opposite direction than the rest and its gene phylogeny is not in agreement with the species phylogeny, suggesting that it has acquired the seed by convergence.

results in *C. elegans* and *P. pacificus* are in excellent agreement with previously published data (Ahmed et al. 2013; Shi et al. 2013; Sarkies et al. 2015). From this we conclude that our experimental design was suitable to reproduce earlier findings, and therefore our new data on the Strongyloididae species are comparable with the existing literature on other systems.

Classes of Small RNAs Identified in Strongyloididae

In free-living adults of all three Strongyloididae species, most of the reads from 5′-monophosphate samples are derived from miRNAs (on average 79%, 55%, and 80% of all mapped reads for *S. ratti*, *S. papillosus*, and *P. trichosuri*, respectively; fig. 2*A*), which is comparable with *C. elegans* and *P. pacificus*. As in *C. elegans* and *P. pacificus*, the length of miRNAs peaks at 22–23 nt in all three species (fig. 2*B*) and they have a very strong 5′U bias (supplementary fig. 2, Supplementary Material online). The proportion of reads mapping to rRNA or tRNA sequences was comparable in all five species tested. However, unlike in *C. elegans* and *P. pacificus*, we found no peaks corresponding to the 21 U and the 26 G RNAs among the reads mapping to "other" parts of the genome in Strongyloididae (fig. 2*A–C*). These peaks were, however, clearly present in our *C. elegans* and *P. pacificus* data sets.

In adults of all five species tested, reads mapping to "other" parts of the genome make up a much greater proportion in the 5′-all-phosphate samples than in the 5′-monophosphate data sets. In *C. elegans* and in *P. pacificus*, the TAP-enriched fraction consists predominantly of the 22 G RNAs. In contrast, the TAP-enriched sRNAs of all three

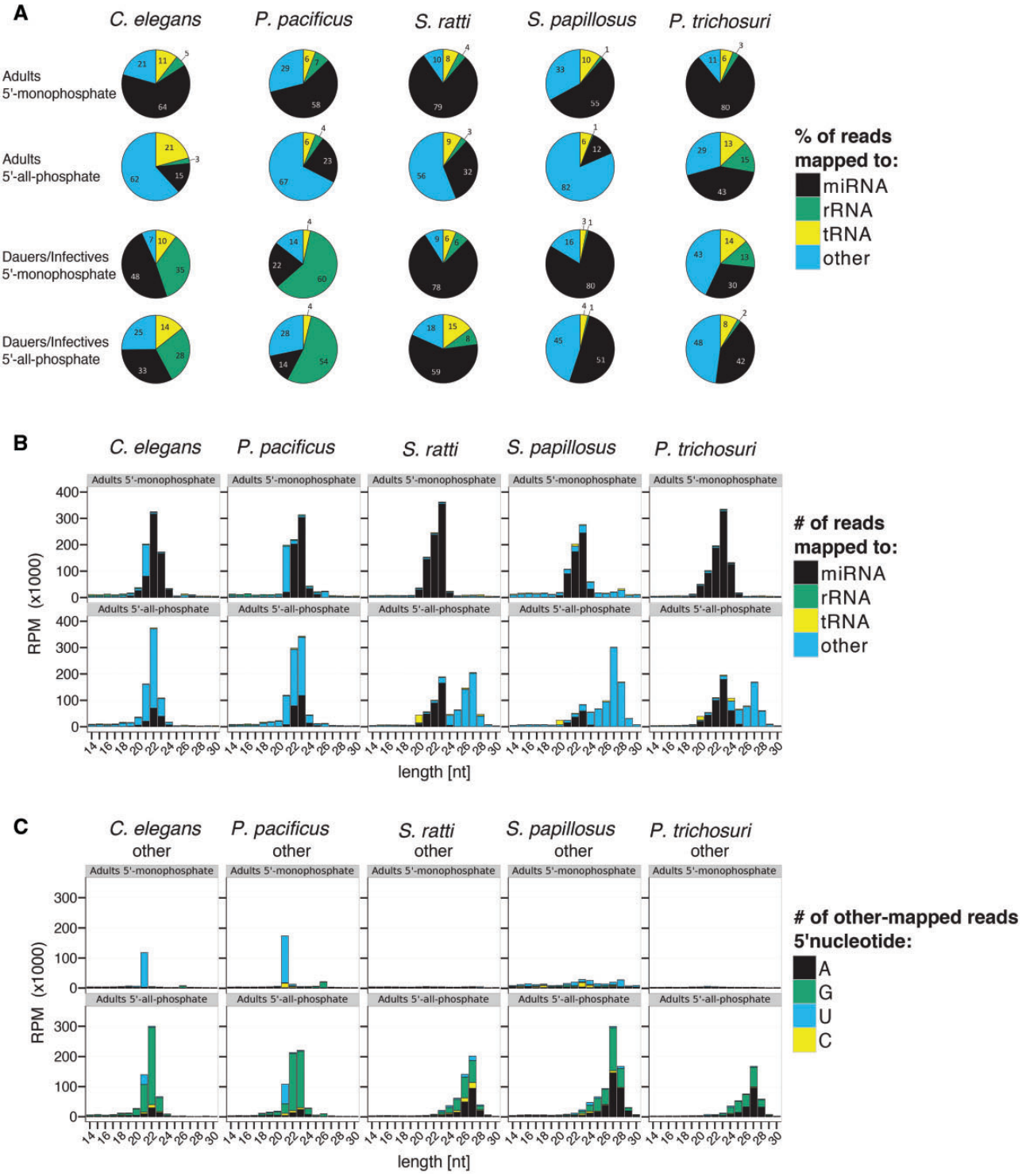

FIG. 2.—sRNA profiles. (*A*) Piecharts representing the percentage of all mapped reads classified as miRNAs (black), rRNA-derived (green), tRNA-derived (yellow), or other (blue). (*B*) Barplots showing the length distribution of small RNA classes. The color code is the same as in (*A*). (*C*) Barplots of the length distribution and 5′ nucleotide of sRNAs classified as other. Reads starting with A, G, U, or C are represented in black, green, blue, and yellow, respectively. In all cases, the average of the three biological replicates is given. In B and C only the results for adults and in C only the results for the class "other" are given. For the corresponding plots for dauers/infectives and for the other sRNA classes see supplementary figure 2, Supplementary Material online. 5′-Monophosphate RNA not treated with TAP before library construction; 5′-all-phosphate RNA treated with TAP before library construction; RPM: reads per million; length[nt]: read length in nucleotides.

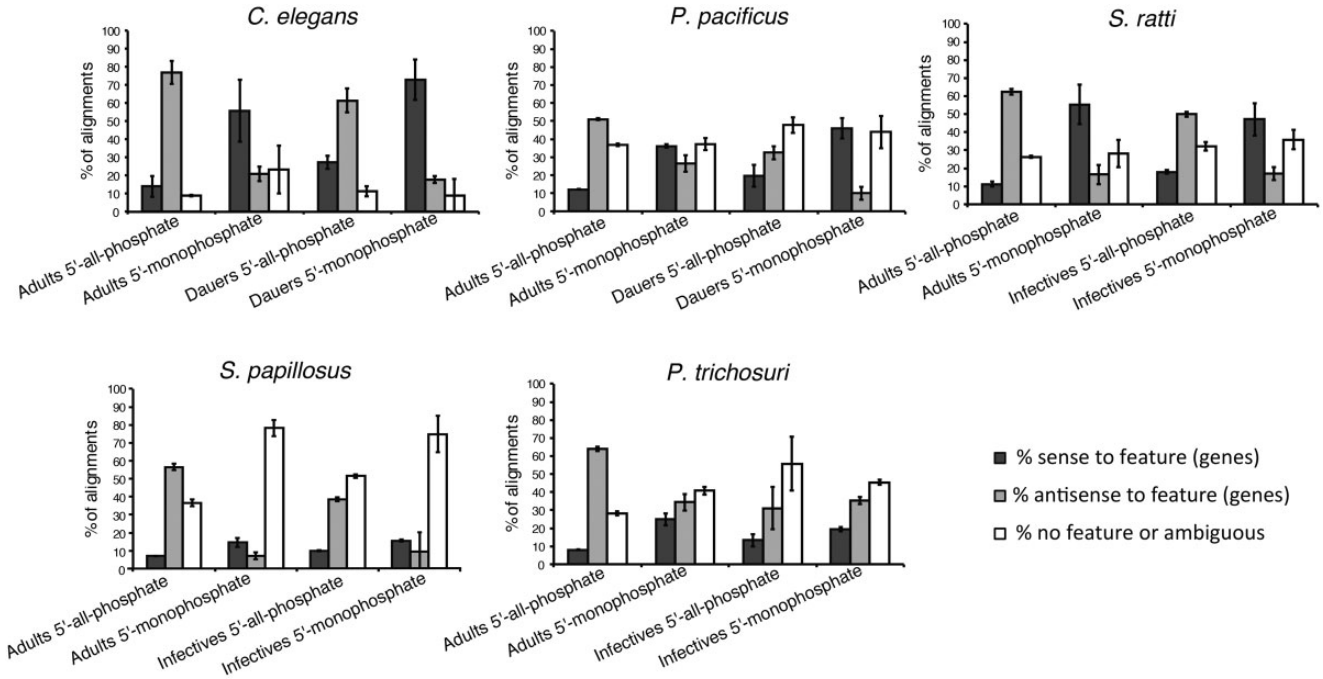

Fig. 3.—TAP-treatment enriches for reads that align antisense to annotated genes in all five species studied. Barplots showing the percentage of other mapped reads that map to annotated features (genes) in either sense (dark gray) or antisense (light gray) orientation or reads that map ambiguously or to regions of the genome without annotated features (white). Notice that the proportion of reads that does not map to annotated parts of the genome is highly dependent on the quality of the genome annotations, which varies strongly between the five species under study. Therefore only the ratio of sense mapped reads to antisense mapped reads is relevant. Given are the means of the three biological replicates. The errorbars indicate the standard deviations.

Strongyloididae species form a novel class of sRNAs, with a length peak at 27 nt (fig. 2*B*) and 5′G and 5′A being similarly abundant (27GA RNAs) (fig. 2*C*). Qualitatively, all findings in the adults described above also hold true for dauer/infective larvae (fig. 2*A* and supplementary fig. 2, Supplementary Material online). However, the enrichment of other-mapped reads in the 5′-all-phosphate samples is less pronounced in this developmental stage. The biological replicates of all species and developmental stages are consistent. The sRNA profiles are presented separately and with full size range (up to 50 nt) for each biological replicate in supplementary figure 3, Supplementary Material online.

Putative Targets of the TAP-Enriched 27GA RNAs

In *C. elegans*, the 22 G RNAs have been shown to be secondary siRNAs mapping mainly antisense to transposons and other genes. We speculated that the TAP-enriched 27GA RNAs are the Strongyloididae equivalent of the 22 G RNAs of *C. elegans*. In that case, one would predict that these RNAs correspond to the noncoding strand of genes, such that they could target the corresponding mRNAs by base pairing. To test this, we counted reads mapping sense or antisense or not mapping to annotated genes with htseq (Anders et al. 2015). We then compared the differences between 5′-all-phosphate and 5′-monophosphate libraries (fig. 3). In all five species analyzed, TAP-treatment led to a strong increase in the proportion of "antisense to gene" reads in adults. In fact, the majority of other-mapped reads (51–77% of unambiguously other-mapped reads) in 5′-all-phosphate adult samples map antisense to annotated genes. The same trend was observed in dauer/infective samples except for *P. trichosuri* (fig. 3). Next we asked what kind of genes are targeted by the TAP-enriched RNAs and performed a differential expression analysis, comparing other-mapped reads from 5′-all-phosphate libraries versus 5′-monophosphate libraries. We then identified the gene predictions targeted by the TAP-enriched RNAs and performed a gene set enrichment analysis. We found that a good portion of the TAP-enriched RNAs target gene predictions containing transposable element-related protein domains in all five nematode species, but also other protein coding genes are putative targets (table 2 and supplementary table 8, Supplementary Material online). Given the limited quality of the annotations of the Strongyloididae genomes, it is difficult to make a more quantitative statement. However, estimates based on two different methods of predicting transposons are provided in supplementary figure 4, Supplementary Material online.

Based on these findings, we hypothesize that these TAP-enriched sRNAs in Strongyloididae represent a class of secondary siRNAs which are produced by RdRPs without 5′ processing, comparable with the 22 G RNAs in *C. elegans*.

Table 2
TAP-Enriched Other-Mapped sRNAs Target Genes with Protein Domains Related to Transposable Elements in all Five Nematode Species

PFAM Domain	Function	*C. elegans* Adults 5′-all-phosphate	*P. pacificus* Adults 5′-all-phosphate	*S. ratti* Adults 5′-all-phosphate	*S. papillosus* Adults 5′-all-phosphate	*P. trichosuri* Adults 5′-all-phosphate
transposable_element		up	not annotated	not annotated	not annotated	not annotated
Rve	Retroviral integrase	—	up	up	up	up
gag-asp_protease	gag-polyprotein putative aspartyl protease	—	up	up	up	up
rve_3	Integrase core domain	—	—	up	up	up
zf-H2C2	binds to histone upstream activating sequence elements that are found in histone gene promoters	—	up	up	up	up
zf-CCHC	Zinc finger	up	up	up	up	—
RVT_1	Reverse transcriptase	—	—	up	up	up
DDE_Tnp_IS1595	ISXO2-like transposase domain	—	—	up	up	up
Asp_protease_2	Aspartyl protease	—		up	up	—
RVP	Retroviral aspartyl protease	—	—	up	up	—
RVP_2	Retroviral aspartyl protease	—	—	up	up	—
DDE_3	DDE superfamily endonuclease	—	—	up	up	up
Asp_protease	Aspartyl protease	—	—	up	up	—
AT_hook	DNA-binding motif present in many proteins	up	—	up	up	—
gag_preintegrs	associated with retroviral insertion elements, lies just upstream of the integrase region	—	—	up	up	—

Indeed, multiple putative RdRP genes are present in the genomes of all three Strongyloididae species (fig. 4*A*).

piRNAs Are Lost in Strongyloididae Nematodes

In *C. elegans* and *P. pacificus*, piRNAs are also known as 21 U RNAs. As described above, we failed to detect a prominent group of "other" mapped TAP insensitive (5′ monophosphorylated) RNAs of 21 or a similar defined number of nucleotides in length with a 5′U in any of the Strongyloididae, although this class of RNAs was clearly present in our *C. elegans* and *P. pacificus* samples (fig. 2*C*). This indicated that these worms either do not possess piRNAs, or that the piRNAs vary in length. In order to investigate this, we asked if we could identify a class of RNAs that showed any of the general properties of piRNAs.

The 21 U RNA genes in *C. elegans* are located in two big clusters on chromosome IV (Ruby et al. 2006), whereas in *P. pacificus*, the 21 U RNA genes are organized in multiple smaller clusters, which are disseminated throughout the genome (de Wit et al. 2009). The tendency to start with a 5′U and to be clustered in the genome is a widespread feature of piRNAs far beyond nematodes (Grimson et al. 2008; Malone et al. 2009; Juliano et al. 2014). Therefore, we analyzed the genomic distribution of all uniquely mapped "other" 20–24 U RNAs in *S. ratti* and in *S. papillosus.* We found no indication for clustering of the genomic regions from which the 20–24 U RNAs originate (fig. 5*F–H*).

In *C. elegans*, each 21 U RNA is transcribed from its own gene and many of these 21 U RNA genes contain a conserved motif (Ruby motif), about 40 nt upstream of the position corresponding to the 5′U of the mature 21 U RNA (Ruby et al. 2006; de Wit et al. 2009; Gu et al. 2012; Weick et al. 2014). A similar conserved motif is also found in *P. pacificus* and the clade V parasitic nematodes *Haemonchus contortus* and *Nippostrongylus brasiliensis* (de Wit et al. 2009; Sarkies et al. 2015). In order to find evidence for a Ruby-like motif in Strongyloididae, we randomly selected 5,000 genomic positions from where "other" mapped 20–24 U RNAs (21 U RNAs for *C. elegans* and *P. pacificus*) originated. We then used the MEME Suite (Bailey et al. 2015) to search for the presence of a conserved motif in the upstream regions. We determined in what proportion of all "other" mapped 20–24 U RNAs the identified motif occurred within 60 bp upstream of the position corresponding to the 5′U. Although we were able to rederive the published Ruby motif from the *C. elegans* and the *P. pacificus* data, we did not find a comparable motif in any of the three Strongyloididae species. Even if we accepted the best identified consensus sequence as a motif, it was present in the upstream region of only a small fraction of all 20–24 U RNA coding regions (fig. 5*A–E* and supplementary table 9, Supplementary Material online).

In other systems, piRNAs specifically interact with PIWI proteins, which are a class of Argonaute proteins that are highly conserved within the animal kingdom (Czech and Hannon 2011). Therefore as a final line of evidence, we asked if genes encoding PIWI proteins are present in the Strongyloididae genomes. To obtain a list of Argonaute proteins in the five species analyzed, we performed a jackhmmer search (HMMER 3.1b1 (Eddy 2011)) on the (predicted)

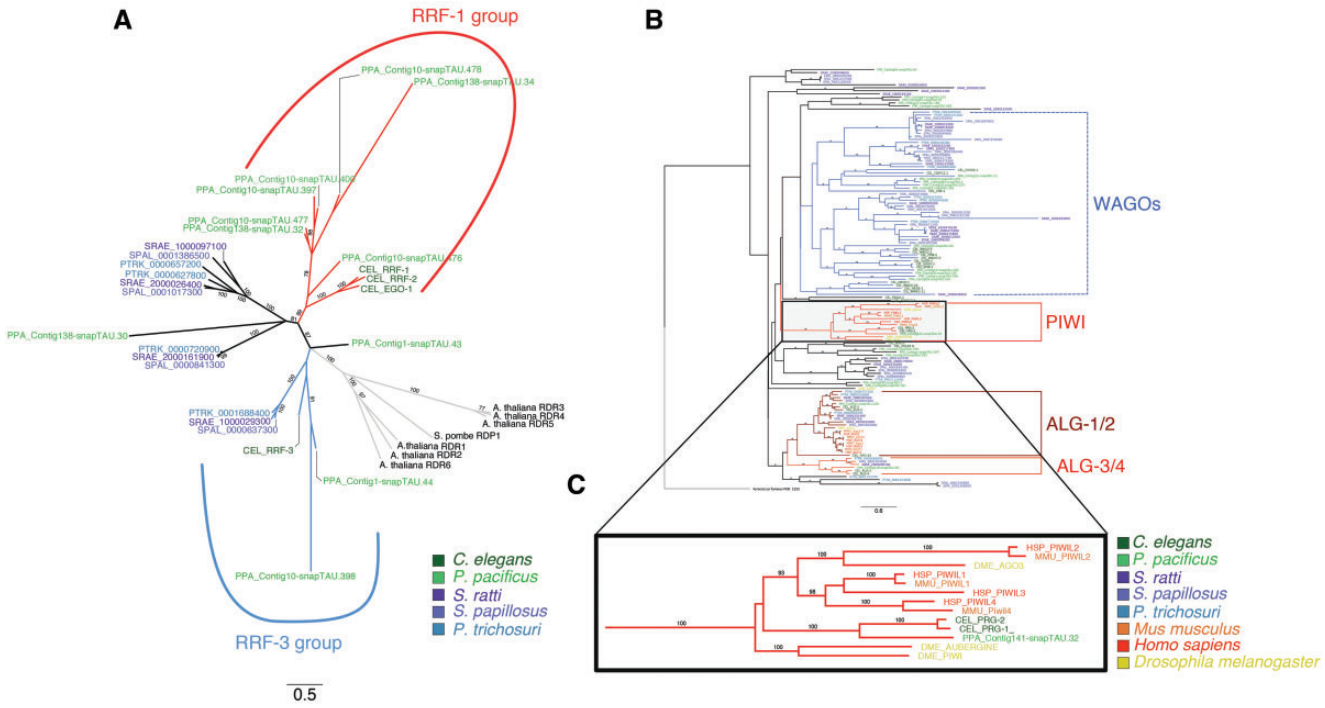

Fig. 4.—Phylogenetic gene trees for RdRPs and Argonautes. (*A*) Unrooted neighbour joining tree of the RdRP genes from the five different nematodes. As outgroups we added RdRPs from *Arabidopsis thaliana* and *Saccharomyces pombe*. The different species are color-coded, and the two previously described subfamilies (RRF-1 and RRF-3) are indicated by colored branches. Only branch support values ≥ 70 from 100 bootstraps are shown. Notice that the RRF-3 group is not supported by a high bootstrap value. (*B*) Neighbour joining tree of the Argonaute genes from the five nematodes under study and *Drosophila melanogaster*, *Homo sapiens*, *Mus musculus* and one argonaute gene from the archean species *Pyrococcus furiosus*. For graphical representation we used FigTree to reroot the tree to the outgroup Argonaute *from P. furiosus*. The different species are color-coded. The conserved subfamilies that contain nematode, mammalian and *Drosophila* members are indicated by colored solid branches (ALG-1/2, ALG3/4, PIWI). The previously described group of nematode (worm) specific Argonautes (WAGOs) are indicated by dashed lines. Notice that monophyly of WAGOs is not supported by high bootstrap values in our analysis. For a high resolution graph of the complete tree see supplementary figure 5, Supplementary Material online. (*C*) Zoom in of the Piwi branch of Argonautes, which is lacking Strongyloididae representatives. Only branch support values ≥70 from 100 bootstraps are shown.

proteins using the protein sequence of *C. elegans* ALG-1 as bait. We accepted Argonaute homologs with an *e*-value $<2 \times 10^{-8}$ and aligned the protein sequences using MUSCLE with default settings (Edgar 2004). As outgroups for the PIWI Argonautes, we used PIWI proteins from *H. sapiens* (PIWIL1 and PIWIL4), *M. musculus* (PIWIL1-2), and *D. melanogaster* (PIWI and AUBERGINE). We then reconstructed unrooted trees with the R package phangorn (Schliep 2011) (fig. 4*B* and *C* and supplementary fig. 5, Supplementary Material online). We identified 25 different Argonaute genes in *S. ratti*. It should be noted that the somewhat higher number in *S. papillosus* is likely an overestimate due to assembly problems in the *S. papillosus* draft genome, which was derived from an outbred population with rather high genetic variability. The number of Argonaute genes in *S. ratti* is very comparable with the number in *C. elegans* (both 25) and *P. pacificus* (23). Several of the major groups contain genes from the two clade V nematodes (*C. elegans* and *P. pacificus*) and the Strongyloididae but cases of clear one to one orthology are rare. This indicates that the Argonaute gene families have independently expanded in the two clades. There are also phylogenetic groups of argonaute genes that appear entirely absent from either of the two clade V species or from the Strongyloididae. Interestingly, one of the two groups not present in Strongyloididae are the PIWI coding genes. Overall, from these results we conclude that "standard" piRNAs are absent from Strongyloididae as is the case for a number of other nematode species (Sarkies et al. 2015).

A Conserved Set of miRNA Families Is Differentially Expressed in Dauers/Infectives versus Adults

In order to identify developmentally regulated miRNAs, we quantified the miRNA expression (as described in Materials and Methods) and compared the miRNA expression levels in dauer/infective larvae and in free-living adults. Only those miRNAs that had at least 1 read per million (miRNA mapping reads) in at least three samples were included in the analysis. Up- and downregulated miRNAs were considered significantly differentially expressed if they showed at least a 2-fold change in expression (log2 fold change ($\log_2$FC) of ≥1 or ≤–1) and an FDR <5%. Of all expressed miRNAs, we found 49% (109/224)

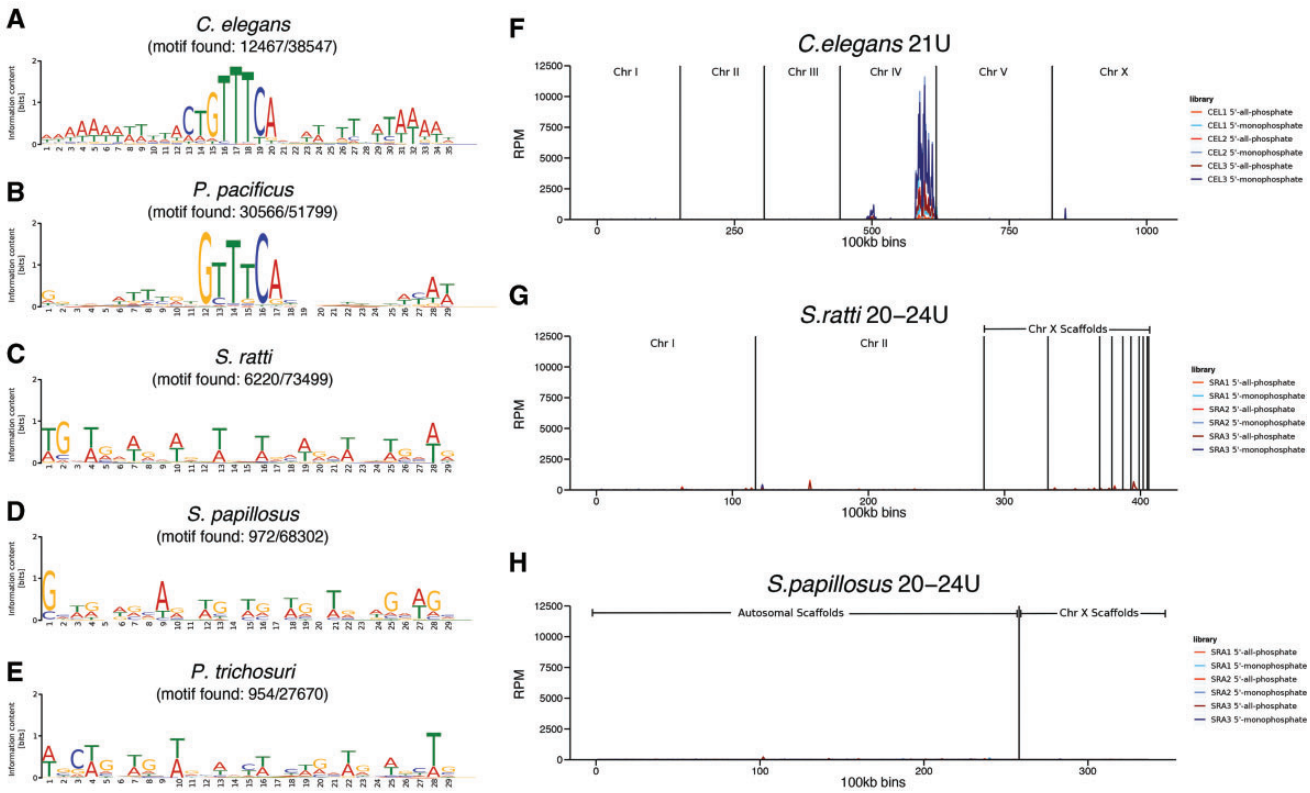

Fig. 5.—Absence of a Ruby-type motif and no genomic clustering of 20–24 U RNAs in Strongyloididae. (*A–E*) Sequence logos of de novo predicted motifs based on 5,000 randomly selected 60 bp regions immediately upstream of aligned 21 U (*A, B*) or 20–24 U (*C–E*) other-mapped sequences. The numbers beneath the species indicate the number of places in the genome to which 21 U or 20–24 U reads align that do have the motif in the upstream region/total number of places to which 21 U or 20–24 U reads align. (*F–H*) Plots representing the genome coverage by 21 U or 20–24 U other-mapped reads from adult worm replicates along the chromosomes/scaffolds (100kb bins) for *C. elegans* (*F*), *S. ratti* (*G*), and *S. papillosus* (*H*). The different biological replicates and treatments are color coded. The two strongest peaks in *C. elegans* correspond to the known major and minor piRNA gene clusters. RPM reads per million.

in *C. elegans*, 44% (167/381) in *P. pacificus*, 47% (65/139) in *S. ratti*, 52% (73/140) in *S. papillosus*, and 61% (99/163) in *P. trichosuri* to be significantly differentially expressed (supple mentary table 3, Supplementary Material online). Roughly equal numbers of miRNAs were up- and downregulated in dauers/infectives compared with adults across all species, with 54 up and 55 down in *C. elegans*, 75 up and 92 down in *P. pacificus*, 37 up and 28 down in *S. ratti*, 41 up and 32 down in *S. papillosus*, and 43 up and 56 down in *P. trichosuri*.

For some of the species, we investigated (*C. elegans*, *P. pacificus*, and *S. ratti*), Ahmed et al. (2013) had already compared the expression of miRNAs in dauers/infectives and in mixed stages. In total, these authors had looked at 177 miRNAs (of which 152 were also present at high enough levels to be included in our analysis) in *C. elegans*, 331 (of which 291 are also included in our data set) in *P. pacificus*, and 106 (of which 100 are also included in our data set) in *S. ratti* (supplementary fig. 6 and table 10, Supplementary Material online). In order to compare our data with Ahmed et al. (2013), we categorized the miRNA expression changes into upregulated, downregulated, and unaffected, made a contingency table and performed a χ^2 test. The two data sets show a highly significant correlation of the categorized expression changes in dauers versus mixed/adult stages for all three species ($P = 1.29 \times 10^{-10}$, $P < 2.2 \times 10^{-16}$, and $P = 2.72 \times 10^{-8}$ for *C. elegans*, *P. pacificus*, and *S. ratti*, respectively, supplementary fig. 6A, C and E, Supplementary Material online). We plotted the $\log_2$FC values of dauers/infectives versus mixed stage/adults of the two studies against each other and performed a linear regression (supplementary fig. 6B, D and F, Supplementary Material online). In general, the expression changes of miRNAs detected in both data sets are comparable and only very few miRNAs defined as up- or downregulated in one of the data sets show the opposite trend in the other data set. This again demonstrates that our findings are consistent with previously published data as far as such data exists, adding credibility to our data about not previously analyzed species.

Among the 26 miRNA seed families that are shared between all five nematode species, we found ten that showed

consistent differential expression in all five nematode species. We considered differential expression to be consistent if at least one seed family member from each species is differentially expressed in the same direction (up- or downregulated in dauers/infectives), as the majority of the miRNAs that are members of the same seed family. Eight seed families were upregulated (lin-4, mir-124, mir-231, mir-232, mir-251, mir-50, mir-51, and mir-86) and two families were downregulated (let-7 and mir-35) in dauers/infectives versus adults in all five nematode species (fig. 1*B* and *C* and supplementary fig. 7, Supplementary Material online). For each of the conserved miRNA seed families, we constructed seed-constrained multiple sequence alignments with LocARNA (Will et al. 2007, 2012), which takes the predicted secondary structure of the miRNAs into account. These alignments were then used to infer gene trees shown in figure 1. Furthermore we analyzed the conservation of the preference for the 5p- or 3p-arm giving rise to the mature miRNA for each seed family, and found that most of the miRNAs members of the different seed families share the origin (5p- or 3p-arm) of their mature miRNA (fig. 1*B–D* and supplementary fig. 7, Supplementary Material online). Almost all exceptions were found for miRNAs that were phylogenetically more distant from the rest of the members in a seed family and had possibly acquired the same seed by convergence. These findings indicate that certain miRNA seed families perform stage specific functions that are conserved over large evolutionary distance. An interesting case among the 26 seed families is the mir-34 family (fig. 1*D*). Against the general trend that Strongyloididae have fewer miRNAs, we found three paralogs in the Strongyloididae, two in *C. elegans* and only one in *P. pacificus*. Further, the *C. elegans* and the Strongyloididae mir-34 family genes are more closely related to each other than to the single *P. pacificus* gene, and in *P. pacificus* the mature miRNA is on the 5p-arm, whereas in the others the mature miRNA is on the 3p-arm. All of this indicates that the *P. pacificus* mir-34 is of different phylogenetic origin (convergent acquisition of the seed). Fittingly, the *P. pacificus* mir-34 is differentially expressed in the other direction suggesting a different function.

No Signs of Circulating Parasite-Derived sRNAs in the Host Blood

There are more and more reports of parasite-derived sRNAs circulating in the blood or other tissues of animal hosts. Circulating miRNAs were found in the blood of hosts infected with the filarial nematodes *Dirofilaria immitis* and *Onchocerca volvulus* (Tritten et al. 2014) and in hosts infected with trematodes of the genus *Schistosoma* (Cai et al. 2015). For the intestinal parasitic nematode *Heligmosomoides polygyrus*, it has been shown that it secretes vesicles containing miRNAs as well as a nematode Argonaute protein (Buck et al. 2014). We sequenced sRNAs from whole blood of three rats that were infected with *S. ratti*, and compared their sRNA expression to two sRNA data sets from uninfected rats, to see if any sRNAs from *S. ratti* are circulating in the blood of their host animal (supplementary table 2, Supplementary Material online). We could not find any *S. ratti*-specific miRNAs (or any other sRNAs) that were present in the infected rats but not the uninfected ones. Either there are no circulating sRNAs that are secreted from *S. ratti*, they are identical in sequence to host sRNAs, or they are present in such low amounts that our method was not sensitive enough to detect them. As expected, TAP-treatment made no difference for rat-derived sRNAs except for a strong TAP-dependent peak of 39 nt entirely derived from 5 S rRNA and a slight enrichment of tRNA fragments (supplementary fig. 8, Supplementary Material online).

Discussion

The nematodes are probably the most species rich animal phylum and differ in many ways not only from other phyla but also from each other (Lee 2002). One example are various aspects about their noncoding small RNAs. Here, we present the first global characterization of the sRNA profiles of three members of the parasitic nematode family Strongyloididae. In a previous study that compared sRNAs in various nematodes (Sarkies et al. 2015), the closest relative of the Strongyloididae that had been included was *Globodera pallida*, a plant parasite that although part of the same major clade (clade IV) of nematodes (Blaxter et al. 1998), is as phylogenetically distant as is possible within a clade. This is the first global characterization of sRNAs in any animal parasite of clade IV. Therefore, the Strongyloididae represent almost certainly an independent transition to parasitic life style from the few other parasitic nematodes for which such an analysis had been done.

Like in other nematodes, the Strongyloididae sRNA libraries that were limited to RNAs with 5′ monophosphates were dominated by miRNA-derived sequences. Comparable with other systems, Strongyloididae have conserved and taxon-specific miRNAs. Among the 157 different miRNA seeds identified in Strongyloididae, only 48 were shared amongst all three species tested. Interestingly, more than half of them (26) were also found in *C. elegans* and *P. pacificus*. Also for these two clade V species, the number of seeds shared between them but not with the Strongyloididae was smaller than the number of seeds shared among all five species of the two clades. A substantial fraction (10/26) of the miRNAs with conserved seeds, showed conserved differential expression between free-living adults and infective/dauer larvae, indicating that in spite of the large phylogenetic distance, these miRNAs might still fulfill similar functions. Consistent with a largely conserved miRNA system, we identified Argonaute proteins in all three species which clearly belong to the families of Argonautes involved in miRNA function in other systems.

Similarly to other nematodes of clades III–V (Wang et al. 2011; Sarkies et al. 2015), we identified a population of RNAs in Strongyloididae only detectable upon TAP treatment. However, differently from all other previously characterized nematodes where these RNAs are around 22 nt long (24 nt in the clade IV representative *G. pallida*) and in their overwhelming majority start with 5′ G (hence called 22 G RNAs), in Strongyloididae the TAP-enriched RNAs are longer, around 27 nt, and are about as likely to have 5′ As as 5′ Gs (27GA RNAs). This difference suggests that Strongyloididae also have different RdRPs for their biosynthesis and different Argonautes capable of binding these longer RNAs. Consistently, we identified four putative RdRP genes in each of the three species of Strongyloididae. For two of them, clear one to one orthologs were present in all three species analyzed. For the other two, there are one to one orthologs in the two *Strongyloides* species, whereas both genes in *P. trichosuri* are more closely related to one of the *Strongyloides* paralogs. For none of the Strongyloididae RdRPs, a clear orthology relationship to any of the four *C. elegans* RdRPs was detectable (fig. 4*A*). We found about the same number of Argonaute genes in Strongyloididae as in *C. elegans*. However, cases of clear orthology are rare and largely limited to the two conserved families (ALG-1/2 and ALG-3/4, involved in miRNA and primary endogenous siRNA function, respectively) (fig. 4*B*) (Conine et al. 2010; Buck and Blaxter 2013). This indicates that most of the expansion of the Argonaute family occurred after the phylogenetic separation of *C. elegans* and Strongyloididae. We showed that a large portion of the 27GA RNAs has the potential to target transposable elements (supplementary fig. 4, Supplementary Material online), like 22 G RNAs do in *C. elegans* (Gu et al. 2009). The fact that they appear to be made by RdRPs, comparable with 22 G RNAs, leads us to hypothesize that the 27GA RNAs correspond to the 22 G secondary siRNAs in *C. elegans*. The 22 G RNAs in *C. elegans* are secondary siRNAs triggered by the primary endogenous siRNAs (26 G RNAs) and 21 U RNAs (Pak and Fire 2007; Sijen et al. 2007; Gu et al. 2009; Vasale et al. 2010; Ashe et al. 2012; Lee et al. 2012; Shirayama et al. 2012;). Although in our data 26G RNAs are visible as small peaks in the *C. elegans* and *P. pacificus* samples (fig. 2*C*), no such peaks are visible in the samples derived from Strongyloididae. Also in Sarkies et al. (2015) no 26 G peaks are visible in the samples from *Brugia malayi* (clade III) and *G. pallida* (clade IV). However, 26 G RNAs have been found in the clade III parasitic nematode *Ascaris suum*, where they are strongly enriched in the male gonad (Wang et al. 2011). We speculate that primary endogenous siRNAs, which trigger the production of the 27GA RNAs do exist in Strongyloididae but they might be more heterogeneous in length compared with clade V nematodes and *A. suum*. We cannot exclude the presence of an unknown chemical modification that prevents certain sRNAs from being included in the sequencing libraries. In *C. elegans* portions of the 26 G and the 21 U RNAs have been shown to be 3′-end methylated (Kamminga et al. 2012). We do not think that this particular modification is present in the Strongyloididae because in none of the genomes we found a homolog of HENN-1, the methyltransferase responsible for this modification. This is consistent with earlier studies in which neither the gene nor 3′-end methylation have been found in nematodes outside the clade V (Wang et al. 2011; Sarkies et al. 2015).

We found no indication of the presence of piRNAs or Piwi proteins in Strongyloididae. This suggests that this taxon lacks this widely conserved class of small RNAs normally involved in silencing transposable elements in the germline. The absence of piRNAs had already been postulated for various other nematodes of multiple clades based on the absence of recognizable Piwi genes in the genomes (Wang et al. 2011; Sarkies et al. 2015). Among them is *Panagrellus redivivus*, a relatively closely related free-living nematode (Srinivasan et al. 2013). In fact, although piRNAs are widely conserved, within nematodes they have so far only been found in one of the major clades (clade V), which contains *C. elegans*. Although the exact phylogeny of nematodes is under debate, all phylogenetic studies we are aware of Blaxter (2011, 2007), Holterman et al. (2006), and Meldal et al. (2007) agree that Strongyloididae are more closely related to *C. elegans* than to some of the nematode species shown to lack piRNAs. Given the wide conservation of piRNAs among animals, the assumption that the last common ancestor of all nematodes had piRNAs appears reasonable. If we accept that the last common ancestor of all nematodes had piRNAs, the absence of piRNAs in all nematodes outside of clade V studied so far can only by explained by independent losses during evolution in multiple branches of nematodes, as proposed by Sarkies et al. (2015). However, maybe one should not prematurely exclude an early loss followed by a regain of piRNAs in clade V, possibly through horizontal gene transfer.

For some parasitic nematodes, it has been recently described that they secrete small RNAs and in a few cases, parasite-derived sRNAs were found circulating in the host blood (Cai et al. 2015; Tritten et al. 2014). Therefore, we sequenced 5′-all-phosphate and 5′-monophosphate sRNAs isolated from blood of rats which were infected or mock infected with *S. ratti*. We failed to detect *S. ratti*-derived sRNAs, indicating, that if such RNAs are present in the blood, they are either extremely low abundant or they are identical with host sRNAs. This finding does not exclude that parasite-derived sRNAs are present locally in the small intestine of the host and might play a role in the host–parasite interaction.

Taken together, we identified a novel class of small RNAs termed 27GA RNAs in the Strongyloididae, a family of parasitic nematodes, which includes the human pathogen *S. stercoralis*. This class of RNAs is only detectable in RNA seq experiments if the RNA is treated with tobacco acid pyrophosphatase, indicating that these RNAs carry triphosphates at their 5′ ends and are made by RdRPs. Correspondingly,

Strongyloididae possess different subfamilies of RdRPs and Argonaute proteins. A large fraction of the 27GA RNAs have the potential to target transposable elements. We hypothesize that 27GA RNAs correspond to the 22 G secondary siRNAs in *C. elegans*. Further, Strongyloididae lack piRNAs and Piwi-type Argonaute proteins.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

Declarations

Animal Experiments

For animal experiments, all relevant national and international animal welfare regulations and guidelines were followed. The experiments were approved by the Regierungspräsidium Tübingen (AZ: 35/9185.82-5/15.07.2015).

Availability of Data and Materials

All data sets generated and analysed in this study are available at the European Nucleotide archive (ENA) under the accession PRJEB21191. It is policy of miRBase to only assign definite miRNA names after acceptance of a publication. Therefore, the final names for the newly described miRNAs will be assigned by miRBase according to their schedule.

Funding

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Authors' Contributions

A.H. and A.S. designed the experiments. A.H. collected the samples and constructed the sRNA libraries. A.H. performed the analysis pipeline. A.H. and A.S. interpreted the data. A.H. prepared the figures and tables with input from A.S. A.H. and A.S. cowrote the manuscript. Both authors approved the final draft.

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