The Reproductive Biology of *Strongyloides* Nematodes - Sex Determination, Chromatin Diminution and Germ-line Organization

Dissertation
der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Arpita Kulkarni
aus Pune, Indien

Tübingen
2015

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen

Tag der mündlichen Qualifikation:	07.09.2015
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	PD Dr. Adrian Streit
2. Berichterstatter:	Prof. Dr. Nikolaus Blin

List of publications

Teile der vorliegenden Arbeit wurden bereits veröffentlicht oder wurden zur Veröffentlichung eingereicht:

***Parastrongyloides trichosuri* suggests that XX/X0 sex determination is ancestral in Strongyloididae (Nematoda)**

Arpita Kulkarni, Anna Dyka, Linda Nemetschke, Warwick Grant and Adrian Streit:

Parasitology (2013), 140, 1822-1830. doi:10.1017/S0031182013001315

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

Arpita Kulkarni, Anja Holz, Christian Rödelsperger, Dorothee Harbecke and Adrian Streit:

Chromosoma, in press (2015)

The genomic basis of parasitism in the *Strongyloides* clade of nematodes

Vicky Hunt, Isheng J Tsai, Avril Coghlan, Adam J. Reid, Nancy Holroyd, Bernardo Foth, Alan Tracey, James A. Cotton, Eleanor Stanley, Helen Beasley, Hayley Bennett, Karen Brooks, Bhavana Harsha, Rei Kajitani, **Arpita Kulkarni**, Dorothee Harbecke, Eiji Nagayasu, Sarah Nichol, Yoshitoshi Ogura, Michael Quail, Nadine Randle, Diogo Ribeiro, Alejandro Sanchez-Flores,

Tetsuya Hayashi, Takehiko Itoh, Dee Denver, Warwick Grant, James B Lok, Haruhiko Murayama, Jonathan Wastling, Adrian Streit, Taisei Kikuchi, Mark Viney, Matthew Berriman

Nature Genetics, in revision (2015)

Germ line organization in *Strongyloides* nematodes reveals alternative differentiation and regulation mechanisms

Arpita Kulkarni, James W Lightfoot and Adrian Streit:

Chromosoma, submitted (2015)

Identification and cloning of the boundaries of chromatin diminution in the nematode males of *Strongyloides papillosus*

Arpita Kulkarni, Christian Rödelsperger and Adrian Streit:

Manuscript in preparation

Acknowledgements

Adrian you were a good boss! Thank you for this opportunity and for giving me the freedom to always explore my ideas.

I am very grateful to **Prof. Ralf J. Sommer**, for all the help, support and encouragement that he has always extended to me over these past years. I also thank **Prof. Nikolaus Blin** for agreeing to be my co-supervisor and for taking the time for the evaluation of this thesis.

A big danke schön to **Melanie Mayer** for her help in proof reading this thesis and for translating the summary into German.

I am grateful to all my collaborators at the **Sanger Institute** and **Dr. Warwick Grant** (La Trobe University, Australia) for being so supportive and for allowing me to be a part of so many exciting projects.

Likewise, a big thank you goes to **Dr. Christian Rödelsperger** and **Dr. James Lightfoot** for investing so much time and energy in helping me with my projects. I am especially grateful to Dr. James Lightfoot and **Dr. Christian Liebig** for introducing me to confocal microscopy! A big thank you goes to the ever enthusiastic **Dr. Matthias Flötenmeyer** and **Iris Koch**, for their help with the TEM and for always lending me a helping hand! You guys were incredible!

Much of my success was due to the fact that I had great co-workers. **Anja Holz**! I could not have asked for a more cheerful and supportive co-worker than you! Likewise, I thank **Anna Dyka** for spending hours, always laughing and never complaining, helping me collect dung from cattle and pigs and for help with its (smelly) processing!

I felt instantly at home in Germany because of some incredible people and their support. Amongst the most important of people for me to thank is **Julia Hildebrandt**. You have helped me on more occasions than I can count and made my life and time in Germany comfortable/enjoyable/happy. Thanks for all the lovely dinners at Halloween, Easter, Birthdays and every other possible celebratory occasion. You and **Vlado** bring laughter and cheer into all situations!

Sören! Thank you for being so patient and for always giving me stellar advice. Plus thank you for all the fun times at basketball. **Vahan** and **Vladislav**, thank you for being such strong and supportive friends!

Things went smoothly for me in the lab because of our wonderful technicians: **Doro**, **Metta**, **Hanh**, **Heike, Tobias, Gabi** and **Walli**! Thank you for all your help and support. Likewise, hats off to our cleaning ladies **Evi**, **Aida** and **Gertrude** and the media kitchen for help with lab reagents, autoclaving and for providing (almost magically) ready-made bottles of any media one desires.

A big thank you to everyone in Dept. 4 past and present, especially **Kostadinka**, **Melanie**, **Josephine**, **Bogdan** (Спасибо за обучение меня русская!), **Masa**, **Nermin**, **Edu**, **Cam**, **Erik**, **Simone**, **Isabella**, **Lisa**, **Amit, Martin, Jan, Igor, Dhananjay**, **Gaurav**, **Neel**, **Suryesh**, **Praveen** and especially the Streit group members **Alex**, **Anja**, **Tegegn** and **Siyu**!

Lastly, I thank my family. Without your love and support, this PhD was impossible! I dedicate this thesis to aai, baba and tai.

Contents

Summary

Nematodes of the genus *Strongyloides* are obligate parasites of various vertebrates (including man) and are thus of economic and medical significance due to their disease-causing ability. Interestingly, the life cycle of *Strongyloides* nematodes consists of 2 distinct generations, a parasitic one within the vertebrate host and a free-living one outside the host. This free-living generation is almost unique among nematode parasites of vertebrates. Ease of access and methods for cultivating this generation are proving important for understanding the molecular basis of true parasites. For the purpose of my thesis, I have chosen to work on 3 different aspects of the previously poorly understood reproductive biology of *Strongyloides*, namely:-

I. sex determination, with an aim to create a better understanding of sex chromosome evolution within this genus; II. identifying the boundaries and mechanisms of chromatin diminution in the males of *S. papillosus*; and III. a detailed characterization of the *Strongyloides* spp. germ line in comparison to the well-studied nematode *C. elegans*.

There are 3 sex determining systems described for *Strongyloides* species: the XX/X0 system, where females have 2 X-chromosomes but males only one; sex specific chromatin diminution, wherein males are determined by the loss of an internal portion of a chromosome; and an environmental one, with no karyotypic differences between the sexes. I could show that XX/X0 sex determination is ancestral within Strongyloididae, suggesting that chromatin diminution in particular is a derived state. This has given us an understanding of the rapid evolution of sex determining mechanisms and sex chromosomes within this genus by allowing us to correlate the evolutionary life histories of reproduction with chromosome structure. Further, through molecular and genetic tools, I have identified the boundaries on the chromosome where chromatin diminution occurs in the males of *S. papillosus* and speculated about the cellular mechanisms that might govern this event. In the process, I have contributed significantly in sequencing, annotating and improving the existing *S. papillosus* genome assembly. Finally, I have characterized the germ line of 3 closely related Strongyloididae members (*S. ratti*, *S. papillosus* and *P. trichosuri*). Specifically, I have studied differential chromatin amplification in some germ nuclei, the absence of stem cell populations and germ cell divisions, differential control of gametogenesis and the differences in germ line chromatin, i.e histone modification patterns. My results reveal striking differences in development, organization and fluid dynamics of the Strongyloididae germ line, both between the different Strongyloididae species and in comparison to *C. elegans*.

This study thus showcases *Strongyloides* as a promising genus for basic biological and evolutionary research. More importantly it provides the much needed understanding of the reproductive biology of these emerging and medically relevant parasitic nematodes.

Zusammenfassung

Nematoden der Gattung *Strongyloides* sind obligate Parasiten vieler Vertebraten (einschließlich Menschen) und daher wirtschaftlich und medizinisch von Bedeutung aufgrund ihrer Fähigkeit Krankheiten zu verursachen. Interessanterweise besteht der Lebenszyklus von *Strongyloides* Nematoden aus zwei unterschiedlichen Generationen, einer parasitären innerhalb des Vertebratenwirts und einer freilebenden außerhalb des Wirts. Diese freilebende Generation ist unter Nematodenparasiten von Vertebraten nahezu einzigartig. Zugänglichkeit und Methoden zur Kultivierung dieser Generation erweisen sich als wichtig für das Verständnis der molekularen Basis echter Parasiten. In dieser Arbeit untersuche ich drei verschiedene Aspekte der bisher nur schlecht verstandenen Fortpflanzungsbiologie von *Strongyloides*, nämlich:

I. Geschlechtsdetermination mit dem Ziel, das Verständnis der Evolution der Geschlechtschromosomen in dieser Gattung zu verbessern; II. Bestimmung der Grenzen und Mechanismen der Chromatindiminution bei *S. papillosus* Männchen; und III. eine ausführliche Beschreibung der *Strongyloides* spp. Keimbahn im Vergleich zum gut erforschten Nematoden *C. elegans*.

Es gibt drei Geschlechtsdeterminationssysteme, die bei *Strongyloides* Arten beschrieben wurden: das XX/X0-System, wobei Weibchen zwei, aber Männchen nur ein X-Chromosom haben; geschlechtsspezifische Chromatindiminution, wobei Männchen durch den Verlust eines internen Teils eines Chromosoms bestimmt werden; und ein umweltspezifisches ohne karyotypische Unterschiede zwischen den Geschlechtern. Ich konnte zeigen, dass die XX/X0-Geschlechtsdetermination anzestral innerhalb der Strongyloididae ist, was darauf hinweist, dass insbesondere die Chromatindiminution einen abgeleiteten Zustand darstellt. Dies hat zu unserem Verständnis der schnellen Evolution der geschlechtsdeterminierenden Mechanismen und Geschlechtschromosomen innerhalb dieser Gattung beigetragen, indem es uns erlaubte die evolutionären Reproduktionslebensgeschichten mit der Chromosomenstruktur zu korrelieren. Außerdem ermittelte ich mithilfe molekularer und genetischer Werkzeuge die Grenzen auf dem Chromosom, wo die Chromatindiminution bei *S. papillosus* Männchen stattfindet und spekulierte über die zellulären Mechanismen, die dieses Ereignis möglicherweise steuern. Dabei habe ich maßgeblich zur Sequenzierung, Annotation und Verbesserung des existierenden *S. papillosus* Genoms beigetragen. Schließlich beschrieb ich die Keimbahn dreier nah verwandter Strongyloididae Arten (*S. ratti*, *S. papillosus* und *P. trichosuri*), indem ich die differentielle Chromatinamplifikation in manchen Zellkernen der Keimbahn, die Abwesenheit von Stammzellpopulationen und Zellteilungen in der Keimbahn, die differentielle Kontrolle der Gametogenese und die Unterschiede im Chromatin der Keimbahn, d.h. Histonmodifizierungsmuster, untersuchte. Meine Ergebnisse zeigen markante Unterschiede in der Entwicklung, Organisation und Strömungsdynamik der Strongyloididae Keimbahn, sowohl zwischen den verschiedenen Strongyloididae Arten als auch im Vergleich zu *C. elegans*.

Somit präsentiert diese Arbeit *Strongyloides* als eine vielversprechende Gattung für grundlegende biologische und evolutionäre Forschung. Zudem liefert sie das dringend benötigte Verständnis der Fortpflanzungsbiologie dieser aufkommenden und medizinisch relevanten parasitären Nematoden.

1. Introduction

1.1 The Phylum Nematoda

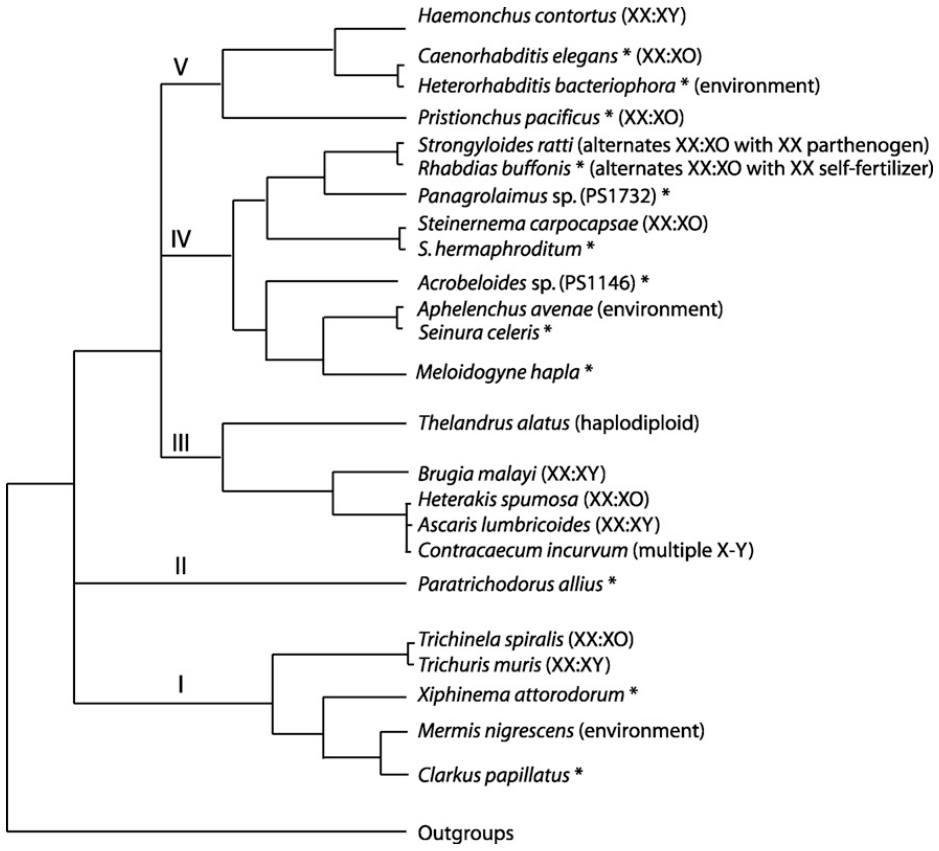
(from the Greek "nema" for thread and "eidos" for form)

Nematodes or 'roundworms' as they are commonly referred to, constitute the animal phylum of Nematoda. This is amongst the most diverse of animal phyla, with a species estimate of around 1-10 million (found as either free-living or parasitic), thus being a close competitor to the species-rich phylum Arthropoda (Lambshead, 1993; http://en.wikipedia.org/wiki/Nematode). Species belonging to Nematoda have successfully inhabited every imaginable ecosystem and ecological niche; aquatic (from marine to freshwater) and terrestrial (from polar to the tropics). Further, they are found living successfully at any geographical elevation, ranging from the high mountains to the deep trenches of our seas (Platt H.M, 1994; Lambshead, 1993).

This ability to survive and thrive in any habitat results in their sheer dominance in numbers over other animals. Their presence at various tropic levels is a clear indicator of the important role they play in many ecosystems (Platt HM, 1994). This role may be perceived as being beneficial or detrimental in nature. On the one hand, nematodes are the causative agents behind many animal (including human) and plant diseases, and millions of people die due to nematode infections across the world. Similarly, some studies show that we experience about a 15% loss of crops per annum globally due to nematode infections (http://www.apsnet.org/EDCENTER/K-12/NEWSVIEWS/Pages/Nematodes.aspx). On the other hand, studies on nematode species have led to several breakthroughs in biological and medical sciences. In 1998 *Caenorabhditis elegans* was the first multi-cellular animal to have its genome fully sequenced (see 'Genome sequence of the nematode C. elegans: a platform for investigating biology' by the *C. elegans* sequencing consortium). Whether beneficial or not, nematodes have a great impact on our life and that of our surroundings, making their study essential.

Blaxter et al. in 1998 clustered the phylum Nematoda into 5 major clades based on the sequencing of the small ribosomal subunit. According to this study, the genus *Strongyloides* (in particular *S. ratti* and *S. papillosus* as the main focus of this thesis) is a clade IV member. For comparison, *Caenorabhditis* species are several hundred million years away and belong to clade V. Each clade has examples of both free-living and parasitic forms indicating that parasitism has evolved multiples times (atleast 7

times: 4 times within animal parasitic nematodes and 3 times within plant parasitic nematodes) independently within this phylum (Blaxter et al., 1998).



from daSilva 2007

Fig.1 Phylogenetic tree showing all 5 nematode clades based on *SSU* sequencing. The genus *Strongyloides* is represented here by its most well studied member, *S. ratti*. In brackets are the modes of sex determination in the respective species.

1.2 The nematode genus *Strongyloides*

Nematodes of the genus *Strongyloides* were discovered (first description goes back to 1856 by Carl Wedl) to parasitize the gastrointestinal tract of various vertebrate animals including humans (Dorris et al., 2002; Spears, 1989; Streit, 2008). *Strongyloides* infection is in fact relatively common in wild and domestic animals. The genus currently consists of about 53 "true" described species belonging to the nematode clade IV (Speare, 1989; Blaxter, 2008) and has gained importance due to the species *S. stercoralis*, discovered in 1876, which is known to infect man. This relevance to humans has encouraged further work on this genus. Like some other gastro-intestinal nematode parasites, *Strongyloides* spp. also reproduce in the host gut and then lay eggs that eventually pass out of the host feces into the environment.

Strongyloides spp. follow this basic plan with some additional but significant differences. One of the main differences is that *Strongyloides* has a life cycle with an alternating free-living (facultative) and parasitic (obligate) generation (Speare, 1989). Female-only adult worms form the parasitic stages of *Strongyloides* (Viney and Lok, 2007; Streit, 2008). Further work on the parasitic stage (at least for the *Strongyloides* species studied so far) proved that the mode of reproduction in this generation is asexual.

In addition, there is a dioecious generation that occurs outside of the host and reproduces sexually. This free-living generation is almost unique among nematode parasites of vertebrates. The parasitic and free-living generations of *Strongyloides* are so morphologically distinct that they were mistakenly described as being different species of *Anguillula* (Bavay, 1876; Bavay, 1877), until they were discovered to be separate stages of one life cycle and then brought together as *Strongyloides* (Grassi, 1879).

Depending on the *Strongyloides* species in question, an infection is primarily (re)established when the infective larvae actively penetrate the host skin (reviewed in Streit, 2008) or in rare cases when the host ingests the nematode eggs and/or infective larvae, although this mode of infection is debated. Following a complex larval migration route involving multiple host organ systems, the larvae are then thought to establish themselves in the host gut mucosa and develop into actively reproducing adults thus completing the cycle (Speare, 1989; http://en.wikipedia.org/wiki/Strongyloidiasis).

Given that it is very hard to distinguish nematodes of this genus based on morphological features alone, earlier researchers defined some species of *Strongyloides* solely based on the host they were found in, due to their supposedly high host specificity (Augustine, 1940; Speare, 1989; Eberhardt et

al., 2008; Viney and Lok, 2007).

As a consequence, it was not uncommon to have taxonomic trouble within this genus, which resulted in different nomenclatures for the same species by different authors. This confusion has now been easily overcome by the advent of molecular identification (based on the SSU rDNA) supplemented by identification based on specific morphological features (Blaxter, 1998; Dorris, 2002; Eyualem and Blaxter, 2003; Floyd 2002; Hermann 2006a,b; Holterman, 2006; Eberhardt et al., 2008). Morphological features currently used for accurate identification of parasitic females include the shape of the stoma in apical view, the type of ovary, the shape of the tail and the number of lobes on the circumoral elevation. In the free-living generation, features such as the perivulval in females and the nature and shape of the spicule and its ventral membranes in males have become important. However, the list of informative characters given here is not exhaustive (for a comprehensive description refer to Little, 1966 and Speare, 1989).

A host infected with *Strongyloides* often remains in good health, with the worms merely living as innocent commensals and the infection being asymptomatic. An infection does however have the potential to become problematic under poor health conditions, in immuno-compromised patients or when it is massive, thereupon causing disease or even death.

Side Note

The genus *Strongyloides* belongs to the family Strongyloididae and super family Rhabdiasoidea (Speare, 1989). Other members of this family include the genera of *Parastrongyloides* (found in Austrialian possums) and *Leipernema* (found in pangolins, elephants, hippopotamus etc), neither of which are reported to naturally infect man. Members of these 3 genera are distinguished using their parasitic stages (Speare, 1989). *Parastrongyloides* and *Leipernema* are not discussed further in this thesis.

1.3 *Strongyloides* parasites of humans

Only three species of *Strongyloides* are known to infect humans naturally: *S. stercoralis, S. fuelleborni* and *S. fuelleborni kellyi.*

The first *Strongyloides stercoralis* nematodes were discovered in 1876 by a French doctor named Louis Normand who observed worms in the stools of his French troops suffering from what they called

"diarrhea de la Cochinchine" (Cochinchine is a region in southern Vietnam). The nematodes were named *Anguillula stercoralis* by Bavay but were soon rechristened in 1879 by Grassi as *Strongyloides stercoralis*, worms belonging to the new genus of "*Strongyloides*". Since then *Strongyloides stercoralis* is considered to be the type species for this genus (a historical perspective can be found in Speare, 1989).

S. stercoralis has a wide distribution in tropical and subtropical regions of the world (Schad, 1989). In addition to humans, *S. stercoralis* infections have also been reported in other mammals such as cats, dogs and captive primates. Some ambiguity still exists, as some investigators claim that the species found in dogs is typically *S. canis* and not *S. stercoralis* (Brumpt, 1921).

S. stercoralis is known to cause "Strongyloidiasis" which is currently considered the 4th most important nematode infection in the world. Strongyloidiasis is a chronic disease of the gastro-intestinal (GI) tract that lasts several decades. *S. stercoralis* also has the unique ability of autoinfection, wherein infective larvae develop within the host body and re-infect it, thereby continuing and self-sustaining the cycle of infection within the same host (Speare, 1989).

It is estimated that some 100-200 million individuals are infected worldwide with Strongyloidiasis. Given that these infections can be difficult to detect in normal healthy individuals, with infections being asymptomatic or as mild as dermatitis or diarrrhoea, these numbers could be gross underestimates (Schär et al., 2013; Crompton, 1987; Albonico et al., 1999) {For a more comprehensive list of the range of symptoms caused by *Strongyloides* in humans refer to Speare, 1989}.

Fatal 'Strongyloidiasis' in immuno-compromised patients is however rapidly becoming a huge medical problem in some parts of the developing world, mostly due to the large reproductive capacity of these worms within the weakened host, causing disseminated Strongyloidiasis (Keiser PB et al., 2004; Mejia R et al., 2012; http://en.wikipedia.org/wiki/Strongyloides_stercoralis). This marks the urgent need to study the basic biology of these parasites in greater detail.

Although it is difficult to generalize, the prevalence of *S. stercoralis* infections of humans is often quite low (around 10% or less) in endemic regions and usually affects younger individuals and children (Viney and Lok, 2007). This trend to affect younger individuals seems to be the norm for many other soil-transmitted helminth infections. There is an occasional occurrence of higher prevalence in countries of the developing world attributed to poor sanitation; and like mentioned above, also in immuno-compromised patients (Viney and Lok, 2007).

The anti-helminthic drug Ivermectin is currently used to treat uncomplicated Strongyloidiasis, although Albendazole and Thiabendazole may also be effective (Gompels et al., 1991; Mejia and Nutman, 2012; http://en.wikipedia.org/wiki/Strongyloidiasis). Strongyloidiasis is difficult to cure, and studies indicate that this may require life long treatment, as it is not uncommon for dormant stages of *Strongyloides* to live inside a host, even after decades of effective treatment (http://www.dva.gov.au/sites/default/files/files/publications/health/strongyloides_brochure.pdf).

For comparison with *Strongyloides*, global human infection with the gastro-intestinal nematodes *Ascaris lumbricoides, Trichuris trichiura* and *hookworms (Ancylostoma duodenale* and *Necator americanus)* are 1.4, 1.0 and 1.4 billion, respectively (Albonico et al., 1999). According to more recent reports by the World Health Organization, these numbers, not surprisingly, may again be underestimates (Deworming for health and development. Report of the third global meeting of the partners for parasite control [database online] Geneva: World Health Organization; 2005).

S. fuelleborni is the second *Strongyloides* species that infects humans. *S. fuelleborni* occurs in African primates where infection can be shared with humans (Pampiglione and Ricciardi, 1972; Hira and Patel, 1980). Non-human primates are typically infected with *S. fuelleborni* and *S. cebus*.

S. fuelleborni kellyi , the third species causing human infections, occurs exclusively in humans in New Guinea (Ashford and Barnish 1989; Ashford et al., 1992) {Note: Most of our current knowledge about *Strongyloides* of humans comes from *S. stercoralis* and not the latter two species}

In addition, some other *Strongyloides* spp. may be able to survive transiently in humans if infected artificially under laboratory conditions, e.g. *S. rasomi* and *S. procyonis* have been known to cause brief patent infections in man (in ~30% of cases) if infected experimentally (Speare, 1989). These results are from experiments that were done on human volunteers in those times.

1.4 *Strongyloides* parasites of animals

Most *Strongyloides* spp. commonly infect domesticated and wild animals, as summarized in the table below (modified from Speare R., 1989). Only species relevant to this thesis (*S. ratti* and *S. papillosus*) are then discussed in detail in further sections (Remark: Original references for the species given below can be found in Speare, 1989)

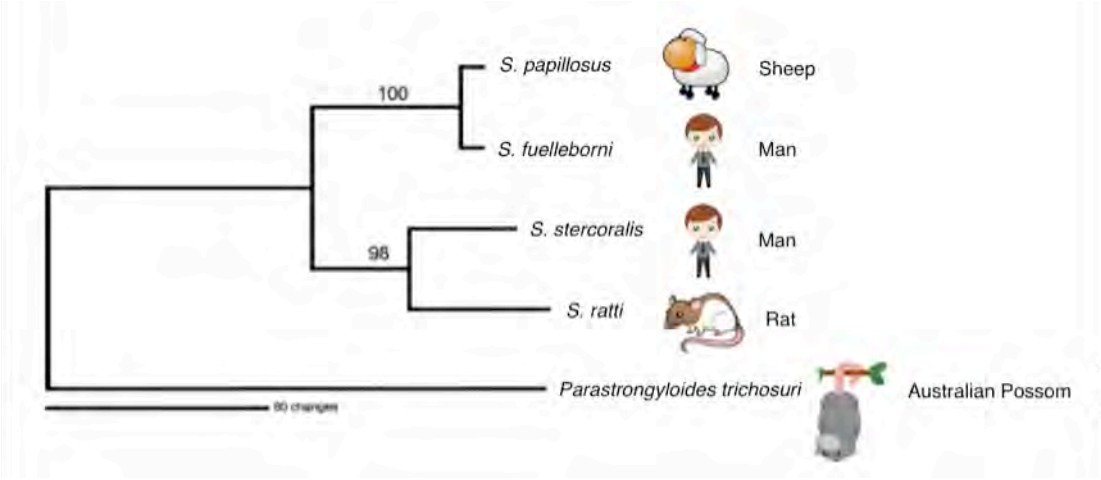
Species	Authors	Scientific name of host	Common name of host
S. agoutii	Griffiths, 1940	*Dasyprocta agouti*	Golden rumped agouti
S. akbari	Mirza & Narayan, 1935	*Crocidura coerula*	Musk rat
S. amphibiophilus	Perez Vigueras, 1942	*Peltophryne peltocephala*	toad
S. ardeae	Little, 1966b	*Nyctanassa violacea*	Yellow crowned night heron
S. avium	Cram, 1929	*Gallus gallus*	Domestic fowl
S. bufonis	Rao & Singh, 1954	*Bufo melanostictus*	Malayan toad
S. carinii	Pereira, 1935	*Leptodactylus gracilis*	frog
S. cebus	Darling, 1911	*Cebus capucinus*	White-throated capucin
S. chapini	Sandground, 1925b	*Hydrochoerus hydrochaeris*	capybara
S. cruzi	Rodrigues, 1968	*Hemidactylus mabouia*	skink
S. cubaensis	Perez Vigueras, 1942	*Butorides virescens maculatus*	Cuban green heron
S. darevskyi	Shapilo, 1976	*Lacerta saxicola*	skink
S. dasypodis	Little, 1966b	*Dasypodis novemcinctus*	armadillo
S. elephantis	Greve, 1969	*Elephas indicus*	Indian elephant
S. erschowi	Popova, 1938	*Nyctereutes procyonoides usuriensis*	Racoon dog
S. felis	Chandler, 1925a	*Felis catus*	Domestic cat
S. fuelleborni	Von Linstow, 1905	*Pan troglodytes* *Papio cyanocephalus*	Chimpanzee Yellow baboon
S. gulae	Little, 1966b	*Natrix cyclopyon cyclopyon*	Green water snake

Species	Authors	Scientific names of host	Common name of host
S. herodiae	Boyd, 1966; 1967	*Ardea herodias herodias*	Great blue heron
S. lutrae	Little, 1966b	*Lutra canadensis*	Common otter
S. martis	Petrov, 1940a	*Martes zibellina* *Mustela ermina*	Sable Stoat
S. minimum	Travassos, 1930b	*Anas bahamensis*	duck
S. mirzai	Singh, 1954	*Zamensis mucosus*	Rat snake
S. mustelorum	Cameron & Parnell, 1933	*Mustela ermina*	stoat
S. mycopotami	Attigas & Pacheco, 1933	*Myocaster coypus*	Coipu rat
S. nasua	Darling, 1911	*Nasua narica panamensis*	coatimundi
S. ophidiae	Pereira, 1929	*Drymobius bifossatus*	snake
S. oswaldoi	Travassos, 1930a	*Gallus gallus*	Domestic fowl
S. papillosus	Wedl, 1856; Ransom, 1911	*Ovis aries*	Domestic sheep
S. pavonis	Sakamoto & Yamashita, 1970	*Pavo muticus*	Green peafowl
S. pereirai	Travassos, 1932	*Elosia rustica*	frog
S. physali	Little, 1966a	*Bufo valiceps*	Wiegman's toad
S. planiceps	Rogers, 1939;1943	*Felis catus*	Domestic cat
S. procyonis	Little, 1966b	*Procyon lotor*	raccoon
S. putorii	Morosov, 1939	*Mustela putorius*	polecat
S. quiscali	Barus, 1968	*Quiscalus niger caribaeus*	bird

Species	Authors	Scientific name of host	Common name of host
S. ransomi	Schwartz & Alicata, 1930	*Sus scrofa*	Domestic pig
S. ratti	Sandground, 1925b	*Rattus norvegicus*	Brown rat
S. ratti v. ondatrae	Chandler, 1941	*Ondatra zibethicus*	Musk rat
S. robustus	Chandler, 1942	*Scirius niger rufiventer*	Fox squirrel
S. rostombekowi	Gamzemlidse, 1941	*Erinaceus europea*	hedgehog
S. serpentis	Little, 1966b	*Natrix cyclopyon cyclopyon*	Green water snake
S. sigmodontis	Melvin & Chandler, 1950	*Sigmodon hispidus*	Cotton rat
S. spiralis	Grabda-Kazubska, 1978	*Rana esculenta* *Rana lessoni*	Edible frog Edible frog
S. stercoralis	Bavay, 1876; Grassi, 1879	*Homo sapiens*	man
S. stercoralis v. vulpi	Mirza & Narayan, 1935	*Vulpus alopex*	Arctic fox
S. thylacis	Mackerras, 1959	*Isoodon macrouris*	Short nosed bandicoot
S. tumefaciens	Price & Dikmans, 1941	*Felis catus*	Domestic cat
S. turkmenica	Kurtieva, 1953	*Himantopus candidus*	stilt
S. venezuelensis	Brumpt, 1934	*Rattus norvegicus*	Brown rat
S. vulpis	Petrov, 1940b	*Vulpes vulpes*	Red fox
S. westeri	Ihle, 1917	*Equus caballus*	Domestic horse

1.5 Phylogenetic relationships between *Strongyloides* species

Since working directly with *Strongyloides* of humans represents an ethical problem, many other *Strongyloides* species have emerged as ideal candidates for laboratory studies due to their ease of handling and maintainance. These include *Strongyloides ratti* and *Strongyloides papillosus*. Their phylogenetic relationships are shown below along with their natural hosts:



Modified from Eberhardt et al., 2008 and A. Hino et al., 2014

Fig. 2. Phylogenetic relationships between different *Strongyloides* spp. along with their natural hosts (right).

1.5.1 *S. ratti*

S. ratti is found to naturally infect wild rats, making it easy to maintain this species in its natural host under laboratory conditions {although gerbils also act as permissive hosts} (Horii et al., 1992; Byeong-Kirl Baek et al., 2002). In a study in the UK, the prevalence of *S. ratti* in wild rats was discovered to be as high as 62% (Fisher and Viney, 1998), showing their natural abundance. In addition, *S. ratti* is phylogenetically closely related to *S. stercoralis* (see Fig. 2 above). Therefore, it is an excellent model system for studying the whole genus in particular and parasitic nematodes in general.

Strongyloides nematodes and their biology has been understood on most part due to the tools and techniques that have been developed in *S. ratti*. This includes: a short life cycle and a completely

sequenced and annotated genome (see submitted genome manuscript below). This is currently the second most complete nematode genome after *C. elegans*. Transgenic lines have been established (Shao H et al., 2012; Lok JB and Unnasch TR, 2013) and newer technologies like CRISPR may be possible in the near future. Furthermore, a genetic linkage map is available (Nemetschke et al., 2010), isofemale lines have been established (Viney et al., 1992), an expressed sequence tags (EST) database exists and microarray analysis has been done (Evans et al., 2008; Thompson et al., 2006).

(Note: More on the chromosomes of this species in the chapter on Sex determination and chromosome evolution)

1.5.2 *S. papillosus*

S. papillosus was the first species of *Strongyloides* to be described in 1856 by Carl Wedl and could well have become the type species for this genus. However, it was not officially recognized as belonging to the genus *Strongyloides* until 1911 (by Ransom, 1911), by which time *S. stercoralis* was already well established. This is unfortunate since *S. stercoralis* is unique in many regards, as has been pointed out before (Speare, 1989). Additionally, *S. papillosus* is phylogenetically closely related to *S. fuelleborni* (Hino et al., 2014; see Fig. 2 above). Luckily, the two most common infections of farm ruminants are with *S. papillosus* (sheep and cattle) and *S. vituli* (only cattle) {Brumpt, 1921; Eberhardt et al., 2008; Lentze, 1999; Peinaar, 1999; Zaffagnini, 1973}, making access to this species relatively easy. As a huge advantage, rabbits make good permissive hosts for *S. papillosus* and therefore this species can also be readily kept in the laboratory. Some basic questions about the biology of this species have already been answered (Nemetschke et al., 2010; Eberhardt A.G. et al., 2007,2008; Albertson et al., 1979; Zaffagnini 1973). An EST database has also been established (Sanger Institute, UK; Genome sequencing center St. Louis, USA), and full genome sequencing data will be available soon (see submitted genome manuscript below). However, working with *S. papillosus* is currently harder than with *S. ratti*. This is mostly due to the fact that not many genomic tools have been established in this species yet and the genome sequence quality is not yet comparable to that of *S. ratti*. Additionally, single worm infections are difficult if not impossible to establish in the suboptimal permissive hosts (e.g. rabbits).

1.6 Life history and reproductive biology of *Strongyloides* species

Sandground on *Strongyloides* (1926)

"…there remains a number of unsolved problems connected with the life-history of this genus, which to the biologists as well as to the parasitologist is undoubtedly one of the most interesting among the nematodes."

Given that we have now made significant progress in establishing genetic tools and sequencing the genomes of multiple species of the genus *Strongyloides* (*S. papillosus, S. ratti, S. stercoralis*, *S. venezuelensis*) and of its sister taxon *Parastrongyloides*, it is now a good time to address and solve some of the open questions that surround the life history and reproduction in this genus (V.L. Hunt et al 2015; Grant et al 2006a,b; Guiliano and Blaxter 2006; Li et al 2006; Viney 2006; Lok 2007). As briefly mentioned above, the life cycle of *Strongyloides* nematodes is bi-phasic with a parasitic generation occurring inside a host and a free-living generation occurring out in the environment. The finer aspects of the life cycle are discussed in detail below.

The parasitic generation consists only of adult females. No parasitic males have been found in *Strongyloides* species so far. A parasitic infection is established when infective *Strongyloides* larvae (all female) infect a new vertebrate host by skin penetration (Streit, 2008). Following a complex migratory route, these larvae finally establish themselves into the mucosa of the small intestine, where they develop into adult parthenogenetic females (Viney and Lok, 2007; Streit, 2008). Reproduction in this generation was shown to be clonal and all parasitic *Strongyloides* species are so far known to reproduce asexually by a genetically mitotic form of parthenogenesis (Viney, 1994). Interestingly, even though this reproduction is clonal, embryos of both sexes are produced.

The male and female embryos then emerge into the environment through the host feces and constitute the facultative free-living generation. The male embryos have only one life style option, to develop into adult free-living males. In contrast, the female embryos have a choice of either developing into adult free-living females and remaining in the environment or becoming infective. The developmental decision to become infective is made around the L1 stage and termed the 'homogonic' or direct cycle. Infective larvae must necessarily then find a host to complete their life cycle.

Auto-infections have been exclusively noted only for *S. stercoralis* and *S. felis*. Auto-infections (as briefly mentioned in earlier sections) are when some larvae of these species develop precociously into

infective larvae or iL3s within the host and then reinfect the same host, therefore giving rise to what has been described as a self-sustaining autoinfective cycle (Streit, 2008; Schad, 1989; Speare, 1989; Keiser and Nutman, 2004). Because this aspect seems to be exclusive to only *S. stercoralis* and *S felis*, it is not discussed further in this thesis.

The female larvae that do not become infective must necessarily go on to form free-living females that mate with the males. Reproduction in the free-living generation is sexual (Viney, 1994; Eberhardt et al., 2007). Oddly, this sexual reproduction results in the production of only one kind of embryo and the formation of all female infective progeny. The free-living generation, consisting of males and females, is termed as the 'heterogonic' or indirect cycle. The infective larvae arising from both the direct and indirect cycle need to find a vertebrate host to complete their life cycle.

In order to avoid the temptation to speculate, it must be noted here that there is no genetic difference between the parasitic and free-living females of *S. stercoralis* (Hammond and Robinson, 1994), *S. ratti* (Nigon and Roman, 1952; Zaffagnini, 1973; Triantaphyllou and Moncol, 1977; Viney, 1994) or *S. papillosus* (Albertson et al., 1979; Eberhardt et al., 2007).

Free-living *Strongyloides* species show 4 external molts before adulthood, whereas infective larvae have 2 external molts in the environment followed by 2 internal molts in the vertebrate host before becoming parasitic adults (Basir, 1950). The developmental decision to become infective is made early in development, typically before the second molt. The exact molecular mechanisms that control this fate remain currently poorly understood. Thus, several researchers are now working on understanding the genetic and molecular basis of this homogonic/heterogonic switch in *Strongyloides* spp. Recently studies have shown that the application of delta7 dafachronic acid prevents the formation of L3 infective larvae in *S. papillosus, S. ratti* and *S. stercoralis* (Ogawa A. et al., 2013). In *C. elegans*, low delta7 dafachronic acid levels act as a signal for DAF-12 to be in its ligand-free form, leading to a dauer fate (Motola et al. 2006). The conclusion that was drawn based on these studies was that the infective L3 formation in *Strongyloides* species and dauer formation in *C. elegans* share a conserved endocrine regulatory module, possibly due to the fact that they are homologous processes in these species (Ogawa A. et al., 2013).

Below is the generalized schematic representation of the life cycle of *Strongyloides* species: (modified from Streit, 2008)

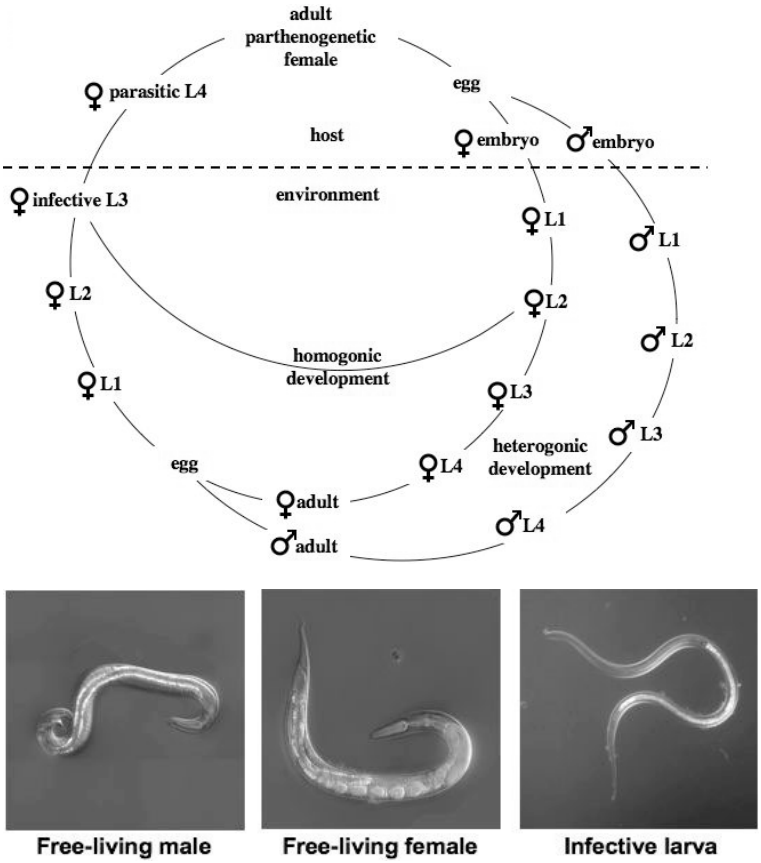


Fig. 3. (Top) Generalized life cycle of *Strongyloides*. (Bottom) Differential interference contrast picutres of an adult male (left), adult free-living female (centre) and an infective iL3 (right) of *S. papillosus*.

1.6.1 Reproduction in the free-living generation

It is now a generally accepted fact among nematologists that the free-living generation of most *Strongyloides* spp. consists of both males and females and that reproduction in this generation is sexual {reviewed in Streit, 2008}.

However this was not the widely held belief until of late. The mere presence of males was not a sufficient criterion for earlier researchers to believe that reproduction in the free-living generation was sexual. Infact, many scientists believed pseudogamy to be the dominant mode of reproduction (Triantaphyllou and Moncol, 1977). This meant that the adult males and females did mate and mature sperm was transmitted to the oocyte, but the male and the female pronuclei did not actually

fuse after fertilization. The male pronucleus was expelled just in time to prevent its fusion with the female pronucleus. This resulted in no genetic contribution from the father to the offspring, but sperm was nevertheless needed to trigger development of the embryos (Hammond and Robinson, 1994; Triantaphyllou and Moncol, 1977; Streit 2008). If true, pseudogamy would prove to be an elegant way to account for the resulting 'all female' progeny. It must however be noted here that all these observations were only cytological in nature and no genetic analyses were done.

This view was in time challenged and discarded by molecular work done by various authors on *S. ratti* and *S. papillosus* showing conclusively that fathers in fact contributed genetically to their offspring. For example, in *S. ratti*, the use of microsatellites clearly demonstrated that males pass on their genetic information to their offspring (Harvey et al., 2001; Viney et al., 1993). Similarly, paternal inheritance of SNP markers was shown in *S. papillosus* (Eberhardt et al., 2007) and in *S. ratti* (Nemetschke et al., 2010).

The genus however consists of many more species than the 2 discussed above, and it must be pointed out that what happens in *S. ratti* and *S. papillosus* may not represent the genus in its entirety. For example, recent studies indicate that no free-living males are observed in *S. venezuelensis*, and reproduction in the free-living generation is thought to be by mitotic parthenogenesis instead (A. Hino et al., 2014) {Note: *S. venezuelensis* is more closely related to *S. papillosus* than to *S. ratti*}

1.6.2 Reproduction in the parasitic generation

Reproduction in the parasitic generation does not require males and occurs by mitotic parthenogenesis. Previous researchers have reached this consensus based on numerous observations:

No parasitic males have ever been discovered in this genus (except for Kreis, 1932; Faust, 1933; whose work in this regard is now disregarded by the *Strongyloides* community). In addition, researchers have failed to isolate mature sperm from the reproductive organs of parasitic females in *S. ratti* (Streit, 2008; Chitwood and Graham, 1940), *S. papillosus* (Streit, 2008; Chang and Graham, 1957) and recently in *S. venezuelensis* (Akina Hino et al., 2014). A parasitic adult female even lacks a spermatheca entirely (see Speare, 1989).

Presence of a vitelline membrane in early embryos is considered a direct result of fertilization. The absence of any vitelline membrane in the developing embryos within the parasitic females of *S. ratti* is

further indication that no fertilization occurs in this generation. In contrast, a vitelline membrane was shown to be present in the embryos resulting from the free-living generation (Chitwood and Graham, 1940).

Successful iso-female-lines have been established for both *S. ratti* (Graham, 1936 and 1938; Viney et al., 1992) and *S. papillosus* (Nemetschke et al., 2010). This means that an infection was established in a natural or permissive host using just one infective L3 larva (all female) and offspring of both sexes (future free-living generation) and directly developing infectives (iL3) were recovered by sampling the host feces (Graham, 1936 and 1938; Viney et al., 1992; Viney, 1994).

Cytological observations showed that no meiosis occured in maturing oocytes in *S. ratti* (Chitwood and Graham, 1940; Nigon and Roman, 1952), *S. papillosus* (Zaffagnini, 1973; Triantaphyllou and Moncol, 1977) and *S. ransomi* (Triantaphyllou and Moncol, 1977).

Additionally, mitotic parthenogenesis implies that all offspring produced are full clones of their mother and therefore identical to each other. This was found to be true by genetically comparing the offspring (male and female) to their mother in both *S. ratti* (Nemetschke et al., 2010; Viney, 1994) and *S. papillosus* (Nemetschke et al., 2010).

These observations clearly indicate that male parasitic worms are not required for successful reproduction in the parasitic generation of *S. ratti, S. papillosus* and *S. venezuelensis*.

Side Note

As is noted above, the development of the embryos arising from the parasitic females seems to be the result of two discrete developmental decisions (Harvey et al., 2000). One of these decisions involves the male/female sex determination switch and the other involves a decision between becoming either a free-living adult female or a homogonically developing infective female (iL3) {Harvey et al 2000}. Clearly these developmental switches are of paramount importance, as this choice affects the reproductive success of the animal (Viney et al., 2002).

When these developmental decisions were studied further in some *Strongyloides* species, they were found to be atleast in part under environmental control. The switch between the alternate female morphs in *S. ratti* was influenced by the host immune status and by the temperature experienced by the developing larvae outside of the host. In contrast, the male/female switch was affected solely by

the host immune status (Harvey et al., 2000; Harvey et al., 2001). However, host immune status and temperature at development are not the only environmental factors that affect this decision. Non-immunological stressors (arising from infections in atypical hosts) have been documented in *S. ratti* (Crook and Viney, 2005). What is clear is that, of whatever nature this environmental factor(s) is, it somehow interferes with gametogenesis in the parasitic female (Harvey et al., 2001). However, it is not yet clear if this interference is a direct result of the environmental factor alone, or due to the mother's response to this factor, or a combination of both (reviewed in Streit, 2008).

In addition to these, some more stressors in multiple *Strongyloides* species are documented below (modified from Streit, 2008).

Condition favouring homogonic	Condition favouring heterogonic	Species studied	Reference
Lower temperature	Higher temperature	*S. fuelleborni*	Hansen *et al.* (1969); Premvati (1958*b*)
Temperature <20 °C or >30 °C	Temperature 20 °C – 30 °C	*S. fuelleborni*	Premvati (1958*a*)
Lower temperature	Higher temperature	*S. papillosus*	Nwaorgu (1983)
Lower temperature	Higher temperature	*S. ratti*	Harvey *et al.* (2000); Viney (1996)
Temperature >31 °C	Temperature 15 °C – 31 °C	*S. stercoralis*	Nolan *et al.* (2004)
Non-neutral pH	Neutral pH	*S. ransomi*	Moncol and Triantaphyllou (1978)
pH 5–6	pH 7–9	*S. fuelleborni*	Premvati (1958*a*)
Low food	High food	*S. fuelleborni*	Premvati (1958*a*)
Low food	High food	*S. simiae*	Beach (1936)[a]
Culturing in water/little food	Culturing on filter paper/high food	*S. fuelleborni*	Little (1962)
Culturing in water	Culturing in Loke's solution[b]	*S. simiae*	Beach (1936)[a]
Fully aerobic	Semi-aerobic	*S. fuelleborni*	Hansen *et al.* (1969)
High population density	Low population density	*S. fuelleborni*	Hansen *et al.* (1969)

Table 2. Examples of non-host environmental factors described to influence the choice between direct and indirect development in *Strongyloides* species. (Remark: original references shown in the table above can be found in Streit, 2008).

1.6.3 Advantages of working on *Strongyloides*

Not many parasitic species offer the advantage of easy maintanance and access to a free-living generation like *Strongyloides* does. Additionally, these species are known for their small genome size of ~40-50Mb (for comparison, the genome size of *C. elegans* is ~100Mb {The *C. elegans* genome sequence consortium, 1998} and *P. pacificus* is ~160Mb {Dieterich C et al., 2008}). Many *Strongyloides* spp. now have a fully sequenced genome (see submitted genome manuscript below), making working with them easier. An interesting advantage also comes from the phylogenetic position of this genus,

as *Strongyloides* species have close relatives representing different modes of reproduction (e.g. with relatives being gonochorists, parthenogens, hermaprodites etc) and different life-styles (e.g. free-living, facultative and obligatory animal parasites, or entomopathogenic and even plant parasitic). This genus therefore displays great potential for becoming important for comparative evolutionary studies. Infact, closely related members within this genus operate on completely different sex determining systems, making it possible to co-relate their evolutionary histories of reproduction with their chromosome structures (see below). Finally, the reproductive biology of these species is currently poorly understood and there are thus many open and interesting questions about parasitism and drug discovery, evolution, development and cellular biology that can be answered using the genus *Strongyloides* as a model system.

1. 7 Sex determination, sex chromosomes and sex chromosome evolution in the genus *Strongyloides*

The life cycle of *Strongyloides* spp. was researched extensively by the early 20th century, mainly for parasitological interest. The most intriguing questions were of both ecological and evolutionary nature, for instance how the choice between clonal and sexual reproduction is made, how these species choose their sex and how they make the decision to become parasitic versus free-living (Streit 2008).

How do we explain the discrepancy that was generated from the large amount of conflicting data on reproduction in this genus? Disregarding false cytological observations, it might well be possible that observed differences are due to actual biological differences within species and isolates of a species. Due to the limitations in correct species identification based on morphology within this genus (Augustine 1940; Speare 1989), it might well be possible that different researchers worked on different species or isolates altogether, resulting in contrasting findings (Streit 2008).

Other aspects of life history and reproductive biology have been discussed in detail in earlier sections. Therefore, sex determination and chromosome number are discussed here. There are currently 3 described sex determining sytems among *Strongyloides* spp: XX/X0 sex determination, with females having 2 X-chromosomes and the males only one; sex-specific chromatin diminution, where males lose an internal part of a chromosome to become male; and lastly (based on old literature but remaining currently illusive) an environmentally based system, with no karyotypical differences between the sexes.

Even though it was shown that sex determination in *Strongyloides* is atleast in part influenced by the environment, sex chromosomes have been shown to exist in *S. ratti* and *S. stecoralis*. Cytological analysis of these two species has revealed the presence of two different karyotypes in the embryos of the parasitic generation. One set of embryos has a karyotype of 2n=5, whereas the other has a karyotype of 2n=6. Based on further molecular and genetic studies, it was shown that the embryos with 6 chromosomes became females, while the embryos with a karyotype of 5 developed as males (Harvey and Viney 2001; reviewed in Streit 2008). Thus the females have 2 pairs of autosomes and a pair of X chromosome (XX) and the males have 2 pairs of autosomes and only a single X chromosome (X0) {reviewed in Streit 2008; Harvey and Viney 2001}. This suggests that in *S. ratti*

and *S. stercoralis* sex determination works using an XX/X0 system. As such, XX/X0 sex determination is very common among nematodes (including *C. elegans*) and is speculated to be the ancestral mode of sex determination (Pires daSilva 2007).

Furthermore, atleast 2 *Strongyloides* species have been shown to lack an independent X chromosome altogether, namely *S. ransomi* and *S. papillosus* (Albertson et al., 1979; Triantaphyllou & Moncol, 1977; Nemetschke et al., 2010). As part of this thesis I showed that this is also the case for *S. vituli* (Kulkarni et al., 2013), which is discussed in detail below.

1.7.1 Different karyotypes in the genus *Strongyloides*

Cytological observations indicate that there are 3 pairs of roughly equally sized chromosomes in *S. ratti* and *S. stercoralis,* which correspond to the 2 pairs of autosomes and the pair of X chromosome of these species. As discussed above, males of these species lack 1 X chromosome (also refer to fig. 4 below).

In *S. ransomi*, *S. papillosus* and *S. vituli,* there are only 2 pairs of chromosomes with one pair being almost twice as large as the other. For *S. ransomi* and *S. papillosus* these chromosomes were commonly referred to in literature as 2 large (2L) and 2 medium (2M) chromosomes (refer last two karyotypes in fig. 4 below). This 2L2M karyotype was hypothesized to be the result of a chromosome fusion event, in which the X chromosome fuses with one of the autosomes, resulting in a pair of long chromosomes, the 2L (Triantaphyllou and Moncol 1977). This hypothesis was later proved to be true, at least in the case of *S. papillosus* (Nemetschke et al., 2010 and this work {Kulkarni et al., 2013}). Recently, the karyotype of *S. venezuelensis* was also shown to be 2n=4, the same as in *S. papillosus*. For the time being, it is unclear if this too is the result of a fusion event as in *S. papillosus* (Akina Hino et al., 2014).

Some previous authors have speculated that the males and females of *S. papillosus* and *S. ransomi* infact do not differ in chromosome number (Triantaphyllou and Moncol 1977). This was proved false in the case of *S. papillosus* in which males undergo chromatin diminution resulting in a different karyotype to that of the females of this species. Thus, the males finally have a karyotype of 1 Large, 3 Medium and 1 Small chromosome (1L3M1S) {Albertson et al 1979; Nemetschke et al 2010}. It appears unlikely (but remains unclear) that *S. venezuelensis* also undergoes chromatin diminution (Akina Hino et al., 2014) {Chromatin diminution is discussed in detail in further sections}

This is depicted in the figures below (modified from Nemetschke et al., 2010).

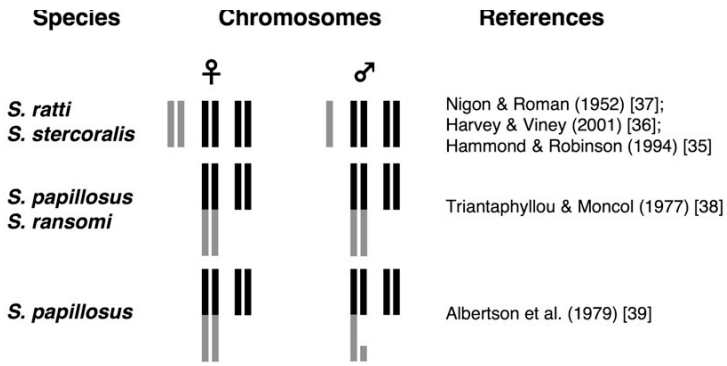


Fig. 4. Chromosomal configurations of *Strongyloides* spp. as described in older literature. Black, autosomes or regions derived from ancestral autosomes; gray, X chromosomes or regions proposed to be derived from ancestral X chromosomes.

Additionally, it was shown that *S. papillosus* males lose one copy of what is X chromosomal in *S. ratti*, meaning that chromatin diminution helps create a hemizygous region in these males (Nemetschke et al., 2010). Refer to the schematic below to understand the chromosomal rearrangements that have taken place between *S. ratti* and *S. papillosus* (modified from Nemetschke et al., 2010).

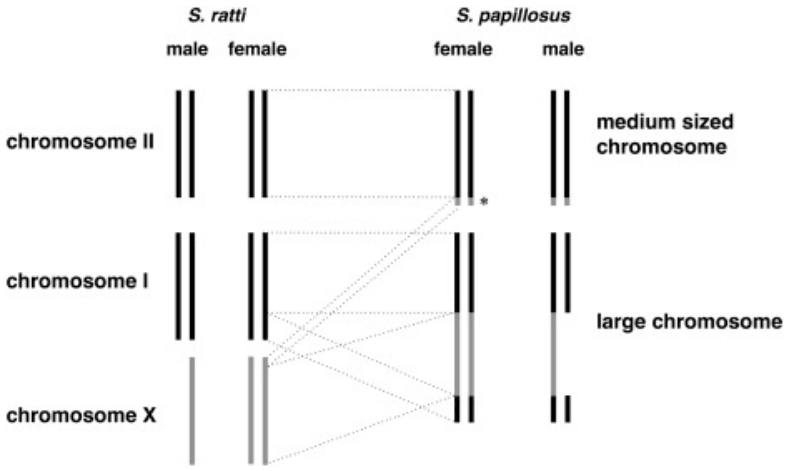


Fig. 5. Model for the evolutionary relationship between *S. papillosus* and *S. ratti* chromosomes. Black, autosomes in *S. ratti* or regions evolutionarily related to *S. ratti* autosomes in *S. papillosus*; grey, X chromosome in *S. ratti* or regions evolutionarily related to the *S. ratti* X chromosome in *S. papillosus*. Note the modification in karyotype for *S. papillosus* from that described by older authors in Fig. 4.

Given that closely related species of *S. ratti* and *S. papillosus* show differences in their sex chromosomes and the sex determining systems that they use, this opened up a number of questions regarding the ancestral mode of sex determination within this genus. This information was crucial, as it would also shed light on how sex chromosomes have evolved in this genus.

In this Thesis

My main interest was to correlate the evolutionary history of reproduction in *Strongyloides* spp. with their chromosome structures, given the variety of both within this genus. In the course of answering this question I have shown for the first time the karyotypes of 2 different nematode species, namely *S. vituli* and *P. trichosuri.* Additonally, I demonstrate the existance of male-specific chromatin diminution in *S. vituli.* I also show that *P. trichosuri* uses an XX/X0 sex determining system, with both generations undergoing sexual reproduction. Earlier workers had proposed the X chromosome-autosome fusion without strong arguments for what was ancestral. Here I provide evidence that fusion of chromosomes rather than fission is the most likely scenario and that chromatin diminution is then a mechanism of restoring an XX/X0 state in *S. papillosus* (Nemetschke et al., 2010; Kulkarni et al., 2013). This implies that XX/X0 is the ancestral mode of sex determination in this genus (see fig. 6 below). Finally, this study is further relevant as it answered important and missing clues in the field and was included in a recently published hypothesis (see figure 7 below) for the evolution of *Strongyloides* spp. and related nematodes (Streit 2014).

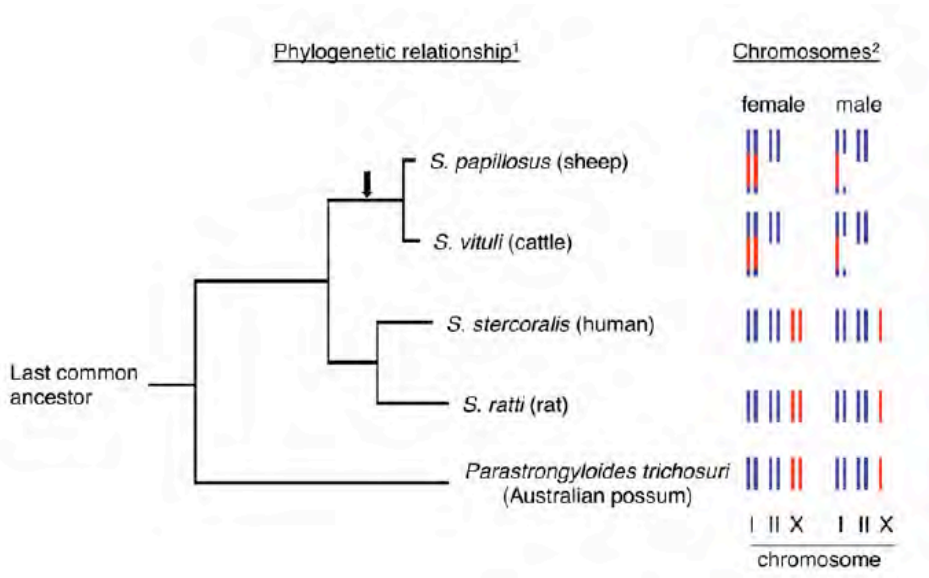


Fig. 6. Different kaytotypes of the related *Strongyloides* spp. (modified from Kulkarni et al., 2013) showing that XX/X0 sex determination is likely ancestral in this genus.

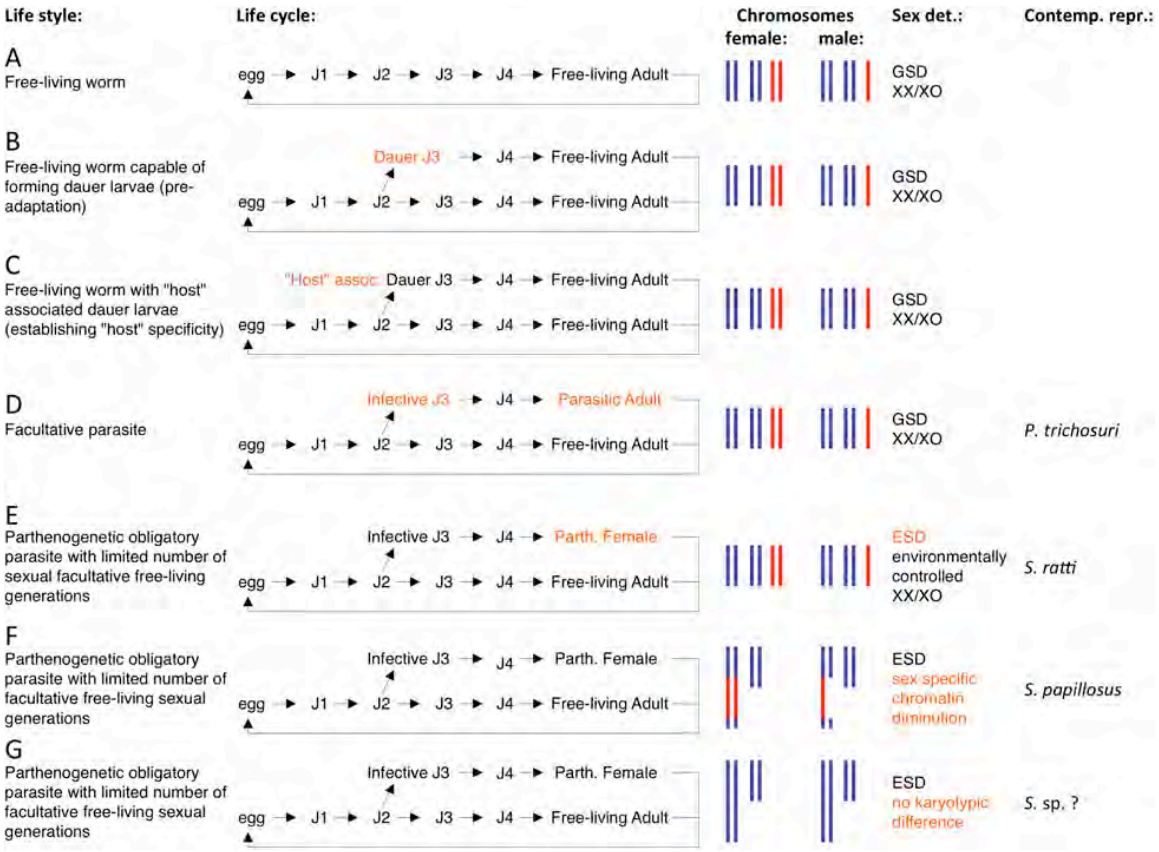


Fig. 7. Hypothesis for the evolutionary history of *Strongyloides* spp. going from an ancestral free-living nematode with a simple life cycle and genetic XX/X0 sex determination (Life style A) to a species of *Strongyloides* with its alternative life cycles, with no genetic differences between the sexes and environmental sex determination (Life style G) as put forth by Streit, 2014. Evolutionary novelties are highlighted in orange (bold grey in the print version). Chromosomes and parts of chromosomes present in two copies in males and in females are represented in blue (black in the print version). Chromosomes and parts of chromosomes present in only one copy in males but two copies in females are represented in red (grey in the print version). For further detailed explanation of individual evolutionary steps please refer to Streit, 2014 {this figure is modified from Streit, 2014}.

1.8 Chromatin diminution

(L. *diminuere*: to diminish or to reduce to small pieces)

It has been shown that the males of *Strongyloides papillosus* undergo the process of chromatin diminution (Albertson et al., 1979; Nemetschke et al., 2010), but not much is understood about this event in this species. Since our current understanding of chromatin diminution comes from multiple species that include non-nematode models, other species are first discussed here to provide the necessary background for later comparison with *S. papillosus*.

The DNA constancy rule suggests that all cells of an organism must have essentially the same identical genome, both qualitatively and quantitatively (Tobler, 1986). This means that when a cell divides mitotically, the resulting daughter cells have the same DNA as the original mother cell. This also implies that all organisms protect and govern genomic integrity carefully at every cellular division.

Interestingly, it was in the late 19th century that a German scientist named Theodor Boveri discovered the first exceptions to this central rule. His work in 1887 (at the University of Würzburg) on the nematode *Ascaris megalocephala* (initially renamed *Parascaris equorum var. univalens* and now *Parascaris univalens)* resulted in the surprising observation that the full complement of chromosomes was retained only in a subset of cells in the *Ascaris* embyro. In contrast, the chromosomes in some other cells underwent a curious process of fragmentation and elimination that came to be known as chromosome or chromatin diminution (Herla, 1893).

Chromatin diminution thus came to be defined as a developmentally regulated process, wherein parts of chromosome(s) were deliberately lost at certain (mainly early) cell divisions, albeit by unknown cellular mechanisms. Due to the event of chromatin diminution, the resulting daughter nuclei contain considerably less chromatin when compared to the nuclei of cells that undergo normal mitosis (Tobler and Muller, 2001).

In subsequent years, to everyone's surprise, a series of other organisms were discovered to undergo the process of chromatin diminution. These included rotifers and ciliates, crustaceans and insects, nematodes, mammals and other chordates, and even some plants (Tobler et al., 1995; Tobler et al., 1986; Pimpinelli et al., 1989; Kubota et al., 1993; Goday et al., 1993; reviewed in Kloc and

Zagrodzinska, 2001). Given this vast diversity, chromatin diminution is rightly thought to be of polyphyletic origin (Tobler et al., 1986; reviewed in Tobler and Muller, 2001).

For the above nematode species that have been studied, it is known that chromatin diminution removes a considerable number of genes from somatic cells (Streit, 2012; Wang et al., 2012; Smith et al., 2012). In other animals like ciliates (*Oxytricha*), up to 95% of genomic DNA is thought to be lost during a diminution event (Nowacki et al., 2011).

Side note

The process of chromatin diminution is not to be confused with 'whole chromosome elimination', which implies the loss of an entire chromosome at cellular division, for example as seen in the development of Sciarid flies (clarified in later sections) or with the process of 'ribonucleoprotein shedding', whereby large chunks of ribonucleoproteins (but not DNA) are discarded during early divisions, as found in the case of some Lepidopteran oocytes (Tobler et al., 1986).

In addition to chromatin diminution and chromosome elimination, other exceptions to the DNA constancy rule include gene amplification, gene magnification, gene under-replication, gene loss, and gene rearrangement (Mueller et al., 1995). These are not discussed further.

Chromatin diminution in other (non-nematode) animals

Although rare, a number of species across the animal kingdom do display the process of chromatin diminution, arguing for its polyphyletic origin. This diversity is depicted in the picture below. Even though all these species undergo diminution, it must be noted that the elimination of DNA in the different species differs in many aspects, such as developmental stage, cell and tissue type, stage of cell cycle, type of cell division (mitosis or meiosis), type of chromosomes and amount of eliminated material (Mueller et al., 1995). This implies that different animals must employ different mechanisms to regulate this process. It may also be speculated that chromatin diminution carries out different functions in different animals. Some of the best understood examples are briefly discussed below.

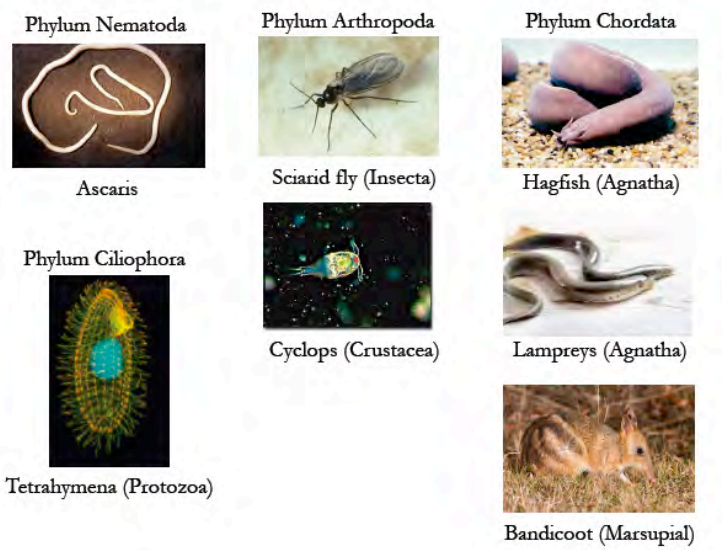


Fig. 8. Examples of species from different animal phyla undergoing chromatin diminution.

1.8.1 Chromatin diminution in ciliates e.g. *Tetrahymena*

Biological processes in ciliates have been studied extensively during the last two centuries, mainly because of their large size (sometimes >100μm) and transparency. Ciliates were also amongst the early models for studying epigenetic programming of DNA rearrangements (Meyer E. and Duharcourt S., 1996).

Ciliates are unique in that they posses two nuclei per cell, a germ line micronucleus (MIC) and a somatic macronucleus (MAC). These nuclei differ not only in appearance but also in function. Most ciliates being unicellular, the MIC and the MAC become analogous to the germ line - soma distinction of metazoans (Meyer E. and Duharcourt S., 1996). The MIC contributes to only germ line function (transfer of DNA to sexual progeny) while the MAC remains transcriptionally active contributing to the cells' mRNA production (Nowacki et al., 2011).

The MAC, after chromatin diminution, consists basically of an edited germ line genome that has amplified and repaired (sometimes through gene unscrambling) its remaining DNA (Nowacki et al., 2011). This gene unscrambling, which is the linking and shuffling together of DNA pieces created after diminution, results in genome rearrangements in the MAC by bringing together previous genetically unlinked loci (DH Ardell et al., 2003).

Broadly speaking, DNA elimination in ciliates occurs through two mechanisms, an imprecise mechanism that involves removal of repetitive DNA sequences between genes and a precise mechanism that refers to the removal of intragenic IESs (for 'internal eliminated sequences'). DNA breakage is known to occur at specific sites in the genome (>6000 sites for IESs) of ciliates. These regions share a 15bp sequence called Cbs for 'chromosomal breakage sequence' that was found to be necessary and sufficient for breakage to occur (Yao MC et al., 1986,1987,1990,2005; King BO, 1982). Additionally, this Cbs is suspected to be species-specific in ciliates.

A direct link between RNAi and DNA deletion (acting via an epigenetic modification of histone) has been shown in *Tetrahymena* (Meister G et al., 2004; Meyer and Chalker, 2007). Sequences around IESs are first thought to be transcribed bidirectionally leading to the formation of long dsRNA (Mochizuki K, 2004) before being processed to small RNAs by Dicer-like enzymes (Nowacki et al., 2011). These small RNAs in turn recognize their original transcription site, leading to DNA deletion at these regions. Up to 20% of the genome is deleted in *Tetrahymena* in this way.

Other ciliates undergoing chromatin diminution include *Oxytricha, Euplotes* and *Stylonychia.* Estimates of DNA lost range between 85%-98% in these species (Prescott DM, 1994; Nowacki et al., 2011), making ciliates the most extreme examples of chromatin diminution.

1.8.2 Chromatin diminution in Copepods e.g. *Cyclops* and *Mesocyclops*

Chromatin diminution is well documented in 17 copepod species and has been shown to be absent in at least 10 (Dorward and Wyngaard, 1997). Depending on the species, the amount of DNA lost ranges between 50% (for *Cyclops divulsus, C. furcifer* and *C. strenuus*) to 90% in *Mesocyclops edax* and 94% in *Cyclops kolensis* (Beermann, 1977; Rasch and Wyngaard, 2001; Grishanin et al., 1996). In all Copepods, most of the eliminated DNA is heterochromatic (Beermann, 1977).

Little information is currently available about the nature of DNA that is eliminated in copepods (Rasch and Wyngaard, 2001). What contributes most to this fact is the difficulty to get ample amounts of germ line DNA for cloning and sequencing copepod genomes, given their small size. So far, based on a few studies, it is believed that only heterochromatic DNA, which is enriched in repetitive sequences, is lost in copepods (Kloc and Zagrodzinska, 2001).

Random amplification studies on DNA in *M. edax* indicate a partial elimination of DNA containing (CAAATAGA and CAAATTAAA) repeat sequences in somatic cells during chromatin diminution (Guy Drouin, 2006). Only one other study by Degtyarev et al from 2004 exists that reports the sequence of eliminated copepod DNA, highlighting the need to investigate these species further.

Strikingly in copepods, chromosomes do not remain fragmented after diminution but are rejoined via unknown mechanisms and therefore do not require the addition of new telomeres (Beermann, 1959). It is hypothesized that, just like phage DNA excision from bacterial chromosomes, heterochromatic DNA is excised out of copepods genomes by the formation of DNA loops. This theory has been further backed up by the discovery of chromatin rings in somatic nuclei of *Cyclops furcifer* (Beermann and Meyer, 1980).

Based on these studies, it is speculated that chromatin diminution might have evolved by the repeated recruitment of RNAi-related mechanisms in the genomes of some copepod species.

1.8.3 Chromatin diminution in Chordates e.g. Hagfish and Lampreys

Hagfish and lampreys (jawless fish) constitute vertebrate genomes that undergo programmed loss of DNA in the soma. Hagfish lose between 21%-55% of their genome (Kubota et al., 1993) and lampreys around 20% (Jeramiah J Smith et al., 2009). This magnitude of DNA loss is normally not well tolerated in vertebrate genomes. Lampreys and hagfish are extremely hard to rear in captivity, making their study difficult. Hence, the recent sequencing of the lamprey genome in 2013 (Jeramiah J Smith et al., 2013) presents a giant leap in understanding programmed loss in chordates (hagfish genome sequencing has been initiated). Currently, many specific DNA sequences (eg. *Germ1* in Lampreys, EEPa1 in hagfish*)* and repetitive elements, single copy and transcribed sequences have been shown to be the target of deletion in the somatic cells of lampreys and hagfish (Jeramiah J Smith et al., 2009, 2010; Kubota et al., 1997). Even so, the mechanism of chromatin diminution remains poorly understood in these species.

Some authors speculate that the presence of chromatin diminution in jawless vertebrates may be considered evidence that the common ancestor of all craniates shared this process.

1.8.4 Whole chromosome elimination in Insects and Marsupials

Just like chromatin diminution, whole chromosome elimination has been observed during development in several taxa in the animal kingdom. However, unlike chromatin diminution, whole chromosome elimination is not restricted to only pre-somatic cells but can occur at different stages of development and in many different cell types.

An outstanding example of chromosome elimination and genome imprinting during development is found in Dipterans belonging to the family Sciaridae. Here whole chromosomes of exclusively paternal origin are selectively discarded during three elimination events at different developmental stages. Depending on how many chromosomes are lost in an embryo during these developmental stages, the sex of the embryo is decided (Goday and Esteban, 2001). A recent publication reports that inbreeding promotes paternal genome elimination in the heterogametic sex, in species where males form the heterogametic sex (Gardner and Ross, 2014). Other examples of sex determination through whole chromosome elimination are found in springtails (Dallai et al., 1999,2000).

Marsupials undergo elimination of sex chromosomes during development too, but this loss is not related to sex determination in any way. Bandicoots have been studied in this regard and it is hypothesized that the loss of sex chromosomes in marsupials is instead an extreme form of dosage compensation among the sexes (Kloc and Zagrodzinska, 2001; Watson et al., 1998).

1.8.5 Chromatin diminution in nematodes: an efficient way of germ line/ soma differentiation

Parascaris univalens

(Formerly called *Ascaris megalocephala* and *Parascaris equorum var. univalens*)

Parascaris univalens infects horses. Boveri's understanding of chromatin diminution during early development was possible because *Ascaris* embryos turned out to be an excellent experimental model for his observations. The big and clear cells of a *Parascaris* embryo consist of only a pair of large chromosomes, making their observation easier. Soon he realized that the embryos develop distinct somatic and germ cell lineages during the first few cleavage divisions and therefore could be experimentally manipulated. To this end, Boveri was rightfully called the "first genetic engineer" (Gilbert, 1977).

Given these advantages, Boveri could thus effectively trace and document the fate of the chromosomes in individual cell lineages with accurate precision, bringing him to the conclusion that the full complement of chromosomes were retained only in the *Parascaris* progenitor germ cell and its lineage. This seems logical now, given that the germ cell and its lineage in time would create the future gametes. In contrast, he noted that the chromosomes in the presomatic cells, the source of all other adult tissues of the animal, underwent the curious process of chromatin diminution. Thus he showed that the process of chromatin diminution is closely linked to germ line versus soma differentiation in the early *P. univalens* embryo and in fact serves to cytologically distinguish germ cells from somatic cells.

Chromatin diminution begins in a presomatic cell at the second cleavage division (S_2) leading to the 4-cell stage in a *P. univalens* embryo, when the chromosomes of the so-called S_1 start to behave abnormally by internally breaking into multiple pieces (see figure). At the following anaphase Boveri noted that the daughter cells received only these diminished chromosomes, whereas their distal ends remained condensed and at the equatorial plate. Remaining cytoplasmic, these heterochromatic regions were somehow degraded (Boveri, 1910; Tobler and Muller, 2001). After diminution, it was shown that a somatic cell lost roughly about 80-90% of its DNA (when compared with the germ cell DNA) and consisted of about 60 small individual chromosomes in a diploid set. It must hence be noted that chromatin diminution results in an alteration of the chromosome number in *P. equorum* (Goday and Pimpinelli, 1986) and *P. univalens*.

In total, this process was repeated 4 more times, in the S_2-S_5 cells, while the progenitor germ cell remains unaffected. This is depicted in the figures below: (Modified from Müller et al., 1996)

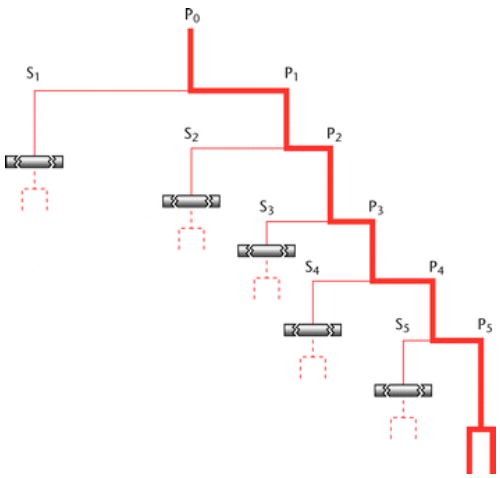


Fig. 9. Cell lineage of the early embryo of *Parascaris univalens*. Chromatin diminution is indicated by a broken bar. The germ line lineage is drawn in thick red lines. Presomatic cell lineages before elimination are represented in thin red lines and somatic cell lineages in broken red lines.

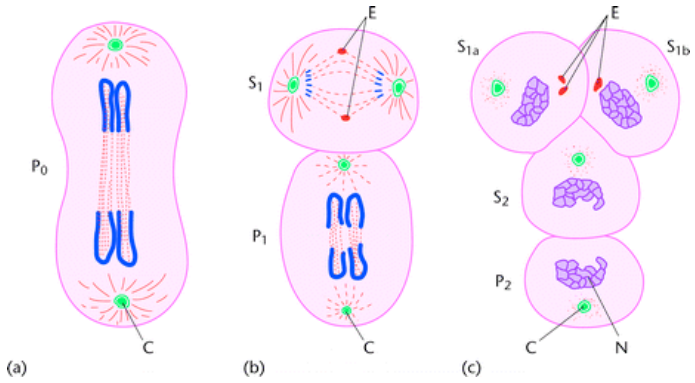


Fig. 10. Chromatin diminution in *P. univalens*. (a) Anaphase of first cleavage division. (b) Anaphase of the second cleavage division. Chromatin diminution occurs in the upper S1 cell but not in the lower P1 cell. (c) Four cell stage after completion of the second cleavage division. The cells S_{1a}, S_{1b} and S2 give rise to the somatic cells, while the P2 cell represents the germ line. P_0, zygote; C, centromere; E, eliminated chromatin; N, nucleus; P_0-P_2, germ line; S1, S_{1a}, S_{1b}, S2, presomatic cells.

Further experiments conducted by Boveri in 1910 concluded that the differential distribution of the egg cytoplasm by assymetrical cleavages controls the early germ versus soma decision in *P. univalens* (Boveri, 1910). This proved that the existence of certain factors (for example localized mRNA or

protein) in the cytoplasm of pre-germinal blastomeres was responsible for the prevention of chromatin diminution (reviewed by Tobler et al., 1992).

Goday and co-authors in 1992 described how this loss is achieved using Transmission Electron Microscopy studies on the fine structure of the centromere and spindle microtubules in chromosomes before and after chromatin diminution. *P. univalens* chromosomes are holocentric (all studied nematodes have holocentric chromosomes) with diffuse centromeres. Their detailed study revealed that the non-eliminated chromosomal regions in all pre-diminution and diminution blastomeres are associated with spindle microtubules. In contrast, no spindle microtubules get associated with the eliminated and heterochromatic distal ends. Plus, these heterochromatic portions are devoid of any kinetochore plates, explaining why upon fragmentation they do not get distributed to the spindle poles but rather are kept at the equatorial plate, making them an open target for degrading enzymes in the cytoplasm. This results in their eventual disintegration (Goday et al., 1992).

Some interesting experiments in *P. univalens* from M.R. Esteban in 1995 have shown that, early in development, cells with symmetric cell division (caused in turn by mRNA localization) eventually take up the 'somatic' fate. These cells will undergo chromatin diminution. Cells with asymmetric cell division will form 'germ' cells, which do not undergo cell division.

Experimentally, certain chemicals can induce chromatin diminution in cells, provided the embryos are exposed to them early in development. Vegetalizing agents (eg. LiCl) cause chromatin diminution, whereas animalizing agents (eg. NaSCN) prevent chromatin diminution. However, once a cell becomes committed to a particular fate (P_5 onward, refer figure 9), use of vegetalizing agents can no longer lead to chromatin diminution. These experiments have shown conclusively that chromatin diminution is an event that is strictly related to somatic cell behaviour in *P. univalens* and that the two cannot be separated experimentally (M.R. Esteban et al., 1995).

Ascaris suum

(Formerly called *Ascaris lumbricoides var. suum*)

Ascaris suum is an intestinal parasite of pigs. It undergoes chromatin diminution and, like *P. univalens*, chromatin diminution in *A. suum* has also been studied extensively. In fact, the molecular mechanisms underlying chromatin diminution have almost exclusively been investigated in *A. suum*

making it an important model for understanding the process of chromatin diminution. There are 3 basic differences in chromatin diminution between *P. univalens* and *A. suum* as reported by Goday and Pimpinelli in 1986. Chromatin diminution is first observed at the third cleavage division in *A. suum*, instead of the second. It is limited to the blastomeres S_2-S_4 as no S_5 is reported in *A. suum*. Lastly, *A. suum* has a 2n = 38A+10X karyotype in females in contrast to 38A+5X in males and the fragmentation of the many small germ line chromosomes results in the loss of their terminal chromatin (Seidl and Moritz, 1997). According to one report (Jentsch et al., 2002), the number of chromosomes in *A. suum* changes post diminution to 2n = 58A+12X in females or 58A+6X in males. In contrast to *P. univalens*, about 13-25% of nuclear DNA is lost from pre-somatic cells in *A. suum* (Wang et al., 2013; Tobler et al., 1986; Moritz et al., 1976; Tobler et al., 1972). *A. suum* is not discussed further in these regards. Chromatin diminution is a complex process involving chromosomal breaks, formation of new telomeres at these breaks and degradation of the DNA fragments. *A. suum* is discussed further in this context.

In *A. suum*, chromosomal fragmentation is specific to certain regions on the chromosome called CBRs, for chromosomal breakage regions (Mueller et al., 1995) that can span several kilobases in this nematode. These CBRs represent unique sequences, meaning that they do not hybridize with each other or with other genomic DNA fragments in a given species. Furthermore, CBRs of different species share little similarity. Recent studies demonstrate the successful cloning and analysis of 52 CBRs from *A. suum* (Wang et al., 2012). To date no DNA motifs have been identified that could act as recognition signals for CBRs and therefore for the cells' elimination machinery (Mueller et al., 1991), implying that CBRs are not recognized by their sequence alone but rather based on their chromatin structure and position.

For *A. suum*, once an internal fragment is eliminated, there is no rejoining of the remaining newly created chromosome ends. Stabilization of these ends occurs by de novo addition of a 2-4kb TTAGGC telomeric repeat sequence (Mueller et al., 1991). [*C. elegans* does not undergo chromatin diminution but was shown to have the same telomeric repeat of TTAGGC (Giuseppina Cangiano, 1993)]. Additionally, these telomeric repeats are added onto all chromosomal ends, including the ones on the DNA fragments that are degraded (Mueller, 1995). This indicates that telomere formation is non-specific in this species, but a process that applies to any broken DNA end (Refer to Side Note on Telomeres and Telomerase below).

Breakage at a CBR is not position specific, but is scattered over a 2-3kb window, as indicated from cloning studies. Telomeric repeat addition at these sites is not directed by a repeated sequence motif

or a preexisting TTAGGC motif either. This indicates that telomere formation is mediated by the activity of a telomerase rather than being a recombination event in this species (Magnenat et al., 1999; Jentsch et al., 2002; Mueller, 1995).

A. suum has a 334Mb genome. Latest sequence analyses have demonstrated a 13% loss in DNA content in somatic cells when compared to germ line cells (Wang et al., 2012). This corresponds to roughly 5% of *A. suum* genes (around 700 genes) being lost during diminution. Both copies of the gene are lost upon diminution, leading to the gene's complete absence in the soma (Wang et al., 2012; Jex et al., 2011).

In addition, Wang et al., 2012 observed the following: The same sets of genes are eliminated in different somatic cells of an animal and also among sexes. A large portion of these eliminated genes code for basic cellular machinaries like translation. Additionally, high-throughput RNA sequencing has shown that eliminated genes are preferentially expressed in the germ line, making diminution a mechanism of differential gene regulation in somatic cells. It must be noted that nearly 70% of eliminated chromatin is "junk" DNA consisting of a 121bp satellite repeat sequence.

Strikingly, most genes that are lost in *A. suum* have paralogs elsewhere in a region of the genome that is not affected by diminution. To date no evidence has been found that a RNA-guided mechanism is involved in DNA elimination in *Ascaris*, unlike that in ciliates (see above).

Side Note: Telomeres and Telomerase

Telomerases (also called terminal telomere transferases) play an important role in cellular mortality, aging and cancer (Greider and Blackburn, 1985). They are ribonucleoprotein complexes with enzymatic activity capable of adding a species-specific repeat sequence at the 3' end of DNA strands. Such sequences called "telomeres" are naturally found at the ends of linear eukaryotic chromosomes (Muller HJ, 1938; McClintock, 1941). Telomeres are repetitive, non-coding DNA sequences that are described to be "capping" structures and protect chromosome ends, among other things, from degradation and cellular damage (http://en.wikipedia.org/wiki/Telomere).

1.8.6 Chromatin diminution in other nematodes

Based on cytological evidence, chromatin diminution is not uncommon among nematodes with a current estimate of 11 different nematode species undergoing the event (Meng-Chao Yao et al., 2005). It must be noted that although common, the process of chromatin diminution is not observed in all species of a given taxon, with at least 13 other nematode species not showing this event (Guy Drouin, 2006; Tobler et al., 1986). All of the nematode species for which chromatin diminution has been described are parasitic and most belong to the family of *Ascarididae* (Tobler et al., 1986; Walton, 1974; Tobler et al., 1992).

Strongyloides papillosus (Albertson et al., 1979; Nemetschke et al., 2010) and *S. vituli* (Kulkarni et al., 2013, and this thesis) are the only known partially free-living nematode species undergoing chromatin diminution. These species are discussed separately below.

Other than *P. univalens* and *A. suum*, chromatin diminution remains poorly understood in nematode species. Chromatin diminution in nematodes has been shown to lead to genome rearrangements and it has been proposed that these genome rearrangements are a precise mechanism to control gene expression and gene dosage (M.R Esteban et al., 1995; Tobler et al., 1992, Goday and Pimpinelli, 1993).

Chromatin diminution in *Strongyloides papillosus*

In all other animals studied so far, chromatin diminution has been found to result in germ-line and soma differentiation. The exception is in the case of *Strongyloides papillosus*. Here, this event is used as a mechanism of sex determination. The females of this species have a pair of equally sized long chromosomes (2n=4). The males undergo a male-specific chromatin diminution event that results in the loss of an internal portion of a chromosome resulting in an altered karyotype of 2n=5. The males are then left with one large chromosome, three medium sized chromosomes and one small chromosome described as 1L3M1S in literature (Nemetschke et al., 2010; Albertson et al., 1979). It has been shown that the internal portion that is lost in males is homologous to the X chromosome of *S. ratti*. It must be noted that, unlike in *Ascaris*, only 1 copy of the gene is lost in *S. papillosus* males. Thus, chromatin diminution creates a dosage difference between the males and females in this species. Additionally, we now know from whole genome sequencing data that a large number of genes are lost in this process (see submitted manuscript below).

1.8.7 Role of chromatin diminution

The main role of chromatin diminution, a phylogenetically wide spread process, is a subject of speculation. Studies on different animal models have led researchers to believe that chromatin diminution has evolved for more than just soma – germ line differentiation. From studies on nematodes and lampreys, it is clear that chromatin diminution works as a highly efficient "throw away approach" to gene regulation (Streit, 2012). This means that somatic cells lose DNA sequences (coding and repetitive) that are not needed for them to fulfill their somatic function. It is even speculated in the case of *M. edax* that chromatin diminution evolves repeatedly in ciliates to eliminate 'nonfunctional tandemly repeated DNA sequences' from somatic tissues every time RNAi – related mechanisms are used to remove repetitive DNA sequences (Guy Drouin, 2006).

It will be interesting to see if species displaying endoduplication in their germ lines (eg. nematodes and copepods) discard different DNA sequences in comparison to species that have the normal DNA content in their germ line nuclei. If this were true, this would suggest another role for chromatin diminution. Based on current analyses, the kind of satellite DNA sequences that are lost seem similar in nematodes, hagfish and copepods, suggesting that this DNA might carry out similar (but currently unknown) functions in these animals. In *S. papillosus*, the loss of DNA from the males is a mechanism for sex determination and a way of creating sex-specific dosage differences. In addition, this loss re-instates the XX/X0 sex determination that is ancestral within this genus (Kulkarni et al., 2013; Streit, 2014).

An interesting evolutionary role of chromatin diminution is linked to partial genome duplication events in nematodes. It is thought that partially duplicated regions in the genome are removed by chromatin diminution for restoring genetic balance and dosage compensation within somatic tissues. Thus the deleterious effects of aneuploidy can be prevented within cells. Lastly, it has been speculated that chromatin diminution is important for nematodes with a parasitic life style, wherein these partial genome duplications provide an as yet unseen advantage for the production of a large number of eggs, thereby playing an important role in the evolution of these parasites (Mueller, 1995).

In this thesis

Other than the fact that *S. papillosus* undergoes male-specific chromatin diminution, not much is known about this event for this species. I have identified and cloned the 2 breakpoints where diminution occurs on the chromosomes in the males of *S. papillosus* and my results indicate that these breakpoints appear to be precisely defined in this species to the basepair (unlike in *Ascaris* as discussed above) but with no detectable telomeric repeats added at the ends of the newly formed chromosomes. This finding raises the important question of how chromosome ends are stabily maintained in this species. In addition, I find that these breakpoints occur in relatively gene dense regions. Taken together, my results support that chromatin diminution in *S. papillosus* is an RNA-mediated process. This is further strengthened by the fact that I find an RNaseH-like superfamily member just base pairs away (60bp upstream) from one of the breakpoints. This identification of breakpoints adds to the existing knowledge in this field and may also be a first step to understanding diminution in *S vituli* (Refer Results and Conclusions section 2.5: Identification and cloning of the boundaries of chromatin diminution in the males of *Strongyloides papillosus*). Finally, I have started to look at the role histone modifications play during the event of chromatin diminution by uncovering the nature of (chromosome-specific) germline chromatin in these species (Refer Results and Conclusions section 2.4: Germ line organization in Strongyloides nematodes reveals alternative differetiation and regulation mechanisms).

1.9 The nematode germ line

The germ line forms a central feature in sexually reproducing metazoans, by giving rise to the next generation and hence being in a way, immortal (Gartner et al., 2008). The nematode gonad is amongst the most suitable systems in which to study fundamental problems in developmental and cellular biology. It has become a great system in which to study stem cell biology, as many features of nematode germ lines are analogous to mammalian stem cell systems (reviewed in Hubbard and Greenstein 2000). Additionally, for disease-causing parasitic nematodes such as *Strongyloides*, it is imperative to gain a thorough understanding of their germ lines first, if the development of effective anti-helminthic drugs is a future goal.

In general, it has been shown that nematode gonads vary dramatically across species (Chitwood, 1950; Sommer R.J et al., 2005). Nematode gonads are tubes containing proliferating germ cells that eventually differentiate giving rise to mature gametes (oocytes or sperm). Therefore among nematodes, gonads differ based on the number of gonad tubes within the body (1 or 2), the relative position of the gonad in the body (anterior or posterior) and the position in the body where these gonads open into the external environment (anteriorly, centrally or posteriorly). Most nematode species are dioecious; therefore gonads follow different developmental programs between the sexes, resulting in distinct adult tissue compositions and morphologies (Rudel et al., 2005). In many nematode species, females/hermaphrodites have a gonad consisting of two arms called gonad arms. Such species are said to possess 'didelphic gonads'. Didelphic gonad arms in turn may be symmetrical or asymmetrical (Felix M.A. et al., 1996). Males are always monodelphic, meaning that they have only a single gonad arm. The female/hermaphrodite gonads open to the exterior through the vulva, the egg laying structure, whereas the male gonads have a spicule as a specialized structure that releases mature sperm into the vulva during mating.

The hermaphroditic gonad of *C. elegans* is the most extensively studied nematode gonad and is described below for later comparison with *Strongyloides* species.

1.9.1 The *C.elegans* life cycle

The typical life span of *C. elegans* is about 2 weeks at 20°C, making working with this nematode model very convenient (Brenner, 1974). After hatching these worms undergo four juvenile stages,

termed L1 to L4 larval stages, separated by 4 external molts before becoming reproducing adults {see figure below} (Cassada et al., 1975). On average, it takes 3.5 days to adulthood at 20°C. The sex determination is XX/X0 based (Nigon, 1949) with self-fertilizing hermaphrodites, which replace females in this species, as XX and the occasional males as X0 (males are 0.1% of the hermaphrodite progeny, formed by nondisjunction). Interestingly, hermaphrodites first produce sperm (from L3-L4), before forming oocytes (L4 on). Males only produce sperm. Thus hermaphrodites can self fertilize (self sperm typically lasts 4 days in a 2 week life span at 20°C), but matings with males also occur. Maximum progeny from self-sperm is about 300, whereas about a 1000 offspring may be obtained through successive matings with males. This helps demonstrate that self sperm and not oocyte number is the limiting factor for self-fertility in hermaphrodites (Pazdernik and Schedl; Hodgkin & Barnes, 1991). When matings occur, it is interesting to note that 50% of the cross progeny will be male.

1.9.2 The *C. elegans* germ line: from embryogenesis to the young adult

Hubbard and Greenstein, 2005 in Wormbook review the following work from multiple authors.

The development of the gonad in *C. elegans* can be understood by looking at its 3 different phases; specification, proliferation (or growth) and maintenance. Just like in most animals, in *C. elegans,* germ line lineages are specified as separate from somatic ones during early embryonic development, as early as the 16-celled embryo (Strome, 2005; Wang and Seydoux, 2012; Kimble et al. 1979). In brief, germ line potential is segregated only to the P blastomere of the developing embryo, mainly because of being rich in P granules, which are maternally provided ribonucleoprotein complexes and the protein PIE-1 (reviewed in Strome S., 2005). In *C. elegans,* these P granules are not sufficient for specifying germ cell fate but their absence causes severe germ line developmental defects. In short, P granules bind RNA in putative germ lineage thereby causing translational control. The role of PIE-1 (a P granule protein) has been shown to be critical in this regard (Mello CC, 1996) and is indespensible for germ line fate. PIE-1 has been shown to be a repressor of transcriptional elongation in the specification phase. The division of the P blastomere ultimately gives rise to the P4 cell, which is the actual founder cell of the future germ line. It divides once to give rise to the Z2 and Z3, the primordial germ cells of the hatching L1 larva (Strome S., 2005).

The L1 gonad thus consists of the primordial germ cells (Z2 and Z3) flanked by Z1 and Z4, the somatic gonadal precursors and these 4 cells are in turn surrounded by a basement membrane. These

4 cells remain mitotically inactive till mid-L1 stage, at which cell divisions begin. By the end of the L1 stage, the somatic gonadal precursors divide to give rise to 12 cells; 2 distal tip cells (DTCs) that are indispensible to germ line growth and act as leaders in gonad arm migration and 10 proximal cells required for further somatic gonad differentiation between the sexes during L2. By the end of the L3 larval stage, the gonad arms have begun to extend (Z2 and Z3 being highly proliferative give rise to many germ cells) by following the signal cues from the DTCs and surrounding sheath cells. Germ line sex determination and the first meiotic prophases begin here. In the L4 to young adult stage, an almost four-fold increase in germ cell numbers in observed, indicating that the germ line is robustly proliferative during this time. This also gives rise to the different zones eventually seen in the adult germ line, with a strong proliferative zone (or stem cell niche) being adjacent to the DTC in each gonad arm (Kimble et al., 1981; Strome, 2005).

The following description of the *C. elegans* gonad is the summary of the work done by multiple authors. A Delta/Notch signal (involving GLP-1) from the DTC keeps cells within a certain radius of itself in a mitotic fate, repressing a meiotic one (Austin et al., 1987). Of course, germ line proliferation and meiotic entry are more complex phenomena in *C. elegans*, with several important genes (GLD-1, GLD-2, FBF-1/2, NOS-3) playing key functions in multiple developmental processes (Kim et al., 2009, 2010; Jones et al., 1996), but discussing this in detail is beyond the scope of this thesis. In short, GLP-1/Notch (from the DTC) and FBF-1/2 (Pumilio) are required for cells to remain mitotic, while GLD-1, GLD-2 and NOS-3 are required for a meiosis transition (Strome, 2005). At any given time, the mitotic region contains nearly 200-250 cells, corresponding to ~20 cell diameters (Crittenden and Kimble, 2008). Gametogenesis typically spans the late L4 to adult stages and is discussed in detail below. In essence, the proliferative period spans from L1- adult, when germ cell numbers go from 2 in L1, to about ~16 at L2, to ~ 60 at L3, to ~ 400 at L4 and ~2000 in adulthood (Note: numbers with both arms combined). Henceforth they are maintained at roughly this number, till the end of the worms' lifespan (summarized in Kimble J. and Crittenden SL., 2005).

The growth of the gonad is represented below (modified from Hubbard, E.J.A and Greenstein, D., 2005).

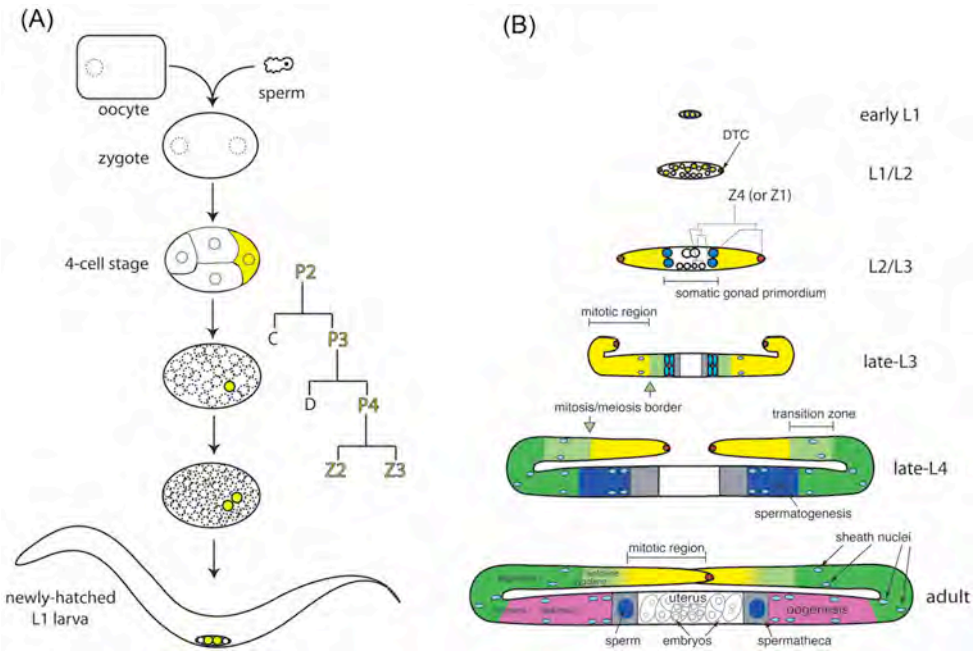


Fig. 11. Cartoon representation of gonadogenesis. (A) Cartoon representation of fertilization and the embryonic germ line. Oocyte and sperm meet, and fertilization initiates embryonic development. Germ line lineages are depicted in yellow. (B) Cartoon representation of post-embryonic hermaphrodite gonad development. Comparative size of gonads at different stages is not to scale. Germ line color scheme: yellow mitotic region, light green transition (early prophase of meiosis I), dark green pachytene, dark blue spermatogenesis, and pink oogenesis. The mitosis/meiosis border is indicated in the late-L3 and late-L4 by light green arrows. In the adult, the mitosis/meiosis border is not sharp (mitotic and meiotic nuclei are interspersed at the border) as indicated here by a yellow/green color gradient. Somatic gonad color scheme: red DTC, blue sheath/spermatheca precursor cells, light blue sheath nuclei, grey spermatheca, and white uterus. Germ line nuclei and their surrounding cytoplasm are often referred to as "germ cells", though they are open to a core of shared cytoplasm (rachis) during much of their development.

1.9.3 The *C. elegans* germ line: the adult hermaphrodite

The layout of the adult *C. elegans* hermaphrodite gonad is organized as a blind-ended U tube with germ cells that are actively proliferating at the closed or distal end and mature gametes at the open or proximal end. Germ cells at intervening stages of germ cell differentiation are found in between these two ends (Hubbard, 2007). This overall layout is similar to that which is found in the gonads of many other animals, for example in *Drosophila* (Margaret de Cuevas, 2015). This is true even in male mammals, if we consider the organization axis of the gonad to go from the basement membrane to

the lumen of the testes tubule (Culty M, 2013; Rossi and Dolci, 2013). The *C. elegans* germ cells are located on the periphery of the gonad in a syncytium, with a rachis or canal running through the center. This is easily visualized if we consider the structure of a corncob, with the outer corn kernels representing the germ cells and the corn rachis forming the central canal (Hubbard, 2007). The germ cells are connected with the central canal, thus facilitating exchange of materials but are prevented from being in direct contact with each other through cellular membranes, thus keeping their immediate cytoplasm separate. This may help adjoining cells to have distinct behaviors (Merritt et al., 2010; Kimble et al., 2013; Hubbard, 2007).

As mentioned in above sections, distinct zones are created along the adult hermaphrodite gonad arm due to the Notch signaling (and PUF - Pumilio and FBF family of RNA regulators) coming in from the DTC (Crittenden S.L and Kimble J., 2003; 2010). The DTC has 2 main functions, a leader function during early larval stages resulting in the U shape of the gonad arms (Hubbard & Greenstein, 2005) and a signaling role to promote a proliferative germ cell fate (in males and hermaphrodites) shown by laser ablations of the DTC (Hedgecock, 1987; Kimble and White, 1981). Proximity to the DTC controls the decision to stay mitotic, with germ cells after a certain distance (~20 cell diameters) of the DTC entering meiosis (Crittenden et al., 2003). Thus, we have a continuously dividing stem cell niche at the distal tips, called the 'proliferative or the mitotic zone', followed by germ cells entering meiosis (Crittenden and Kimble, 2007). These self-renewing mitotic cells (~200-250 cells in wild type animals) continuously replenish the proximal meiotic cell populations for lost mature gametes. This population is thought to have two subsets of cells, ones that remain as germ stem cells (GSCs) and ones that eventually enter meiosis. The two, however, cannot be easily distinguished from each other (Seydoux et al., 2006). This is depicted below (modified from Lints R. and Hall D.H, 2009).

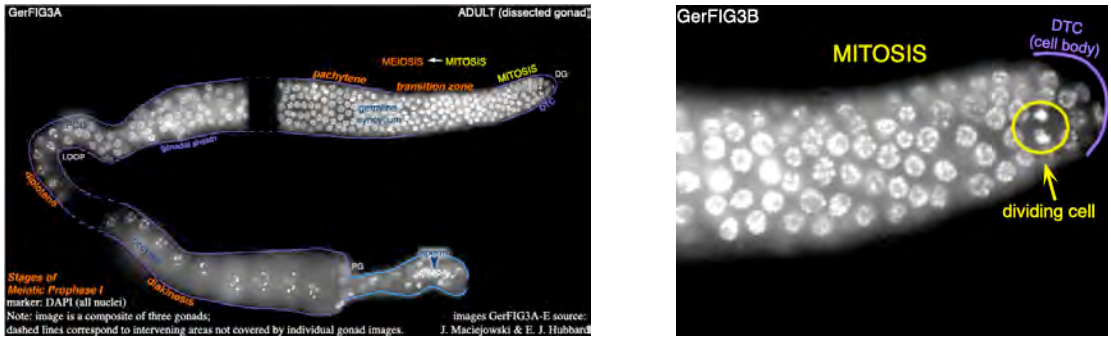


Fig. 12. (Left) Germ line of one gonad arm showing cells in different stages of development (orange color) and the relative positions of the somatic tissue (colored lines). (Right) Zoom in at the distal tip of the germ line showing cells undergoing mitosis.

The zone adjacent to this region is called the 'transition zone', where cells transition from mitosis to prophase I of meiosis (Jaramillo-Lambert et al., 2007). Cells in the transition zone are in prophase, leptotene and zygotene of meiosis. By DAPI staining, in the transition zone, we find the characteristic crescent shaped nuclei, due to chromosomes accumulating at one end (reviewed in Hubbard and Greenstein, 2000). A region of germ cells in pachytene, diplotene and diakinesis follows the transition zone. Homologous chromosome pairing, formation of synapsis and initiation of meiotic recombination occurs in leptotene-zygotene, with the majority of recombination occuring at the transition from pachytene-diplotene.

The 'bowl of spaghetti' appearance of chromosomes is characteristic of the pachytene stage (reviewed in Hubbard and Greenstein, 2000; and in WormAtlas, http://www.wormatlas.org/hermaphrodite/germ%20line/Germframeset.html). Progression of nuclei into diplotene at the loop is brought on by a MAPKinase signal from the surrounding sheath, leading to a single file of organized oocytes in the proximal gonad arm (McCarter, 1999).

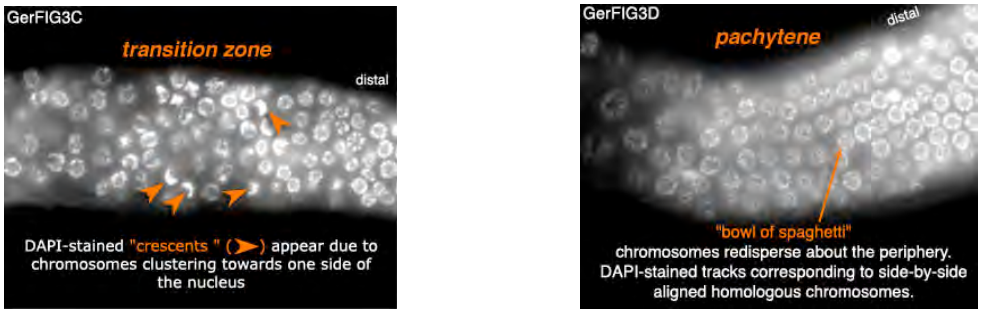


Fig. 13. (Left) DAPI stained dissected gonad showing crescent shaped germ nuclei in the transition zone. (Right) Germ nuclei showing pachytene associated bowl of spaghetti appearance of chromosomes (modified from Lints R. and Hall D.H, 2009).

A string of maturing oocytes with condensed chromosomes is diakinesis. It must be noted that many germ cells enter meiosis, but not all of these become ooctyes. In fact reports indicate that as many as 50% of all oogenic germ cells around the gonadal loop are eliminated as part of 'physiological apoptosis' in the *C. elegans* germ line (Gumienny et al., 1999; Gartner et al., 2008). It is thought that some of these cells function as nurse cells and hence eventually undergo programmed apoptosis during pachytene (Hengartner, 1997). This model has gained considerable support, making physiological apoptosis an important part of oogenesis (Gartner et al., 2008). In addition to physiological apoptosis, germ cells also undergo apoptosis by a second mechanism, called stress-induced apoptosis that is trigerred by a genomic intergrity/DNA damage checkpoint. Thus germ line apoptosis may also provide a means to eliminate oocytes with defective genomes (Gartner et al., 2008).

Thus in oogenesis, pachytene is an extended stage, where among other important processes, germ cells produce the RNAs and factors that contribute to the growing oocytes (Gilbert et al., 1984; Schisa et al., 2001). There is a cytoplasmic flow from the distal to the proximal part of the gonad that helps transport material to growing oocytes (Crittenden et al., 2007; Nadarajan, 2009; Wolke, 2007). In addition, maturing oocytes take up yolk from the intestine (Maddox, 2005; Grant and Hirsh, 1999). This results in enlarged oocytes occupying the entire space within the proximal gonad arm, due to their increased cytoplasmic content and big nucleus size.

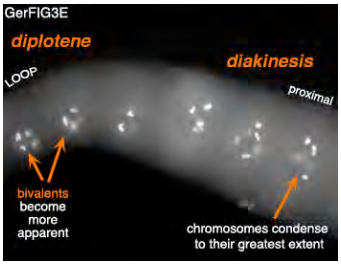


Fig. 14. DAPI stained gonad zoom in showing apparent bivalents at the loop and condensed chromosomes at diakinesis in the proximal part of the germ line (modified from Lints R. and Hall D.H, 2009).

The most proximal oocyte, adjacent to the spermatheca, undergoes meiotic maturation divisions and is ovulated into the spermatheca where it undergoes fertilization, either from stored self sperm or from cross sperm (Greenstein, 2005; McCarter, 1999). It has been shown that oocyte production, maturation and ovulation occur only in the presence of sperm (every ~23 minutes for mated animals or animals having self sperm). In the absence of sperm, oocytes are known to arrest in diakinesis and oogenesis is completely inhibited (Govindan, 2006,2009; Lee 2007; McCarter 1999). Meiosis is completed upon fertilization, and zygotic development begins. The multi-celled embryo is then laid into the environment through the ventrally located vulva, finally hatching as an L1 larva.

In the males, the gonad is arranged exactly similar to that in the hermaphrodites, except for the fact that males have only a single gonad arm, which opens posteriorly into the spicule (Morgan et al., 2010). There is a mitotic cell population at the distal end of the gonad, followed by cells in meiosis towards the proximal end. Gametogenesis in males results in the generation of spermatids, which are stored in the seminal vesicles until copulation. The ameboid spermatozoa (spermatids are activated upon mating, causing them to begin crawling) are then stored in the spermatheca of the hermaphrodite and used for fertilization of oocytes. It has been indicated that before mating, premature sperm is prevented from activation by substances within the male gonad (Stanfield and Villeneuve, 2004).

1.9.4 A brief description of the free-living *Strongyloides* spp. germ line

Speare in 1989 described the *Strongyloides* females as having didelphic gonads with opposed equal uteri and reflexed ovaries, either containing seminal receptacles (if free-living) or not (if parasitic). For the

sake of relevance to my work, this description is limited to the free-living *Strongyloides* generation (see figure below). Just like in *C. elegans* (see above), each gonad arm in *Strongyloides* is essentially a blind-ended U-tube. Sandground (1926), in addition to Nigon and Roman (1952) observed that the *Strongyloides* ovary is divisible into 2 zones. Following the nomenclature by an earlier investigator Schleip (1911), Sandground called the distal zone beginning at the blind end of the ovary the 'budding' zone and the succeeding zone the 'synapse' zone. However, Nigon and Roman instead preferred the words 'vegetative' and 'germinal' for the same zones (Bolla and Roberts, 1968), and their nomenclature continues to this day. The entire distal arm or the 'vegetative zone' near the apical ends of the gonad was shown to contain presumably interphasic giant nuclei with diffuse chromatin in adults (Hammond and Robinson, 1994; Nigon and Roman, 1952; Triantaphyllou and Moncol, 1977). Later, these giant nuclei were also shown to be endoduplicated by microdensitometry studies using fluroscent dyes (Hammond and Robinson, 1994), but without any further clear understanding of the nature of this endoduplication. These researchers also showed that the transiton from the vegetative to the germinal zones was abrupt. The 'germinal zone' contained a band of small and compact nuclei, often densely packed, with a presumably diploid DNA content (Hammond and Robinson, 1994), occupying a small area at the gonadal loop or bend. These authors further noted that some of the nuclei in the proximal part of this zone had the appearance of being in a diakinetic stage of meiosis. A short oviduct follows the germinal zone and contains growing oocytes around a central rachis, most likely for transport of substances into growing oocytes, although this was not proven. These oocytes then were thought to pass through the spermatheca (which contained a considerable number of sperm after matings) and upon fertilization into the uterus. The uterus contained developing embryos that were then laid into the environment through the vulva.

The *Strongyloides* males are slightly smaller than the free-living females. The male gonad arm is monodelphic and much thicker and smaller in comparison to age-matched females. The anterior end of the gonad is always blunt, usually broad but never reflexed (unlike the male gonads of Rhabditoids) and runs to the base of the pharynx. The gonad is also divisible into the 2 distinct zones with the giant and the small nuclei. Proximal to the small nuclei band are spermatogonia in various stages of development in the vas deferens, which finally opens posteriorly through the spicule. An overview of the *Strongyloides* gonad is shown below (modified from Kulkarni et al., 2015 submitted).

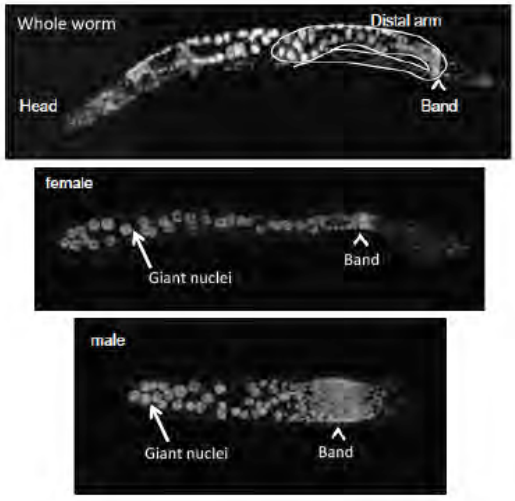


Fig. 15. DAPI stained images of free-living *Strongyloides*. (Top) DAPI stained whole worm (female) with position of posterior gonad arm outlined in body. Distal arm is dorsal. (Middle) Dissected DAPI stained female gonad arm showing the 2 zones, vegetative zone with giant nuclei and the germinal zone with a band of small compact nuclei. (Bottom) Dissected DAPI stained male gonad arm.

Side Note

In essence, the gonad of the parasitic females is similar to that of the free-living female with slight differences. Depending on the species, the parasitic gonad may be spiraled (e.g. *S. papillosus, S. ransomi*) or not (*S. ratti, S. stercoralis*). In fact, this is used as a diagnositic character for *Strongyloides* species identification. In addition, the gonad in parasites being much longer and slender makes the 2 distinct zones longer as well. The parasitic female, being parthenogenetic, lacks a specmatheca. For a detailed description of the parasitic gonad refer to Speare, 1989 and Triantaphyllou and Moncol, 1977.

In this Thesis

1.9.5 Comparative analysis of the germ lines of *Strongyloides* species and *C. elegans*

At a closer look, the germ line organization in *Strongyloides* nematodes is very different from that in *C. elegans*, making it hard to draw direct parallels between these two species (refer figure below). An

understanding of the *Strongyloides* reproductive biology is essential, but has not yet been done extensively.

It was my aim to understand the structure and organization of the *Strongyloides* gonad, since not much is known about it. I wanted to see how the germ line grows and develops and describe parallels between this gonad organization with other well studied nematode gonads like *C. elegans* and *P. pacificus*. In addition, the *Strongyloides* gonad consists of considerably fewer cells than in *C. elegans*. This raises the questions of how germ cell polulations are maintained, what their dynamics or functions are and the possible advantages of evolving such a highly divergent reproductive organ in these species. My work deals with answering some of these questions and uncovers a wide range of differences to *C. elegans*, from endoduplication or differential amplification in the germ line as a mechanism for regulating gene expression, to differential control of gametogenesis among *Strongyloides* spp., and finally the unique germ line chromatin modifications in these species (Refer Results and Discussion sections 2.3 and 2.4).

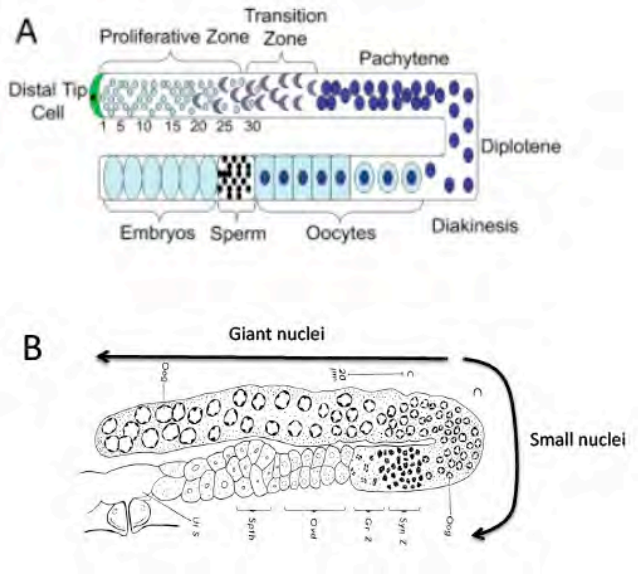


Fig. 16. A schematic representation of the (A) *C. elegans* and (B) *Strongyloides* spp. gonads showing differences in their germ line organizations (*C. elegans* schematic reproduced from Jaramillo-Lambert et al., 2007; *Strongyloides* schematic modified from Triantaphyllou and Moncol, 1977).

1.10 Aim of the thesis

I am interested and fascinated by germ cells and early embryonic development. Therefore, for my thesis, I chose to work on various aspects of germ cell biology and reproductive biology in *Strongyloides* spp. Much of this basic information is currently lacking in the field. The topics I have addressed fall into the 3 basic categories discussed below:

1.10.1 Evolution of chromosomes and sex determination in the genus *Strongyloides*: It was known that *S. ratti* and *S. papillosus* differed in their chromosome numbers and sex determining mechanisms (see above). However, it was not clear if XX/X0 sex determination as in *S. ratti* or sex specific chromatin diminution as in *S. papillosus* represents the evolutionary ancestral state within the Strongyloididae. I addressed this question by investigating the chromosome numbers and sex determining mechanisms of additional species of known phylogenetic relationships with *S. ratti* and *S. papillosus*.

1.10.2 Chromatin diminution in *S. papillosus* males: Based on a small number of markers it had been shown that the chromosomal region undergoing chromatin diminution in *S. papillosus* generally corresponds to the X chromosome in *S. ratti* (see above). By combining quantitative sequencing, molecular cloning and genetic approaches, I aimed at more precisely identifying the genes undergoing chromatin diminution and, in particular, characterizing the boundaries between the diminished and the non-diminished regions. This project was, in part, executed in the context of the efforts of the *Strongyloides* genome consortium, of which our group is a part.

1.10.3 Structure and function of the germ line: The organization of the germ line in free-living *Strogyloides* spp. had been described as rather different from the one of the model nematode *C. elegans*. In particular, no germ line proliferation has ever been observed in adult free-living *Strongyloides* males or females. Instead, the distal portion of the gonad, where mitotic germ cell division takes place in *C. elegans*, is occupied by giant nuclei that are very chromatin rich. To further understand this atypical germ line organization, I set out to first, test by quantitative sequencing if in the giant nuclei the entire genome is evenly amplified or if different regions/chromosomes are present in different copy numbers. Second, I looked at when the germ cells proliferate. Third I asked if the different organization of the germ line is also reflected in different chromatin modification states between *C. elegans* and *Strongyloides* spp. Finally, I wanted to answer if, in spite of the fact that no surviving male progeny is produced by the free-living generation, genetically male determining sperm is ever produced in *Strongyloides* species or not.

2. Results and Discussion

2.1 *Parastrongyloides trichosuri* suggests that XX/X0 sex determination is ancestral in Strongyloididae (Nematoda)

Arpita Kulkarni, Anna Dyka, Linda Nemetschke, Warwick Grant and Adrian Streit

Parasitology (2013), 140, 1822-1830, doi: 10.1017/S0031182013001315

2.1.1 Synopsis:

Strongyloides stercoralis (in man) and *Strongyloides ratti* (in rats), both parasitic roundworms, have been show to employ environmentally controlled XX/X0 sex determination. In both these species, the females have a pair of X chromosomes and a pair of autosomes (2n=6). In contrast, *Strongyloides papillosus* (in sheep) has only two chromosomes and lacks a sex chromosome altogether (2n=4). It was shown in *S. papillosus* that one of its large chromosomes consists of what is the X chromosome plus the autosome number I in *S. ratti*. *S. papillosus* then forms males through chromatin diminution, where an internal portion of one copy of the large chromosome is lost. The portion that is lost is homologous to the X chromosome in *S. ratti*. It was not clear which of these two sex - determining systems is ancestral. I demonstrate for the first time that *S. vituli* (in cattle) has a karyotype similar to *S. papillosus*, with *S. vituli* males also showing male specific chromatin diminution. Whereas *Parastrongyloides trichosuri* (in Australian possums), a closely related outgroup species to *Strongyloides spp.*, shows the same karyotype as *S. ratti* and employs XX/X0 sex determination. Additionally, the X chromosome of *P. trichosuri* is homologous to the X chromosome in *S. ratti*. My data strongly indicates that the last common ancestor of both *Strongyloides spp.* and that of *P. trichosuri* most likely had a pair of X chromosomes and a pair of autosomes in females, with a X chromosome less in males and employed XX/X0 sex determination. The situation with two pairs of chromosomes, as seen in *S. papillosus* and *S. vituli*, is likely derived by the fusion of the X chromosome with an autosome. Thus, chromatin diminution is a way to functionally restore these systems to an XX/X0 state.

2.1.2 Contributions:

Except for some pilot experiments and the identification of a few initial SNP markers, which were done by LN and AS, all experiments in the laboratory were performed by myself or, to a small extent, by a bachelor student (AD) under my guidance. For the *S. vituli* sample collection I was assisted by AD and AS. WG provided samples of parasitic and free-living *P. trichosuri* from Australian possums. Overall my contribution to the experimental work including sample collection amounts to about 90%. I was involved in the writing of the manuscript along with AS and WG. AS supervised all experiments.

2.2 The genome basis of parasitism in the *Strongyloides* clade of nematodes

Vicky Hunt, Isheng J Tsai, Avril Coghlan, Adam J. Reid, Nancy Holroyd, Bernardo Foth, Alan Tracey, James A. Cotton, Eleanor Stanley, Helen Beasley, Hayley Bennett, Karen Brooks, Bhavana Harsha, Rei Kajitani, **Arpita Kulkarni**, Dorothee Harbecke, Eiji Nagayasu, Sarah Nichol, Yoshitoshi Ogura, Michael Quail, Nadine Randle, Diogo Ribeiro, Alejandro Sanchez-Flores, Tetsuya Hayashi, Takehiko Itoh, Dee Denver, Warwick Grant, James B Lok, Haruhiko Murayama, Jonathan Wastling, Adrian Streit, Taisei Kikuchi, Mark Viney, Matthew Berriman

Nature Genetics (submitted 2015)

2.2.1 Synopsis:

Soil transmitted nematode infections, including those caused by *Strongyloides* spp. are among the most neglected tropical diseases. Thus understanding the adaptations that underlie nematode parasitism is of basic biological interest and will help uncover newer ways to control these pathogens. To achieve this understanding, we have produced genome sequences from an evolutionary clade encompassing 4 *Strongyloides spp., Parastrongyloides trichosuri* and *Rhabditophanes sp.*, taxa in which free-living nematode species are thought to transition to parasitic ones. Analysis of this genome shows an extensive expansion of the most common gene families in the transcriptome of the parasitic females, astacin-like metallopeptidase and SCP/TAPS coding genes. We find this to be coincident with the evolution of parasitism in this clade. Further, we find that genes that are upregulated within the *Strongyloides* parasitic female are arranged in physical clusters in the genome called as "parasitism regions".

2.2.2 Contributions:

I was involved in cultivation and collection of parasitic material that was used for sequencing. I provided all the genetic markers (over 100 markers spanning the genome) for improving and annotating the *S. papillosus* genome assembly. I made these markers and each was tested individually in males and females of *S. papillosus* to determine whether they were diminished or not. With the help of these markers and read depth analysis, nearly 18% of the assembly (10.9Mb) was annotated as diminished and 58% of the assembly (35.2Mb) as non-diminished. I have also validated genome sequence in certain regions by long range PCR and inverse PCR experiments, helping to identify, improve and overcome local assembly problems. Lastly, I have imaged the *Rhabditophanes* KR3021 gonad and shown the karyotype in this species for the first time. While I was not involved directly in the writing of this manuscript I did provide comments and suggestions to the sections describing analyses of the materials or markers I had provided. Since this is a many author multi-laboratory effort, a % of contribution cannot be determined {The supplementary materials for this paper is over 130 pages, hence only the main manuscript and relevant supplementary material is included in the publication version attached below}.

2.3 Differential chromatin amplification and chromosome complements in the germ line of Strongyloididae (Nematoda)

Arpita Kulkarni, Anja Holz, Christian Rödelsperger, Dorothee Harbecke and Adrian Streit

Chromosoma (2015), in press

2.3.1 Synopsis:

Strongyloides nematodes are parasites of vertebrates. A closer look at the less studied and thus poorly understood reproductive system of *Strongyloides* spp. presents many interesting differences when compared to the well-studied *C. elegans* gonad. Unlike in *C. elegans*, in the gonad arms of free-living *Strongyloides* spp. of both sexes, the entire region distal to the loop contains fewer than 50 giant nuclei with a very high DNA content. Additionally, around the loop there is a band with very small, compact nuclei numbering up to 150 at peak reproductive age. Both these nuclei sets have interesting dynamics over reproductive age in these worms. Combining quantitative DNA and RNA sequencing I conducted a comparative characterization of the germ lines of free-living males and females of two

species of *Strongyloides*, namely *S. ratti* and *S. papillosus*, and of *Parastrongyloides trichosuri*, a closely related outgroup species that is a facultative parasite of possums. The main findings I present in this regard are: i) there is no mitotic proliferation in the adult *Strongyloides/Parastrogyloides* germ line and there is a significant reduction in giant nuclei number with age in both sexes; ii) in the giant nuclei autosomal sequences are amplified to a much greater extent than X chromosomal ones; there appears to be high transcriptional activity in these cells and autosome encoded mRNAs are much more abundant than X encoded mRNAs. Moreover, I address the reasons for the lack of males in the progeny of the free-living *Strongyloides* spp. and show by sperm sequencing that nullo-X sperm is absent in *Strongyloides papillosus* but present in *Strongyloides ratti*. Additionally I show that *S. ratti* produces unviable male offspring. As control, we use *P. trichosuri*, a species that is known to produce male progeny. This indicates that closely related *Strongyloides* spp. may employ different mechanisms for controlling sex ratios and maintaining sex ratio distortions in the progeny of the free-living generation.

2.3.2 Contributions:

I was involved in all bench work for this project as leading person and my contribution is about 85%. For the isolation of gonads and sperm I was assisted by AH and DH respectively, with help from AH for the construction of the library for RNA sequencing. CR performed all bio-informatics analysis. AS supervised all experiments and offered feedback. I was involved substantially in writing the manuscript together with AS.

2.4 Germ line organization in *Strongyloides* nematodes reveals alternative differentiation and regulation mechanisms

Arpita Kulkarni, James W. Lightfoot and Adrian Streit

Chromosoma (2015), submitted

2.4.1 Synopsis:

Strongyloides nematodes are parasites of medical and economic relevance. Hence it is important to study their reproductive biology. The germ line organization in these nematodes is very different from that in *C. elegans*, making it hard to draw direct parallels between these two species. A closer

look at the poorly understood reproductive system of *Strongyloides* spp. presents many interesting differences when compared to the well-studied *C. elegans* gonad. Unlike in *C. elegans*, the distal gonads of free-living *Strongyloides* spp. of both sexes contain giant nuclei with a very high DNA content. At the gonadal loop region there is a band of small, compact nuclei. Combining electron and light microscopic studies I show that this germ line organization appears to be specific to the Strongyloididae and on further characterization I have uncovered striking differences in the development, organization and fluid dynamics of this germ line compared to *C. elegans*. This is also the first comparative study of its kind to look at germ line chromatin in two species (and among sexes) of *Strongyloides*, namely *S. ratti* and *S. papillosus*, in addition to *P. trichosuri*, a closely related outgroup. In addition, these results are put into an evolutionary context by looking at germ line chromatin in a total of 7 nematode species of varying phylogenetic distances to *S. ratti.* I show: i) the detailed morphology of the gonad in these species, ii) proliferation of germ cells is restricted to early larval stages thus limiting the quantity of progeny in these species; iii) atleast in *S. ratti,* in male meiotic nuclei, autosomes and the X chromosome have distinct chromatin modifications, with H3Pser10 being X chromosome specific. Taken together these results support the notion that Strongyloididae members use alternative differentiation and regulation mechanisms as a result of their atypical germ lines in comparison to the well-studied *C. elegans*.

2.4.2 Contributions:

I designed and performed all experiments for this project and therefore my contribution in 100%. JL and AS supervised all experiments and offered feedback. The manuscript was written in majority by me, with contributions from AS and JL.

2.5 Identification and cloning the boundaries of chromatin diminution in the males of *Strongyloides papillosus*

Arpita Kulkarni, Christian Rödelsperger and Adrian Streit

Manuscript in preparation {included as a draft below}

2.5.1 Synopsis:

S. papillosus undergoes sex specific chromatin diminution. Here we present the identification of regions that are present in two copy numbers in females but only as a single copy in males of *S. papillosus* by combining the inheritance of genetic markers and whole genome re-sequencing data between males and females of 4 different species, namely, *S. papillosus*, *S. ratti*, *S. stercoralis* and the closely related outgroup of *P. trichosuri*. We have further cloned and sequenced these newly formed chromosome ends in *S. papillosus*. We provide an estimate of the number of genes that may be lost in the males during the process of chromatin diminution. Lastly we speculate on how these newly formed DNA ends are protected in the males.

2.5.2 Contributions:

I performed all experiments for this project and therefore my contribution is around 85%. I generated all the sequencing data for read coverage comparison. I generated all molecular markers and performed all cloning experiments. All bioinformatic analyses were done by CR and confirmed by our collaborators at Sanger, namely, BF, NH and MB. AS and I did the experimental design, with AS supervising all experiments and offering feedback.

Identification and cloning of the boundaries of chromatin diminution in the nematode males of *Strongyloides papillosus*

Arpita Kulkarni, Christian Roedelsperger and Adrian Streit

Max Planck Institute for Developmental Biology, Spemannstrasse 35, Tuebingen 72076, Germany

Abstract

S. papillosus undergoes sex specific chromatin diminution. Chromatin diminution is the process where the males of *S. papillosus* lose an internal portion of one of their chromosomes. Here we present the identification of regions that are present in two copy numbers in females but only as a single copy in males of *S. papillosus* by combining the inheritance of genetic markers and whole genome re-sequencing data between males and females of 2 different species, namely, *S. papillosus* and *S. ratti*. We have further cloned and sequenced these newly formed chromosome ends in *S. papillosus*. We provide an estimate of the number of genes that may be lost in the males during the process of chromatin diminution. Lastly we speculate on how these newly formed DNA ends are protected in the males.

Introduction

"The chromosomal theory of inheritance" jointly put forth by Sutton and Boveri in the early 1900s was a fundamental theory, which postulated that chromosomes were the carriers of genetic information (Boveri T. and Sutton W, 1902). This was the first time that chromosomes were held responsible for genetic inheritance, even though the foundations for this idea were laid down by the work of Gregor Mendel and Charles Darwin many years earlier (Mendel G., 1865; Darwin C., 1868). The DNA constancy rule suggests that all cells of an organism must have essentially the same identical genome, both qualitatively and quantitatively (Tobler, 1986). This implies that all organisms protect and govern genomic integrity tightly at every cellular division. Interestingly enough it was Boveri who discovered the first exceptions to this central rule. His work on the roundworms today assigned to the genera of *Ascaris* and *Parascaris* resulted in the surprising observation that the full complement of chromosomes was retained only in the *Ascaris* and *Parascaris* germ cell lineage, the source of future gametes (Boveri 1887). In contrast, the chromosomes in somatic cells, the source of

all other adult tissues, underwent a curious process of fragmentation and elimination known as chromosome or chromatin diminution (Herla, 1893).

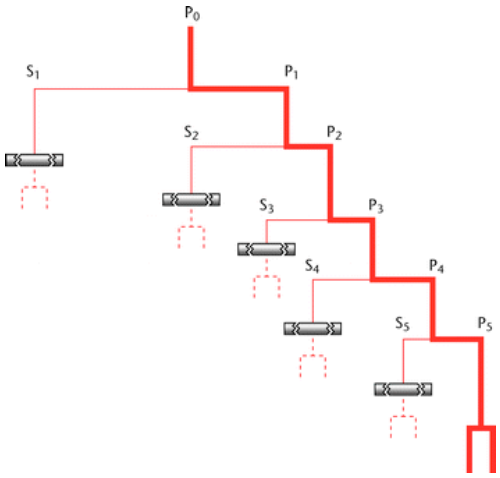


Fig. 1. Cell lineage of the early embryo of *Parascaris univalens*. Chromatin diminution is indicated by a broken bar. The germ line lineage is drawn in thick red lines. Presomatic cell lineages before elimination are represented in thin red lines and somatic cell lineages in broken red lines (modified from Mueller et al., 1996)

Chromatin diminution thus came to be defined as a developmentally regulated process wherein parts of chromosome(s) were deliberately lost at certain cell divisions, albeit by unknown cellular mechanisms (Tobler and Mueller, 2001). Soon after, a series of other organisms were discovered to undergo the process of chromatin diminution. These included rotifers and ciliates, crustaceans and insects, nematodes, mammals and other chordates, and even some plants (Tobler et al., 1995; Tobler et al., 1986; Pimpinelli et al., 1989; Kubota et al., 1993; Goday et al., 1993). Given this vast diversity, chromatin diminution is rightly thought to be of polyphyletic origin (Tobler et al., 1986; reviewed in Tobler and Muller, 2001). To fully illustrate the loss of DNA caused by this event, in some species up to 95% of genomic DNA can be lost during a diminution event (Nowacki M. et al., 2011).

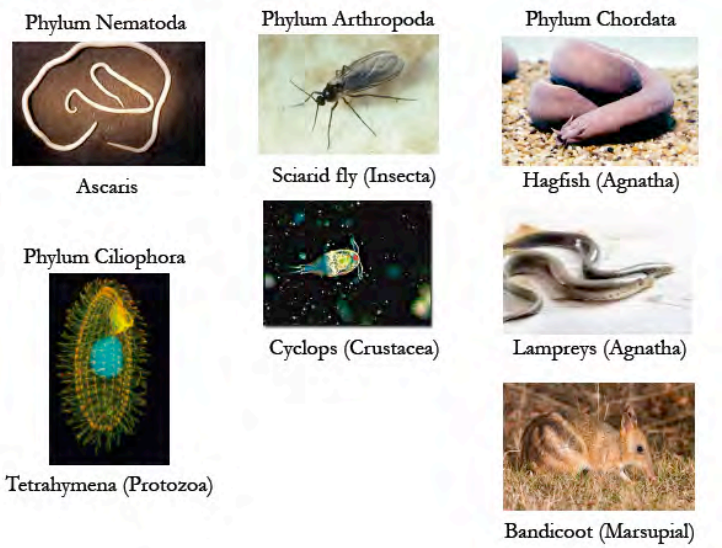


Fig. 2. Examples of species from different animal phyla undergoing chromatin diminution.

Even though previous workers have pointed to the numerous differences that do exist among species undergoing chromatin diminution such as the developmental stage and type of cell cycle, cell and tissue type, type of chromosome and amount of eliminated material, or the number of times the event of chromatin diminution occurs, one thing remains the same (Mueller et al., 1995). For most organisms studied to date, chromatin diminution is a process that differentiates between gonadal and somatic tissue. The only exception is for a species belonging to the nematode genus of *Strongyloides*. *S. papillosus,* a nematode Clade IV (c.f. Blaxter et al., 1998) member, has evolved to use chromatin diminution as a mechanism of sex determination to generate males (Albertson et al., 1979; Nemetschke et al., 2010).

Compared to other nematodes, *Strongyloides* species have a complex life cycle consisting of two distinct generations, one parasitic found inside the mucosa of the small intestine of different vertebrate hosts and one that is free-living found in the environment (Streit, 2008; Viney et al., 2006; Viney and Lok, 2007). The parasitic generation consists of only females; no males have yet been discovered. The mode of reproduction in this generation is by mitotic parthenogenesis. Although this reproduction is clonal, offspring of both sexes are formed. These are laid out into the environment along with the host feces as eggs. The eggs hatch and develop into adult free-living males and females following four molts. The free-living generation has been shown to undergo sexual

reproduction, at least in *S. ratti* and *S. papillosus* (Viney et al., 1993; Eberhardt et al., 2007). The resulting progeny is all female and must necessarily follow the parasitic life style. Thus *Strongyloides* worms are obligate parasites.

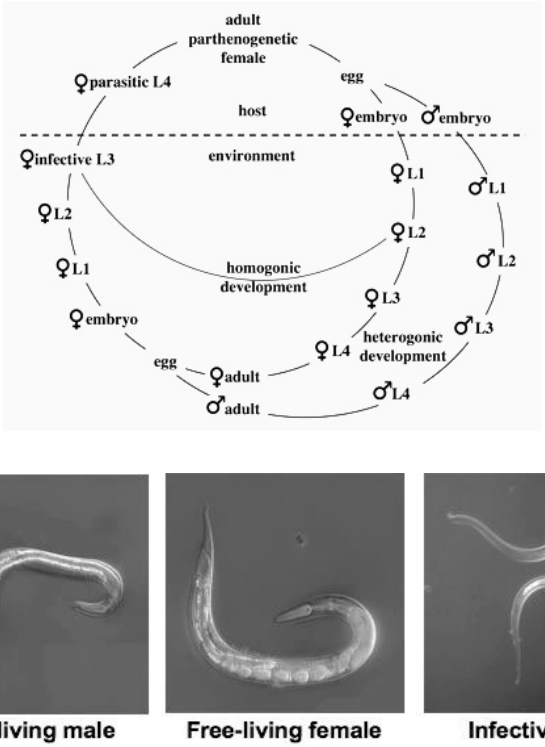


Fig. 3. (Top) Generalized life cycle of *Strongyloides*. (Bottom) Differential interference contrast picutres of an adult male (left), adult free-living female (centre) and an infective iL3 (right) of *S. papillosus* (modified from Streit, 2008)

The different mechanisms of sex determination within Strongyloididae have been recently studied (Striet, 2008; Kulkarni et al., 2013; Nemetschke et al., 2010). It was shown that the karyotype (2n=4) found in *S. papillosus* has arisen through the fusion of the X chromosome with an autosome (the autosome that corresponds to autosome I of *S. ratti* which has 2n=6 in females and 2n=5 in males) {Nemetschke et al., 2010; Kulkarni et al., 2013}. This fusion results in the formation of a pair of long chromosomes and a pair of medium chromosomes (2L2M) in females and, in combination with chromatin diminution, a single long chromosome, three medium chromosomes and a small chromosome (1L3M1S) in males (Albertson et al., 1979; Nemetschke et al., 2010). Further, chromatin diminution in *S. papillosus* males results in the loss of an internal portion of the long

chromosome that is homologous to the X chromosome in *S. ratti* (Nemetschke et al., 2010). Hence, it probably also serves as a mechanism to create dose differences between males and females of this species.

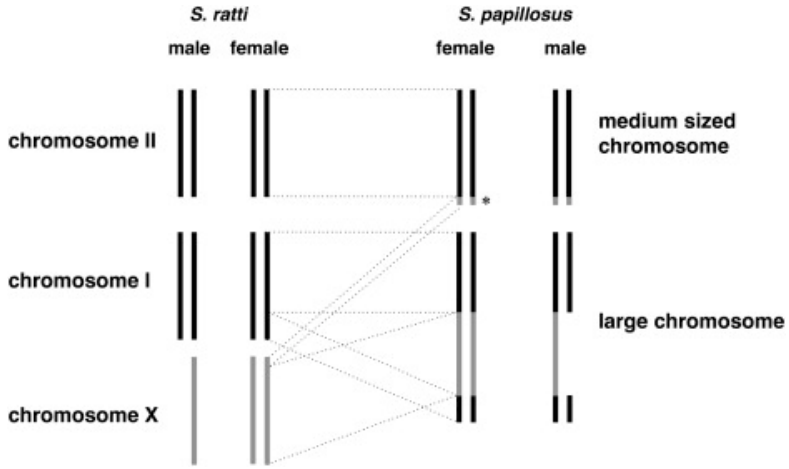


Fig. 4. Model for the evolutionary relationship between *S. papillosus* and *S. ratti* chromosomes. Black, autosomes in *S. ratti* or regions evolutionarily related to *S. ratti* autosomes in *S. papillosus*; grey, X chromosome in *S. ratti* or regions evolutionarily related to the *S. ratti* X chromosome in *S. papillosus* (modified from Nemetschke et al., 2010)

Previous work, by looking at inheritance of SNP markers, has revealed that which of the two long chromosomes undergoes diminution in *S. papillosus* is random (Nemetschke et al., 2010). At first both large chromosomes appear "beaded" but only one of these then undergoes loss of DNA while the other chromosome manages to recover and retain its intact structure (Nemetschke et al., 2010; Albertson et al. 1979). But we understand very little about the exact mechanisms at play during this event in this nematode species, for eg. our understanding about how these boundaries where diminution begins in *S. papillosus* are defined remains poorly understood.

The work that best describes these diminution boundaries in another nematode species was done in *Ascaris* (Mueller et al., 1991; Jentsch et al., 2002; Wang et al., 2012). Authors described that diminution occurs only at specific regions on a chromosome and these regions were then termed as "Chromosomal breakage regions" or CBRs (Mueller et al., 1995). Previous workers found and cloned multiple CBRs in *Ascaris*, 52 to be precise and have shown that these regions can span over several

kilobases (Wang et al., 2012; Meuller et al., 1995). Once these regions were successfully cloned, they provided important information regarding the nature of these breakage sites. Sequence comparison of the different CBRs of *Ascaris* revealed no obvious sequence similarity or presence of any secondary structure among them (Jentsch et al., 2002). This observation ruled for the presence of a telomerase-mediated healing mechanism in *Ascaris* cells. Infact, *Ascaris* has been known to add the telomeric repeats of $(TTAGGC)_n$ to the newly formed chromosomes to prevent them from cellular degradation (Mueller et al., 1991; Wicky, 1996; Zetka and Mueller, 1996; Jentsch et al., 2002). The telomeric repeat of TTAGGC has also been found at *C. elegans* chromosomal ends (Giuseppina Cangiano, 1993). These studies have indicated that telomere length in the worm is clonal, inherited and regulated *in cis* (Raices et al., 2005), similar to telomere length in mammalian cells (Marcela Raices 2008).

Telomerases (also called terminal telomere transferases) play an important role in cellular mortality, aging and cancer (Greider and Blackburn, 1985). They are ribonucleoprotein complexes with enzymatic activity capable of adding a specific repeat sequence at the 3' end of DNA strands, which are naturally found at the ends of linear eukaryotic chromosomes (Muller HJ, 1938; McClintock, 1941). Telomeres are repetitive, non-coding DNA sequences (added by a telomerase) that protect chromosome ends and are described to be "capping" structures and prevent them, among other things, from cellular degradation and damage. So far no extensive research has been done to see if telomerases and telomeres exist in *Strongyloides* spp.

Given that the process of chromatin diminution has evolved several times independently, it is speculated that it must help to serve different functions in different animals (Mueller et al., 1995). But what functions could such programmed genome rearrangement bring about? Increasing evidence from sequencing data hints to partial duplications within the genome being the cause of chromatin diminution (Mueller et al., 1995). The partial duplications are removed from the genome by diminution in order to maintain genetic balance within somatic tissues.

The consequences of eliminating the wrong DNA segments would naturally be disastrous, thus illustrating the need for a precise cellular mechanism for this event. However, the cell machinery that governs this process in different organisms remains largely unknown. Work in ciliates like *Tetrahymena* has shown that micro RNAs might be involved in this process (Meng-Chao Yao et al., 2005; Aronica et al., 2009). In contrast, *Ascaris* shows no indication of the involvement of any such small RNAs. Additionally, a cell has to evolve ways in which to protect and stabilize the newly formed DNA ends.

Here we present the identification of regions that are present in two copy numbers in females but only as a single copy in males of *S. papillosus* by combining the inheritance of genetic markers and whole genome re-sequencing data between males and females of 2 different species, namely, *S. papillosus* and *S. ratti*. We have further cloned and sequenced these newly formed chromosome ends in *S. papillosus*. We provide an estimate of the number of genes that may be lost in the males during the process of chromatin diminution. Lastly we speculate on how these newly formed DNA ends are protected in the males.

Materials and Methods

Identification of the chromosomal break points:

First, adult males and females of *S. papillosus* were hand picked after baermanizing from rabbit fecal cultures as described by Eberhardt et al., 2007 and Nemetschke et al., 2010. Then the genomes of these males and females were quantitatively sequenced using the Illumina platform and mapped to the reference genome assembly. The read depth was calculated for each scaffold and all the scaffolds were classified as diminished/X or non-diminished/Autosomal based on differences in read coverage. In total, sequencing resulted in 118 contigs > 100kb, 647 contigs > 10kb, 5149 contigs< 10kb, 5000 contigs < small contigs/ scaffolds as .tiff files. These were finally narrowed down to 14 candidate contigs/ scaffolds by analyzing where the read coverage in males drops from a ratio of 1.0 compared to females (as expected in non-diminished regions in males and for females) to that of 0.5 (as expected in regions undergoing diminution in males, as males are hemizygous for these regions) (Fig. 5A, B and C). The different regions of these contigs were then tested genetically for their diminution status. To this end new molecular markers were generated and tested in males and in females as described earlier (Eberhardt et al., 2007; Nemetschke et al. 2010). For example: scaffolds such as shown below showing a change in read coverage from 1 to 0.5 were checked manually by making genetic markers to test for diminution in males.

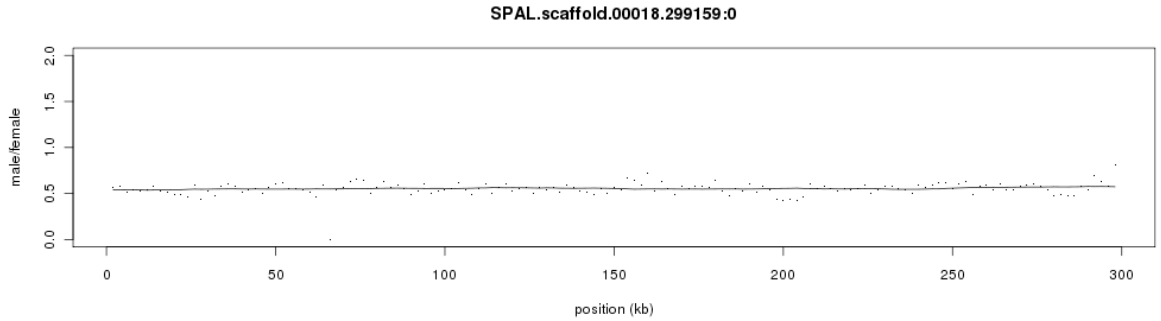


Fig. 5A. Example of a candidate scaffold undergoing diminution based on a read coverage of 0.5. X-axis denotes position along the scaffold, while Y the read coverage ratio between males and females is plotted on the Y-axis.

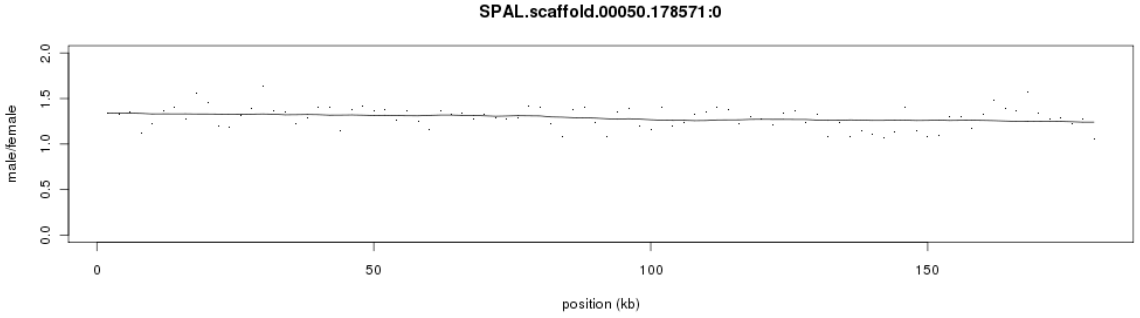


Fig. 5B. Example of a candidate scaffold not undergoing diminution based on the read coverage of 1. X-axis denotes position along the scaffold, while Y the read coverage ratio between males and females is plotted on the Y-axis.

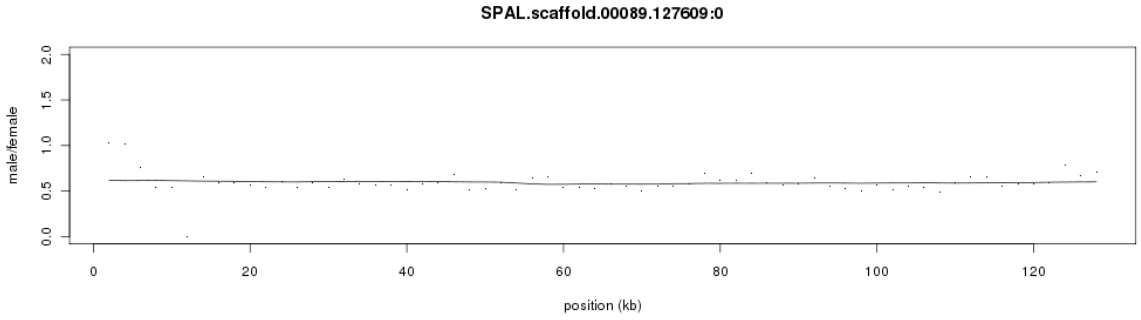


Fig. 5C. Example of a candidate scaffold containing a breakpoint. X axis: position on the scaffold, Y-axis read coverage ration between males and females. Read coverage was analyzed per 2kb window.

Molecular markers for testing diminished or non-diminished status of a contig:

Molecular markers were generated in desired supercontigs as described in Eberhardt et al., 2007 and Nemetschke et al., 2010. These markers were then tested for their diminution status. The rational is that if a marker undergoes diminution in males, SNPs in this region must always and necessarily appear homozygous and never heterozygous. As control, females SNPs in the same region may be homozygous as well as heterozygous.

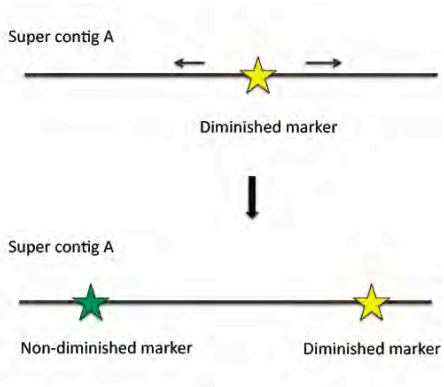


Fig. 6. The general principle used for narrowing the breakpoint was chromosome walking from a diminished to a non-diminished marker.

Once a diminished and a non-diminished marker were successfully identified on a candidate supercontig, making successive markers narrowed this region down (Fig. 6). The aim was then to identify a region with molecular markers that flanked the breakpoint (region with homozygous SNPs and a region with heterozygous SNPs within the same marker in males, as shown in figure 6 above).

Details of molecular markers at the breakpoints:

The details of the breakpoint flanking non-diminished and diminished ytP markers and the primers used to amplify these regions are summarized for ease in the table below.

Marker	Breakpoint	ytP marker	Length	Forward Primer	Reverse Primer	Distance between diminished and non-diminshed markers
Non-diminished marker	I	ytP242	518bp	TCCAGGTATGCAGGTTTCTCC	TAAACTCATGTTGCGCATTG	2.4Kb
Diminished marker	I	ytP243	416bp	TTTGGCTGATTCAGAGAACG	ATGTCACCGACGCAACATAA	
Non-diminished marker	II	ytP228	462bp	GTGACCACCACCTGATCCTT	ATACCACAAGGCCGTTCTCA	350bp
Diminished marker	II	ytP215	678bp	CCACTAATCACAATGAGCAACA	CATGTTTACTTTGAAAAATGCATAAAA	

Cloning of breakpoints:

Principle: Once the breakpoints were narrowed down between two molecular markers, we identified restriction enzymes that cut on either side of the break point, for example enzyme A in the non diminished and enzyme B in the diminished region (Fig below). Purified genomic DNA from males and females (prepared using Illumina DNA extraction kit) was then digested with enzyme A and the ends made blunt with DNA Pol I (Fermentas, digested for 15-20 minutes followed by heat inactivation at 75°C for 20 minutes). The expectation would be that in males we would find the breakpoint followed by telomeric repeats, while in females the sequence would just read through.

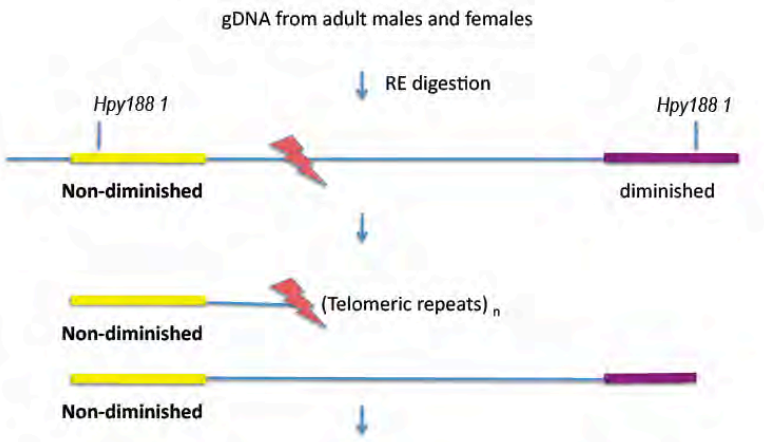


Fig. 7. The two kinds of fragments expected from restriction digestion of gDNA from males (top) and females (bottom)

The resulting DNA fragments were circularized by ligation with T4 ligase (Fermentas) and digested with enzyme B, thereby linearizing the circles derived from non-diminished chromosomes (i.e from females). Next, inverse and nested PCRs were performed only on the intact circularized fragments (i.e from males). These fragments in theory should contain both the breakpoint as well as telomeric repeat sequence of this species. The nested PCR products were then cloned using a TOPO cloning kit and sequenced. All PCR's were done (in triplicates) from different aliquots of genomic DNA (prepared independently from each other) to avoid any bias during amplification. Cloning was done in biological triplicates (as independent experiments) with PCR products from different PCR experiments. Multiple clones were picked each time for sequencing. This cloning strategy is depicted below:

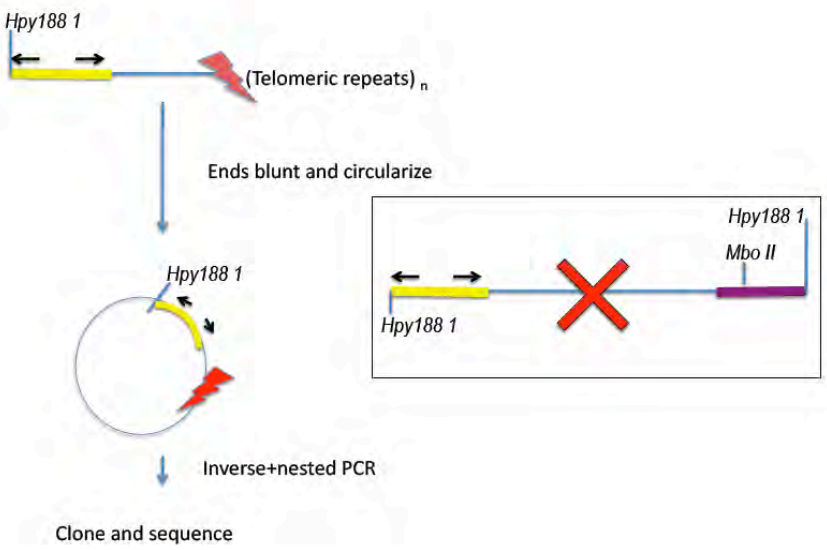


Fig. 8. Circularization and cloning of the male fragment containing the breakpoint and telomeric repeat. The female fragments (shown in box) not containing the breakpoint are eventually not cloned.

Enzyme specifics:

Purified genomic DNA from mixed worm samples and infectives was restricted digested using Hpy188I (Fermentas, buffer 4) overnight at 37°C and then subjected to the exonuclease DNA Pol I for 15-20 minutes followed by heat inactivation at 75°C for 20 minutes. The DNA was then treated with a Ligase for atleast 6hours. The ligase was then heat inactivated at 75°C for 20minutes. Then the gDNA was subjected to MboII (Fermantas, buffer 4) overnight at 37°C. This was then used as a template for inverse PCR. A nested PCR was then performed and this product was then used for sequencing and subsequent analysis.

Breakpoint I:

Inverse Primers used in non-diminished marker ytP242:

Inverse Primer I: TTCCGGGGATCTCCTTTAAC

Inverse Primer II: ATTTTGGCGGATTCGTTAGC

Nested Primers used:

Nested I: ACCAAGACCTCGCCACTAGA

Nested II: ATTTTGGCGGATTCGTTAGC

Nested III: CCATTGAGCAAGGAGGATGT

{Primer I was used either in combination with Primer II or Primer III}

Breakpoint II:

Inverse Primers used on the non-diminished side:

Inverse Primer I: TCTTTTAACATCACGCATCTATTTTT

Inverse Primer II: AGCATATTTCATCATAAAATGTCCCA

{Primer I and II were used in combination}

Inverse Primer III: CATTCTTGAGAACGGCCTTG

{Primers II and III were used in combination}

Nested Primers used:

Nested I: CTGTTGCTACCTCAGTAAG

Nested II: TGAGAACGGGCCTTGTGGTAT

Nested III: CATTCTTGAGAACGGCCTTG

Nested IV: ACCAACAAATGTTCAATTGCT

{Primer I was used in combination with II or with Primer III; Primer II was used in combination with Primer IV}

Overhang (3'OH) detection by Terminal deoxynucleotidyl transferase (Tdt):

To verify the exact nature of the cut made at the breakpoints on the DNA (single stranded, double stranded, or 5'/3' overhangs) we subjected purified genomic DNA from mixed cultures and infective females to the enzyme Tdt. Tdt is a template independent polymerase that adds dNTPs to the 3'OH of DNA molecules. We used a Cytosine specific Tdt enzyme for our experiments with the rational that Cytosine should be incorporated at the 3' end if such an end exists at the breakpoints in males (Fig. X). We specifically wanted to check for 3'OH ends, as telomerase is known to use 3' OH ends to add telomeres. Then we amplified breakpoint regions using primers in our closest non-diminished marker and a poly G primer and finally sequenced cloned nested PCR products. In addition to giving us information of a free 3' end we expected to find sequence and telomere information at these regions using this method. The schematic below describes this protocol:

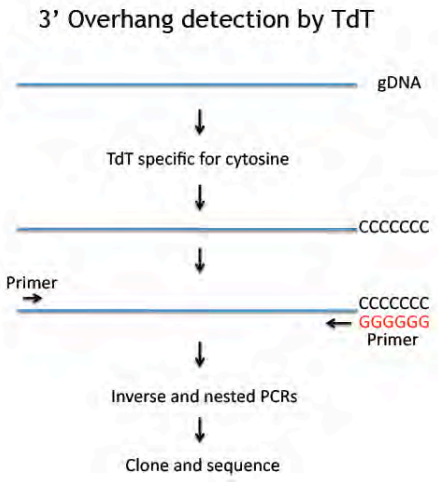


Fig. 9. Overhang detection using the enzyme Terminal deoxynucleotidyl transferase specific for Cytosine.

Results

Characterization of Breakpoint I:

According to *S. papillosus* (v2.2, 2013 genome assembly) breakpoint I lies in scaffold S_000017. This breakpoint is flanked by markers ytP242 (on the non-diminished side) and ytP243 (on the diminished side) and is roughly 2.4kb apart. The ytP marker sequences are as follows (SNP sites used for analysis are indicated as per IUPAC degeneracy nomenclatures in red):

ytP242:

TTTGGCTGATTCAGAGAACGGTATCTCTGAGGATTCTTTGTCAAATTTTGAAGTAGTAGCTGCG
TGTGATGGAAGTGCTTTAGGTGGTAAAGGAATGGCATTTGTAATTTCATATGGGGATAAGATG
TGGAAAGAAA**W**TTTTAAGGTTTATAATAAGGCATCAGCTCAAGATACTGAGGTGTTTGGTTTT
GTGTTATGTTTACTAAAGTTGAAGTCGTTAATTGTAAAAGAAGCTATTGAGAAGAGCAGTAAA
ATAGTTGTGGTAACAGACTCTTCTTATGTTGCTAAAGGGGTTAATAATCATTTACAAACTTGGT
CTGTAAATGGATGGAGAAAATCTAGTGGCGAGGTCTTGGTTCATGCTAATTTCTGGAAAAGAA
TTTGGTTAACGTTAAAGGAGATCCCCGGAATTGAAGTAAGATGGGTGAGAGGTCATAAAACTT
GCTCTGTTAATATTTTGGCGGATTCGTTAGCTAAAGAAGGTGCTCAATCTCAGCAAGTCCACCC
CCACGCCGCCC

ytP243:

TCCAGGTATGCAGGTTCTCCCTATGATATTTAATGAATCTAATTGTTTCCCACTTTAATTCAATT
AACAACTCATAAATAGGGCGTTGGTTAATGAAACCAAGTAATAAATTATTATTTCTGTTTCCTAA
AATAATTTATATTAAACACCAAAAAACAATTGCTATTAACAAAAGCAATAAACAAACCTTTATTG
AAGTAATCTTCAATTTCAGATTCATGCTCTTTCATTAATTTATCTAATTCTGTAAAAGTTACTTTT
GTCATGTTATGATTTTTAAGATTATTCTTAG**W**AAATTCATTGTTTAGAATTGTTAAAAGTTTTG
GAAATTTTAATATACAATATGTATAAATTTTTCTTTGATCCTCGTCAACAGTTTTATTTAAAATTA
TTTGTGTCCTAATAAACGCTAGCAA

Additionally, this region was confirmed to contain the breakpoint by looking at the read coverages of males and females from whole genome sequencing information. The drop in the male coverage from 1 to 0.5 is seen in the middle panel shown in the figure below.

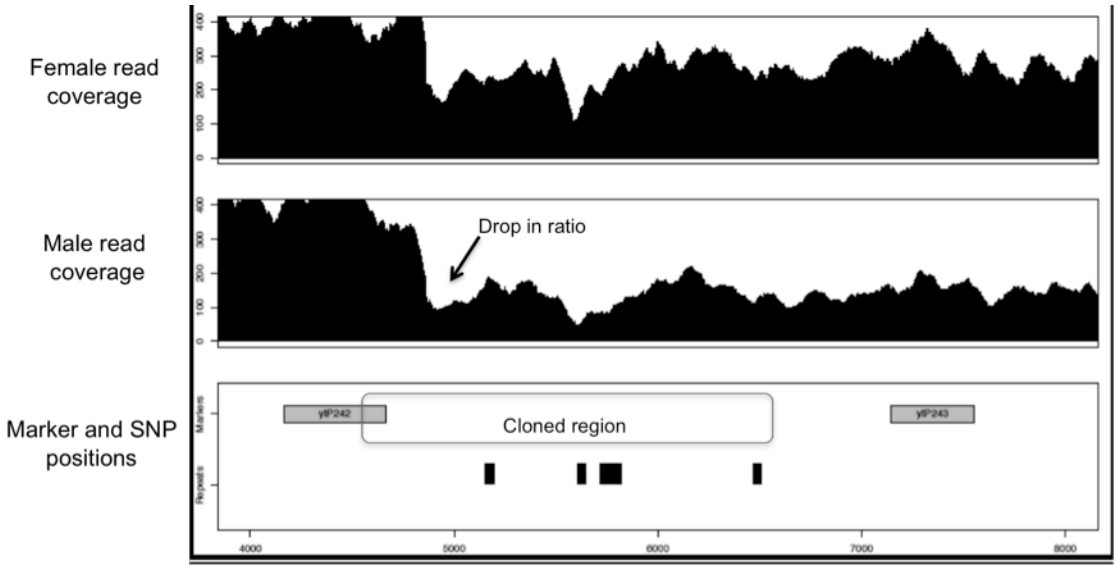


Fig.10. The read coverages of males and females at the breakpoint on scaffold S_000017. X axis is position along the scaffold and Y axis is read coverage. Marker and SNP positions and the region cloned is indicated in the bottom panel.

In order to gain a further understanding of the sequence around the breakpoint we performed a complete analysis of the AT-GC contents of this scaffold. Additional Breakpoint I scaffold details are given in the table below:

Breakpoint scaffold name (v2.2, 2013 assembly)	Total count in bp	Adenine (A) count	Thymine (T) count	Guanine (G) count	Cytosine (C) count	% GC content	Position of breakpoint in bp
S_000017	437,242	166,724	163,116	53,390	53,995	24.6%	At 313,771

We were interested in looking for possible repeats or consensus patterns at or around the breakpoint that could aid in its identification by cellular enzymes, either due to its sequence or due to the formation of secondary or tertiary structures that then get recognized and are cleaved. In order to do this we tested the scaffold sequence using the Tandem repeat Finder Program and came up with 2

consensus patterns, each in 2 copy numbers, placed equidistantly from the breakpoint at either 800bp upstream to the breakpoint or 800bp downstream. This is depicted below:

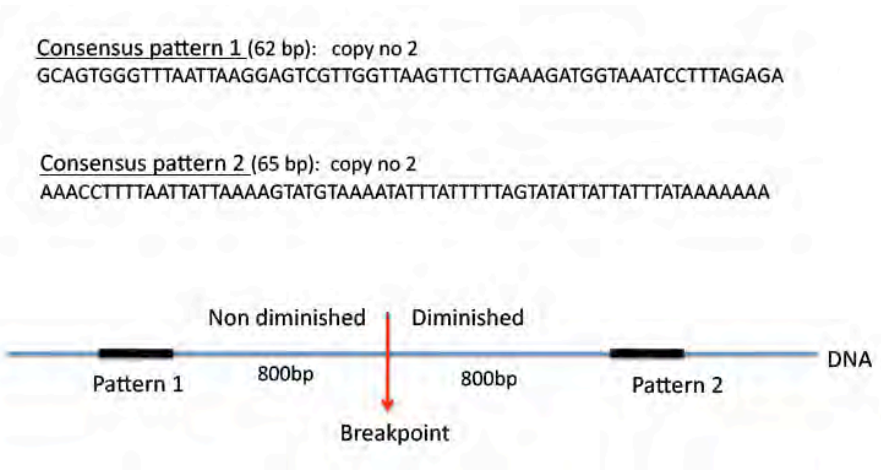


Fig.11. (Top) The sequences for the 2 consensus patterns detected at breakpoint I. (Bottom) A schematic description to show the patterns are equidistantly placed from the breakpoint.

To clone Breakpoint I, genomic DNA was restriction digested first using MboII followed by Hpy188I. The smallest expected size fragment on complete restriction digestion was roughly 1.5Kb. Inverse and nested PCR were then performed after circularization and products were cloned and sequenced. We successfully sequenced 140 clones for this breakpoint. Out of the 140 clones, we surprisingly found 136 clones to have a breakpoint at the same precise position. Of the remaining 4, 2 clones had a breakpoint 2bp upstream of the predicted site (the predicted site being the position found in the 136 clones mentioned above), while the remaining 2 clones had a breakpoint 17bp upstream to predicted site.

Next, we compared the Breakpoint I sequence to other published breakpoint sequences in literature. We found that none of these sequences shared any sequence similarity to that of Breakpoint I. Infact Breakpoint I was also not similar in sequence to the other breakpoint in *S. papillosus* i.e breakpoint II. Inspite of this, there were 2 sequences in the genome with local similarity to the breakpoint I sequence. These sequences had 82% and 99% sequence identity. It remains currently unclear, based on the current sequence assembly, if these sequences represent sequencing and assembly errors or if these sequences are not artifacts of sequencing.

In none of our 140 clones analyzed did we find any sequences that resembled telomeric repeats. For each clone, we read the sequence until the breakpoint (using reference genome assembly as a guide) without problems. After the breakpoint what followed was the TOPO vector sequence in all cases, indicating the absence of any telomeres at these breaks. To further confirm this result we used our reference *S. ratti* genome assembly (which is curently the second best annotated nematode genome next to *C. elegans*) to identify the presence of telomeric repeats of TTAGGC {present in *C. elegans* and *Ascaris*}. Sequencing data shown no such repeats in the *S. ratti* genome. A de-novo approach trying to identify any other telomeric repeats also failed, suggesting the possibility that these nematodes may indeed lack telomeres.

We were also interested in identifying the nature of the cut that was made at the breakpoint. There are 3 different kinds of cuts possible on a DNA strand, a cut leading to a 5' overhang, a cut leading to a 3' overhang or a double stranded break i.e blunt ends. Given our puzzling result for the absence of telomeres, we were specifically interested in checking for free 3' ends if they were generated. This was because the enzyme Telomerase requires a free 3' end to catalyze the addition of telomeres. To this end we performed an overhang detection experiment using the enzyme Terminal deoxynuclotidyl transferase (Tdt) specific for Cytosine. The expectation was that multiple Cytosine residues would be incorporated at any break site in the DNA, and such a region could then be amplified in a PCR reaction using a Poly-G Primer against these Cytosine residues. In sequencing our clones for this experiment we never found any amplification of the breakpoint regions, suggesting that no overhang ends were available for Cytosine addition. We interpret this as a free 3' end may be either absent, protected or chemically modified in this species.

Additionally, we looked at gene predictions in the region upto 10Kb upstream and downstream of the breakpoint. Surprisingly, we found multiple open reading frames (ORFs) in the vicinity, with an example of an upstream gene given below (Fig. 12)

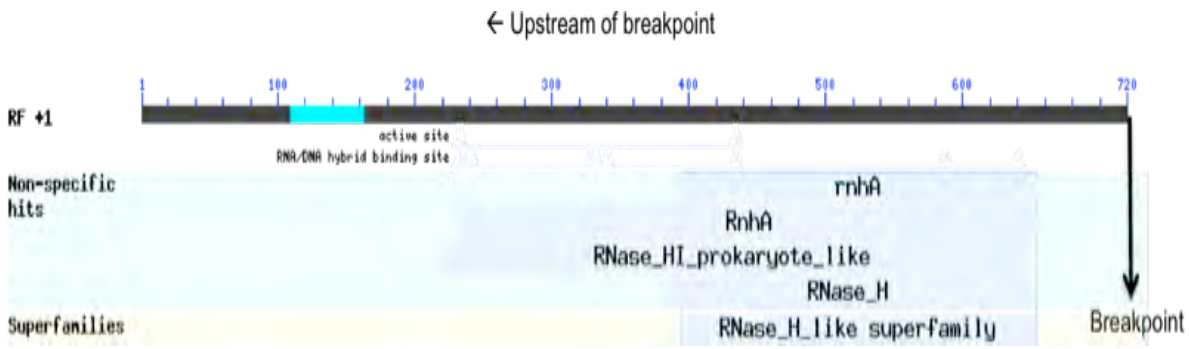


Fig. 12. The predicted RNase H like superfamily member ~60bp upsteam of our predicted breakpoint site.

Additionally we searched extensively for Transposons and Retroelements in the scaffold (~100Kb sequence used) undergoing diminution using the RepeatMasker program. We did not detect any retroelements or transposons in this scaffold, the details of which are given below:

file name: RM2sequpload_1382098912
sequences: 1
total length: 98240 bp (95588 bp excl N/X-runs)
GC level: 25.34 %
bases masked: 2604 bp (2.65 %)

	number of elements*	length occupied	percentage of sequence
Retroelements	0	0 bp	0.00 %
SINEs:	0	0 bp	0.00 %
Penelope	0	0 bp	0.00 %
LINEs:	0	0 bp	0.00 %
CRE/SLACS	0	0 bp	0.00 %
L2/CR1/Rex	0	0 bp	0.00 %
R1/LOA/Jockey	0	0 bp	0.00 %
R2/R4/NeSL	0	0 bp	0.00 %
RTE/Bov-B	0	0 bp	0.00 %
L1/CIN4	0	0 bp	0.00 %
LTR elements:	0	0 bp	0.00 %
BEL/Pao	0	0 bp	0.00 %
Ty1/Copia	0	0 bp	0.00 %
Gypsy/DIRS1	0	0 bp	0.00 %
Retroviral	0	0 bp	0.00 %
DNA transposons	0	0 bp	0.00 %
hobo-Activator	0	0 bp	0.00 %
Tc1-IS630-Pogo	0	0 bp	0.00 %
En-Spm	0	0 bp	0.00 %
MuDR-IS905	0	0 bp	0.00 %
PiggyBac	0	0 bp	0.00 %
Tourist/Harbinger	0	0 bp	0.00 %
Other (Mirage, P-element, Transib)	0	0 bp	0.00 %
Rolling-circles	0	0 bp	0.00 %
Unclassified:	0	0 bp	0.00 %
Total interspersed repeats:		0 bp	0.00 %
Small RNA:	0	0 bp	0.00 %
Satellites:	0	0 bp	0.00 %
Simple repeats:	44	2145 bp	2.18 %
Low complexity:	9	459 bp	0.47 %

Fig. 13. Data generated using the RepeatMasker program showing no predictions in a 100Kb region in the scaffold containing Breakpoint I.

Characterization of Breakpoint 2:

According to *S. papillosus* (v2.2, 2013 genome assembly) breakpoint II lies on scaffold DIM_S_000025#1. The breakpoint is flanked by markers ytP228 (on the non-diminished side) and ytP215 (on the diminished side) which are ~350bp apart. The ytP marker sequences are as follows (SNP sites used for analysis are indicated as per IUPAC degeneracy nomenclatures in red):

>ytP215 (Diminished marker)

CCACTAATCACAATGAGCAACACAATTTATCAATAACTATTGTAAAATTTATAAATAATTACAAA
AGTGTAGGCATTTGAACTAAAATTATGTTAAAATACAAAGCGATACACTAAAATGTTTTATAAA
ATTTATGTAGAGTTTTTTATTCTAGTTAATAGAAAACTCTTTTTGGCTTAAAAACGAATTGATGG
TATATGAAAAGAAGAACTGATAGTCCAAATAGGTAATTTTGTTTGTGGTAAAGTTTTTTAAAAA
TTTCCAACAAGTCAAATTTTTCATTACACATACTGTATAAATTTCCAACAATAACAAATAAATTAA
TATTTTATTATCATTTCAGAGTTGAAATTGTATTCACTTCAATTGCATAAATTATTATGAGTTGC
ACCATTTGTGGCCTTATCTATATTACAGTAAGTTTTCATACATAGAGTAAATTAAAGTTGCTAAG
CATCACATTTGAAATTTATAA**Y**GGAGGATTAAGCTAAAAAAACTTTATGCGAAAATAATATTTA
AATATTATAAAAATAGCCTAAATTTGTGCTAAATGTAAAAATTATTTTTCTATTAAATAAATAAT
TCCATGATCTTGTAATTTAGTAAGGGAAAATATCCTCTACTCTCTCACTATCGCACGATCATTTC
TTCCGA

>ytP228 (Non diminished marker)

GTGACCACCACCTGATCCTTTGCACAAAATTGCTTTTTTTAAATCATTATTTATTAATCTTTTTAA
AATGGTTTCATCTGATTTTTTGAAATTGTTTACCATATGTAGTATTGCGCGTTTTGATACAGTTT
GGGTCACATTGAAGGGGATCCGATTAATTTTAGGTTTAGCAATTGAACATTTGTTGGTATAATA
ATAACGAACTATTCTTAACTTTTTCTCAATAGAATATTTTCTTACTGAGGTAGCAACAGTAGAAA
ATATATGAAAAAAAGTAGTATTGTAAAAATGGGACATTTTATGA**W**GAAATATGCTTTAAACTAA
CTCTTTTAACATCAAGCATCTATTTTTTAATTAATTAAAATATTATAAGATATATCTTTAAAACAG
AAATTTATACATTTGATCAATCTAACAAGATACCTGCACAAGAAAGTATTT

Positional information for this breakpoint

We succeeded in genetically linking the highly biased marker ytP44 (see Nemetschke et al., 2010) to ytP228, our non-diminished marker for breakpoint II. ytP44 is on the shorter side of the autosome, helping us gain positional information on this breakpoint (fig. 13)

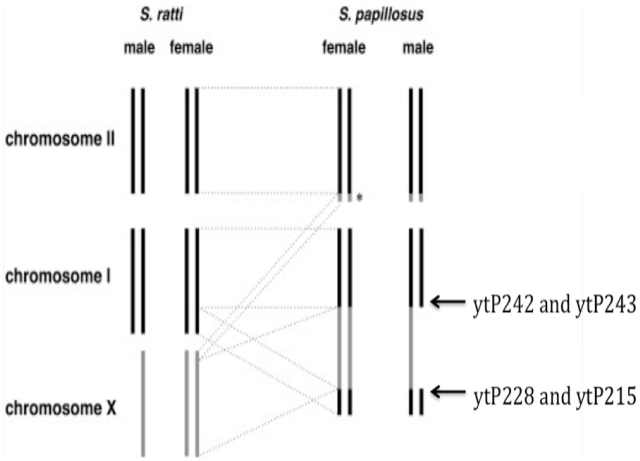


Fig. 13. Orientation of the position of the breakpoints on the large chromosome of S. papillosus (modified from Nemetschke et al., 2010)

Support from sequencing data showing that the breakpoint lies on this scaffold seen by the drop of read coverage ratio from 1 to 0.5 in males in this scaffold.

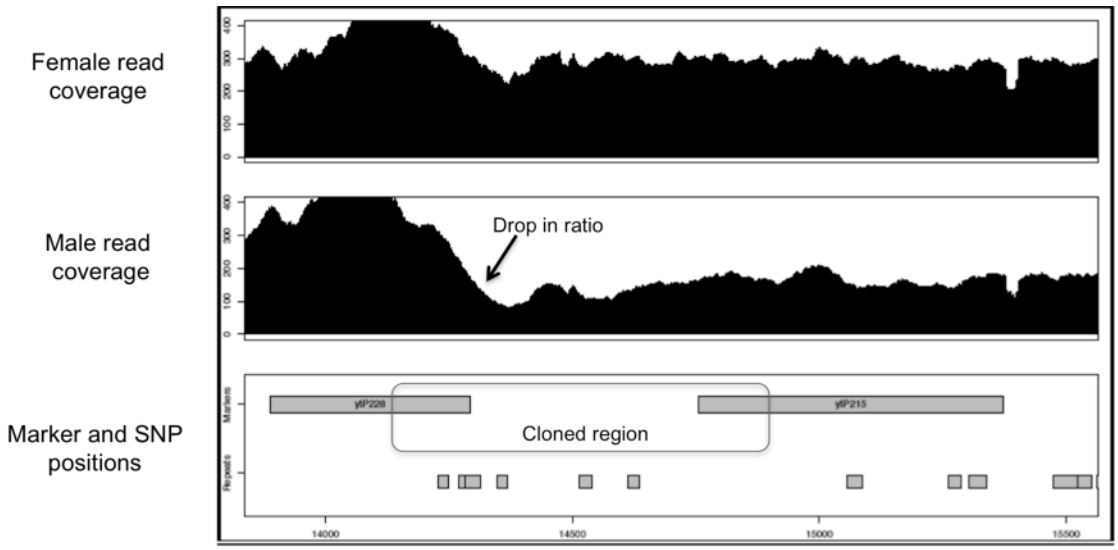


Fig. 14. Drop in read coverage ratio is males (middle panel) indicating the breakpoint. X axis shows position along scaffold and the Y axis shows read coverages. (Bottom) Position information of ytP markers and SNPs in the scaffold.

Further details for the scaffold containing Breakpoint II:

In comparison to Breakpoint I, Breakpoint II lies in a scaffold that has a higher AT content, given its smaller scaffold size.

Breakpoint scaffold name (v2.2, 2013 assembly)	Total count in bp	Adenine (A) count	Thymine (T) count	Guanine (G) count	Cytosine (C) count	% GC content	Position of breakpoint in bp
DIM_S_000025#1	64,121	24,351	24,368	7,721	7,681	24%	At 10,926

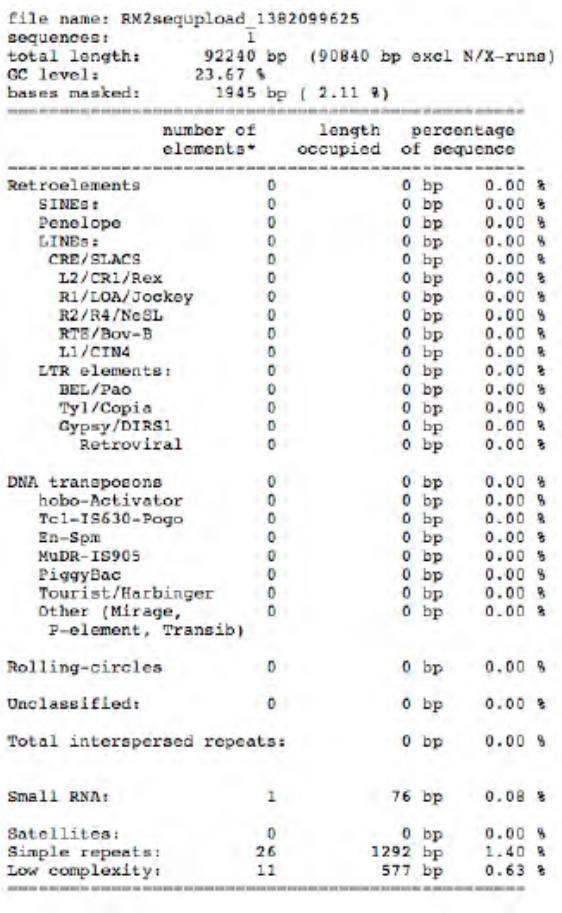
We did not find any meaningful repeats or consensus patterns in this scaffold using the Tandem repeat Finder Program, unlike for Breakpoint I. Breakpoint II was cloned using Hpy188I as the first

restriction enzyme, followed by MboII. The number of clones analyzed for this breakpoint was 21 clones. All clones again had the breakpoint at the same precise position. We attribute the extremely AT rich sequence in the local region at this breakpoint to be a cause for the fewer number of clones we achieved, as this region is hard to amplify. Again no predictable telomeric repeats or a free 3' end was found in any of these clones on sequencing. This is consistent with what we found for Breakpoint I.

This Breakpoint has no similarity to Breakpoint I or to other published breakpoints. This breakpoint contains a large number of collapsed repeats. As a result there are a number of sequences in the genome with local similarity to breakpoint sequence, ~ 36 sequences with 77-90% similarity and 11 sequences with 91-98% similarity. This may represent either sequencing/assembly errors due to local high AT content, or this may be a region with multiple duplications in the genome.

We found no good gene predictions in a 10Kb region upstream of this breakpoint. This is not surprising given the AT rich regions in the vicinity.

Our search for Transposons and retroelements using the RepeatMasker program did not yield any positive results. This is shown below.



```
file name: RM2sequpload_1382099625
sequences:             1
total length:      92240 bp  (90840 bp excl N/X-runs)
GC level:          23.67 %
bases masked:       1945 bp ( 2.11 %)
==================================================
                number of      length   percentage
                elements*    occupied  of sequence
--------------------------------------------------
Retroelements            0           0 bp    0.00 %
   SINEs:                0           0 bp    0.00 %
   Penelope              0           0 bp    0.00 %
   LINEs:                0           0 bp    0.00 %
    CRE/SLACS            0           0 bp    0.00 %
      L2/CR1/Rex         0           0 bp    0.00 %
      R1/LOA/Jockey      0           0 bp    0.00 %
      R2/R4/NeSL         0           0 bp    0.00 %
      RTE/Bov-B          0           0 bp    0.00 %
      L1/CIN4            0           0 bp    0.00 %
   LTR elements:         0           0 bp    0.00 %
      BEL/Pao            0           0 bp    0.00 %
      Ty1/Copia          0           0 bp    0.00 %
      Gypsy/DIRS1        0           0 bp    0.00 %
        Retroviral       0           0 bp    0.00 %

DNA transposons          0           0 bp    0.00 %
   hobo-Activator        0           0 bp    0.00 %
   Tc1-IS630-Pogo        0           0 bp    0.00 %
   En-Spm                0           0 bp    0.00 %
   MuDR-IS905            0           0 bp    0.00 %
   PiggyBac              0           0 bp    0.00 %
   Tourist/Harbinger     0           0 bp    0.00 %
   Other (Mirage,        0           0 bp    0.00 %
    P-element, Transib)

Rolling-circles          0           0 bp    0.00 %

Unclassified:            0           0 bp    0.00 %

Total interspersed repeats:          0 bp    0.00 %


Small RNA:               1          76 bp    0.08 %

Satellites:              0           0 bp    0.00 %
Simple repeats:         26        1292 bp    1.40 %
Low complexity:         11         577 bp    0.63 %
==================================================
```

Fig. 15. Search for Transposons and repeatelements using the RepeatMasker program did not yield any candidates.

Search for Telomerase:

We performed a systematic BLAST (BLASTn, BLASTx, tBLASTn, tBLASTx) search for all described telomerase subunits or components of the shelterin complex from *C. elegans* and yeast in *Strongyloides* nematodes (*S. ratti* and *S. papillosus*). Currently, *S. ratti* is the best-annotated genome after *C. elegans*, which facilitated this analysis. However, we found no significant hits for the main components of the shelterin complex (and also trt-1, Pot-1, Pot-2 and Pot-3 from *C. elegans* and yeast) neither in *S. ratti* or *S. papillosus*. While these results can in part be explained by the current genome assemblies, these finding does support our results of the cloning experiments indicating that these species might infact lack a telomerase altogether.

Discussion

We have identified and cloned the breakpoints on the chromosome where chromatin diminution occurs in the males of *S. papillosus*. These breakpoint sequences represent unique sequences, i.e they do not share any sequence similarity with each other or to other published sequences and thus could not have been predicted before hand. Additionally, based on alignment of the two-breakpoint regions, we could rule out that there is any homology or large conserved motifs that could play a role in the establishment of the breakpoints. In this regard, further experiments need to be done to know if the consensus patterns and collapsed repeats that were identified by us around the breakpoints have any functional significance.

We find that the position of the breakpoint seems to be precisely defined down to the basepair in the males, unlike in *Ascaris* where this break occurs in a region spanning a few hundred base pairs (Mueller et al., 1995; Wang et al., 2012). Such a precise cut on the DNA argues for the possibility of RNA mediated mechanisms at play in these species. To this end, a thorough small RNA sequencing profile needs to be created for this species. The involvement of a RNA-mediated mechanism has been shown for other species undergoing chromatin diminution, for e.g. in *Tetrahymena* and other ciliates (Nowacki et al., 2011). In *Ascaris* however, no evidence has been reported to date for diminution to have a RNA basis (Mueller et al., 2001). In further support of this, we did find a ribonuclease H (RNase H) like superfamily member base pairs upstream from Breakpoint I. RNase H is a family of highly conserved non-sequence-specific endonucleases. The ribonuclease activity of this enzyme is thought to cleave the 3' O-P bond of RNA in a DNA/RNA duplex to produce 3'OH and 5'P terminated products. Additionally, in other biological processes like DNA replication, RNase H is responsible to remove the RNA primer thereby allowing the completion of the newly synthesized DNA molecule (Champoux and Schultz, 2009).

Surprisingly, we could not find any telomeric repeats added at these breakpoints, suggesting the possibility that these newly formed DNA ends are stabilized in other ways, possibly through protein caps. In Insects, researchers have taken advantage of the fact that the enzyme Telomerase has a RNA template within and the sequence of this RNA template corresponds to the telomere sequence of that species. The absence of any predicted telomerase (and hence its RNA template) from whole genome sequencing data in *Strongyloides* spp. prevents us from predicting telomeric sequence and further strengthens our finding that *S. papillosus* may lack telomeric repeats altogether. Additionally,

other experiments we performed for overhang detection at the breakpoint using the Terminal deoxynucleotidyl transferease did not reveal a free 3' end. Such a free 3' end is needed for Telomerases to catalyze the addition of telomeric repeats of these regions. Even in *S. ratti*, which has the second best annotated genome next to *C. elegans*, analyzing whole genome sequencing data did not yield any predictions for telomeric repeats. We are however limited by the current genome assembly atleast for *S. papillosus* and these negative results need to be investigated further, before conclusions are drawn concretely. If it is true that there are indeed no telomeric repeats, as suggested by our experiments, it will be very interesting to uncover the underlying mechanisms of how such a precise cut is made on a chromosome and how the newly formed DNA ends are stabilized and maintained in the cell and its progeny.

Lastly, we know that the DNA sequences that undergo elimination are homologous to the X chromosomal sequences in *S. ratti*. The X chromosome in *S. ratti* is quite gene dense, with an approximate estimate of a substantial number of genes being on this chromosome. Therefore in the current absence of a good genome assembly and gene annotations for *S. papillosus*, if we extrapolate, this number should match the rough prediction for the number of genes lost during the event of chromatin diminution in *S. papillosus* males. This however is only a rough estimate and with the genome sequencing underway for this species will become clear in the near future.

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Lebenslauf/ Curriculum vitae

Arpita Kulkarni

Born on 28th July, 1983

In Pune, India

EDUCATION AND RESEARCH EXPERIENCE:

Max Planck Institute for Dev. Biology Tübingen, Germany

PhD Thesis Dissertation 2010-2015

Thesis Supervisor: Dr. Adrian Streit

Thesis: "Understanding the *Strongyloides* gonad:

Sex determination, Chromatin diminution and Germ-line Organization"

Department of Zoology University of Pune, India

Master's degree in Molecular Biology 2004-2006

Thesis Supervisor: Prof. Dr. D.N. Deobagkar,

Director IBB Pune & Ex-Vice Chancellor, Uni. Of Goa

Thesis: "Characterization of a phosphate solubilizing bacterium"

Department of Zoology Fergusson College, Pune

Bachelor's degree in Zoology 2001-2004

Publications

Parastrongyloides trichosuri suggests that XX/XO sex determination is ancestral in Strongyloididae (Nematoda)

ARPITA KULKARNI[1], ANNA DYKA[1], LINDA NEMETSCHKE[1]†, WARWICK N. GRANT[2] *and* ADRIAN STREIT[1]*

[1] *Department of Evolutionary Biology, Max Planck Institute for Developmental Biology, D-72076 Tübingen, Germany*
[2] *Department of Genetics, La Trobe University, Victoria 3086, Australia*

(*Received 10 April 2013; revised 27 May and 24 June 2013; accepted 4 July 2013; first published online 19 August 2013*)

SUMMARY

The parasitic roundworms *Strongyloides stercoralis* (in man) and *Strongyloides ratti* (in rats) employ environmentally controlled XX/XO sex determination with a pair of X chromosomes and two pairs of autosomes. *Strongyloides papillosus* (in sheep) has only two pairs of chromosomes, one of which combines the genetic material homologous to the *S. ratti* chromosomes X and I. This species creates males through the elimination of one copy of the portion related to the X chromosome (chromatin diminution). It is not clear which one of these two sex-determining mechanisms is ancestral. We demonstrate that *Strongyloides vituli* (in cattle) has two pairs of chromosomes like its very close relative *S. papillosus* whereas *Parastrongyloides trichosuri*, a closely related out-group to *Strongyloides* spp. in Australian brushtail possums, has three chromosome pairs and employs XX/XO sex determination. The X chromosome of *P. trichosuri* is homologous to the X chromosome of *S. ratti*. Our data strongly suggest that the last common ancestor of *Strongyloides* spp. and *Parastrongyloides* spp. had two pairs of autosomes along with two or one X chromosome in females and males, respectively. The situation with two pairs of chromosomes is likely derived and occurred through the fusion of the X chromosome with an autosome.

Key words: *Parastrongyloides* spp., *Strongyloides* spp., sex determination, X chromosome, evolution.

INTRODUCTION

The nematode genus *Strongyloides* consists of small intestinal parasites of various vertebrates (Speare, 1989; Dorris *et al.* 2002; Viney and Lok, 2007). The life cycle of *Strongyloides* spp. (Fig. 1A) has been reviewed and discussed recently (Viney and Lok, 2007; Streit, 2008). In brief, parasitic worms, which are all female, live in the small intestines of their respective hosts. They produce female and male progeny by mitotic parthenogenesis. The young females can develop into infective third-stage larvae (L3i) that infect a new host (homogonic or direct development). Alternatively the females, like all the males, develop into free-living adults that reproduce sexually (heterogonic or indirect development). With very few exceptions (Yamada *et al.* 1991; Streit, 2008), the entire progeny produced by the free-living *Strongyloides* sp. consists of females that develop into L3i.

The presence of easily accessible free-living adults and a number of techniques and resources facilitating experimental work recently developed for multiple species of *Strongyloides* and for their relative *Parastrongyloides trichosuri* (Viney *et al.* 2002; Grant *et al.* 2006*a*, *b*; Eberhardt *et al.* 2007; Nemetschke *et al.* 2010*b*; Shao *et al.* 2012) render this group of parasites highly attractive for genetic analysis.

In all species of *Strongyloides* investigated thus far, the sex of the progeny produced by the parthenogenetic parasitic females is determined by an environmental stimulus (environmental sex determination, ESD) such that an increasing immune response of the host against the worms leads to a higher proportion of males (reviewed in Streit, 2008). Nevertheless, in *Strongyloides ratti* (a parasite of rats) and *Strongyloides stercoralis* (a parasite of humans and dogs), X chromosomes exist (Nigon and Roman, 1952; Hammond and Robinson, 1994; Harvey and Viney, 2001) (Fig. 2). In these species both sexes have two pairs of autosomes, and the females have two but the males only one X chromosome (environmentally controlled XX/XO sex determination). Therefore in these species $2n = 6$ in females and $2n = 5$ in males. In *Strongyloides papillosus* (a parasite of sheep) the genetic material homologous to the chromosomes I and X of *S. ratti* is combined in one chromosome (Nemetschke *et al.* 2010*a*). In males an XX/XO system appears to be functionally restored through a sex-specific chromatin diminution event that creates a hemizygous region corresponding to the X chromosome in *S. ratti*

* Corresponding author: Department IV, Max-Planck-Institut für Entwicklungsbiologie, Spemannstrasse 35, D-72076 Tübingen, Germany. E-mail: adrian.streit@tuebingen.mpg.de
† Present address: Max Planck Institute of Molecular Cell Biology and Genetics, D-01307 Dresden, Germany.

doi:10.1017/S0031182013001315

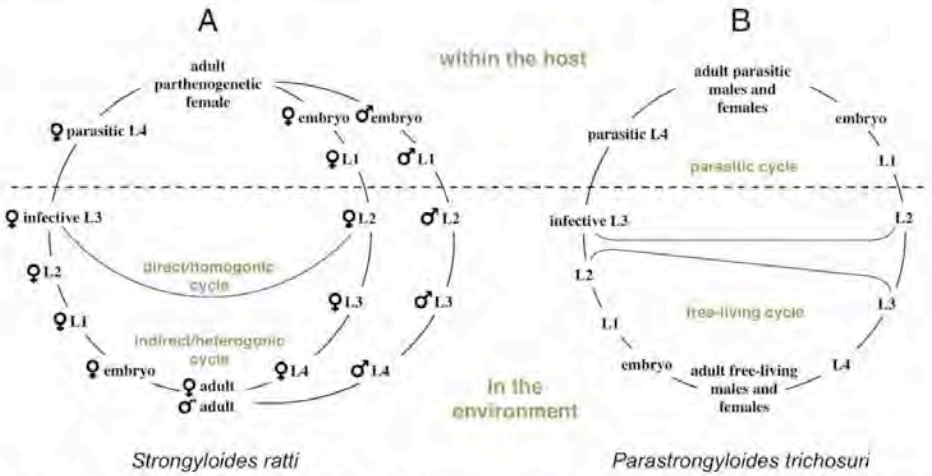

Fig. 1. Life cycles of *Strongyloides ratti* (A) and *Parastrongyloides trichosuri* (B). A also applies for the other species of *Strongyloides* mentioned in this publication except: (i) in *S. papillosus* and *S. vituli* embryonated eggs are passed and the L1s hatch within a short time outside of the host and (ii) in *S. stercoralis* a fraction of the larvae develop into L3i within the host (autoinfective L3i). For further explanation see text.

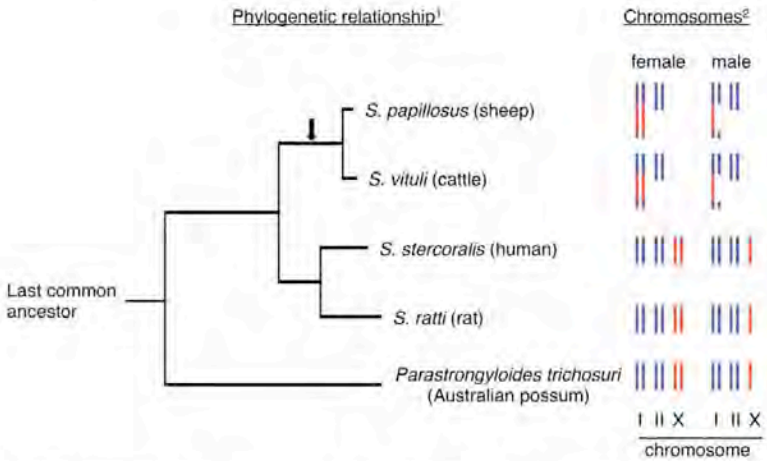

Fig. 2. Phylogenetic relationships and chromosomes in the species discussed. Chromosomes/genomic regions present in two copies in both sexes are depicted in black (print version) or blue (online version), chromosomes/genomic regions present in two copies in females and in only one copy in males are depicted in grey (print) or red (online version). For *Strongyloides stercoralis* it is likely but it has not been demonstrated that the X chromosome is homologous to the X chromosome in *S. ratti*. For *S. vituli* the chromosomes are drawn identical to *S. papillosus* although the exact topology is not known (see discussion). The arrow indicates the postulated X chromosome to chromosome I fusion, which led to the reduction of the haploid chromosome number from three to two. [1]According to Eberhardt *et al.* (2008). [2]The references for the chromosomes are for: *S. papillosus*: Albertson *et al.* (1979), Nemetschke *et al.* (2010*a*); *S. vituli*: this publication; *S. stercoralis*: Hammond and Robinson (1994); *S. ratti*: Harvey and Viney (2001), Nigon and Roman (1952); *P. trichosuri*: this publication.

(Albertson *et al.* 1979; Nemetschke *et al.* 2010*a*) (Fig. 2). Since in the process an internal portion of one chromosome is eliminated and both ends are retained as separate chromosomes this leads to $2n = 5$ in males while in females $2n = 4$. Nemetschke *et al.* (2010*a*) proposed that the different sex-determining

systems within the genus *Strongyloides* may represent different intermediate steps in the transition from XX/XO genetic sex determination (GSD) to ESD with no genetic differences between the sexes. This hypothesis assumes that the situation in *S. ratti* is ancestral and chromatin diminution in *S. papillosus* is derived. However, there is no strong evidence to support or reject this assumption. Knowing the chromosome numbers and the sex-determining systems of closely related species whose phylogenetic position with respect to *S. ratti* and *S. papillosus* is clear, could shed some light on this question (Fig. 2). Therefore, we analysed *Strongyloides vituli*, a species clearly much more closely related to *S. papillosus* than *S. ratti* (Eberhardt *et al.* 2008) and *P. trichosuri*, which is an outgroup to the genus *Strongyloides*.

Strongyloides vituli is a parasite of cattle and is most closely related to *S. papillosus* (Eberhardt *et al.* 2008). Although Brumpt (1921) considered the *Strongyloides* in cattle a species different from *S. papillosus*, and instated the name *S. vituli*, most later authors treated all *Strongyloides* in domestic ruminants as *S. papillosus*. Only recently, molecular diagnostics revealed that in cattle frequently two species of *Strongyloides* co-exist (Eberhardt *et al.* 2008). One of them is more abundant and differs in its 18S RNA sequence from *S. papillosus* found in sheep. For this species the name *S. vituli* was reactivated (Eberhardt *et al.* 2008). The other species is presumably *S. papillosus*, which is the only species of *Strongyloides* described in sheep. Here we show that *S. vituli* females have two pairs of chromosomes ($2n = 4$) and that loci homologous to loci undergoing male-specific chromatin diminution in *S. papillosus* are also hemizygous in *S. vituli* males.

The genus *Parastrongyloides* is closely related to *Strongyloides* spp. (Dorris *et al.* 2002) and *P. trichosuri*, a parasite of Australian brushtail possums, is well established as a laboratory system (Grant *et al.* 2006*a*, *b*). Like *Strongyloides* spp. *P. trichosuri* can also undergo parasitic and free-living life cycles (Fig. 1B). Unlike *Strongyloides* spp., this species is also dioecious in the parasitic generations and it can undergo a large, if not unlimited number of consecutive free-living cycles (Grant *et al.* 2006*b*).

Here we show cytologically and genetically that *P. trichosuri* has three pairs of chromosomes and employs XX/XO sex determination ($2n = 6$ in females, $2n = 5$ in males). We confirm that, unlike all species of *Strongyloides* studied so far, *P. trichosuri* reproduces sexually not only in the free-living but also in the parasitic generation.

Taken together our results strongly suggest that XX/XO sex determination with a haploid chromosome complement of two autosomes and one X chromosome ($n = 3$, $2n = 6$ in females, $2n = 5$ in males) is ancestral in the Strongyloididae.

MATERIALS AND METHODS

Culturing and manipulating Strongyloides *sp. and* P. trichosuri

Strongyloides ratti and *S. papillosus* were maintained as described earlier (Viney *et al.* 1992; Eberhardt *et al.* 2007; Nemetschke *et al.* 2010*b*). *Parastrongyloides trichosuri* was maintained in continuous free-living cycles (Grant *et al.* 2006*b*) on NGM plates (Stiernagle, 1999) with a piece of autoclaved rabbit feces supplemented with *Escherichia coli* OP 50 bacteria.

Strongyloides vituli *isolates*

Feces from a total of seven fattening bulls were collected between May and July 2012 in Tübingen-Unterjesingen, Germany (48°31′11″N 8°58′06″E). The feces were cultivated for 2 days and the worms isolated by the Baerman technique (as described by Eberhardt *et al.* 2007; Nemetschke *et al.* 2010*b*).

Parastrongyloides trichosuri *isolates*

Two re-isolates from the isolate deliberately released at Kahurangi, New Zealand (Cowan *et al.* 2006) and one isolate from Brisbane Australia were used. The isolate Brisbane was isolated by collection of feces deposited by wild, free-ranging brushtail possums on the parapet of a pedestrian bridge in the Walton Bridge Reserve, The Gap, Brisbane (27°26′S, 152°52′E), a suburban park in which brushtail possums had been observed frequently. The faeces were <12 h old at the time of collection.

Parasitic *P. trichosuri* were obtained at necropsy of free-ranging wild brushtail possums captured from Hunterville, New Zealand (39°53′S 175°39′E). The anterior third of the small intestine was removed at the point of capture and kept on ice for 8–10 h. Once in the laboratory, parasites were isolated from the small intestine according to Grant *et al.* (2006*b*). Briefly, the intestinal sample was slit along the longitudinal dimension in warm saline and the internal (mucosal) surface rubbed gently with a fingertip to release the parasites. These were then collected individually from the saline with the aid of a dissecting microscope into a second dish of warm saline. Individual male and female adults were then picked separately from this preparation. The females were cut behind the pharynx and the posterior portions containing the uterus were placed individually on culture plates over night. Hatched progeny was collected individually for genotyping.

Parastrongyloides trichosuri *sequences*

DNA fragments homologous to known molecular genetic markers of *S. ratti* (Nemetschke *et al.* 2010*b*)

were isolated by BLAST searching the est_others database at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) followed by PCR amplification of the corresponding genomic sequence from *P. trichosuri* genomic DNA. Alternatively the corresponding DNA sequences were isolated by degenerate PCR.

Generation of molecular markers

Molecular markers were generated as described (Eberhardt *et al.* 2007; Nemetschke *et al.* 2010*b*). For *S. vituli* the same PCR primers as for *S. papillosus* were used. All markers used are listed in Suppl. file 1 – in Online version only.

Single worm lysis and genotyping

Genotyping of individual worms was done as described (Eberhardt *et al.* 2007; Nemetschke *et al.* 2010*b*).

DAPI staining/microscopy

Adult worms were fixed with ice-cold methanol followed by rehydration through a concentration series of methanol in PBS (80%, 60%, 40%, 20%, 0% [twice]). Then the worms were mounted on polylysine-coated slides in 10 μL of Vectashield containing 1 $\mu g\,mL^{-1}$ DAPI (4′,6-diamidino-2-phenylindole).

RESULTS

Strongyloides vituli *has two pairs of chromosomes and likely undergoes male-specific chromatin diminution*

We isolated 96 free-living female and 169 free-living male *Strongyloides* sp. from 2-day-old bovine fecal cultures from a total of seven fattening bulls (see Materials and methods). The cultures also contained a high number of L3i from the direct cycle and we isolated 282 of them. Sixteen females were set aside for cytological observations. Of the other females, the males and the L3i a portion of the small ribosomal subunit RNA (*SSU*) was sequenced to determine the species (cf. Eberhardt *et al.* 2008). 277 (98·2%) of the L3is were *S. vituli*, the remaining five (1·8%) were *S. papillosus*. Among the free-living females 71 (88·75%) were *S. vituli* and nine (11·3%) were *S. papillosus* and among the free-living males 38 (22·5%) were *S. vituli* and 131 (77·5%) were *S. papillosus*. These numbers confirm the observations by Eberhardt *et al.* (2008) that *S. papillosus* in cattle tends to form much higher proportions of males and heterogonic females than *S. vituli*. None of the in total 531 worms genotyped contained both versions of the *SSU* sequence confirming that these two species, although present in the same host, do not normally hybridize.

The 16 females mentioned above were stained with DAPI and meiotic chromosomes counted (Fig. 3C). In all cases the number of chromosomes was two bivalents ($n = 2$). We managed to recover from the microscope slide and determine the *SSU* sequence for five of the 16 females. Four of them were *S. vituli*, one was *S. papillosus*. From these results we conclude that the haploid number of chromosomes in *S. vituli* is two, as in *S. papillosus*.

Next, we amplified 10 *S. vituli* genomic regions (Suppl. File 1 – in Online version only) that contained single nucleotide polymorphisms (SNPs) homologous to regions in *S. ratti* and *S. papillosus* for which the chromosomal locations were known: five SNP markers that are autosomal in *S. ratti* and do not undergo chromatin diminution in *S. papillosus* and five SNP markers that are homologous to markers that are located on the X chromosome in *S. ratti* and do undergo chromatin diminution in *S. papillosus* (cf. Nemetschke *et al.* 2010*a*, *b*). For the five *S. ratti* autosomal markers we found *S. vituli* males and females that were heterozygous for any two alleles, indicating that both sexes have two copies of these loci (Table 1), whereas for the five *S. ratti* X-linked markers we never observed heterozygous males, while heterozygous females were common (except for a single marker in a single animal; Table 1). Although for some of the markers the number of males genotyped is too small to reach a definitive conclusion for the markers concerned, taken together our results clearly indicate that *S. vituli* males have a hemizygous region, which is homologous to the region undergoing chromatin diminution in *S. papillosus*. Most probably *S. vituli* males undergo sex-specific chromatin diminution, as do the males of *S. papillosus*.

Parastrongyloides trichosuri *has three pairs of chromosomes*

First, we stained free-living females of *P. trichosuri* with DAPI and counted the meiotic chromosomes (Fig. 3D). As in *S. ratti* (Fig. 3A), we found three bivalents ($n = 3$) in *P. trichosuri*. In embryos derived from a continuously free-living culture we found two different karyotypes, one with six chromosomes ($2n = 6$, Fig. 3E) and one with five chromosomes ($2n = 5$, Fig. 3F). These observations showed that the haploid number of chromosomes in *P. trichosuri* is three ($n = 3$) and suggested that it employs chromosomal sex determination.

Parastrongyloides trichosuri *employs XX/XO sex determination and the X chromosome is homologous to the* S. ratti *X chromosome*

We isolated 19 sequence polymorphisms (Suppl. File 1 – in Online version only) in genes for which

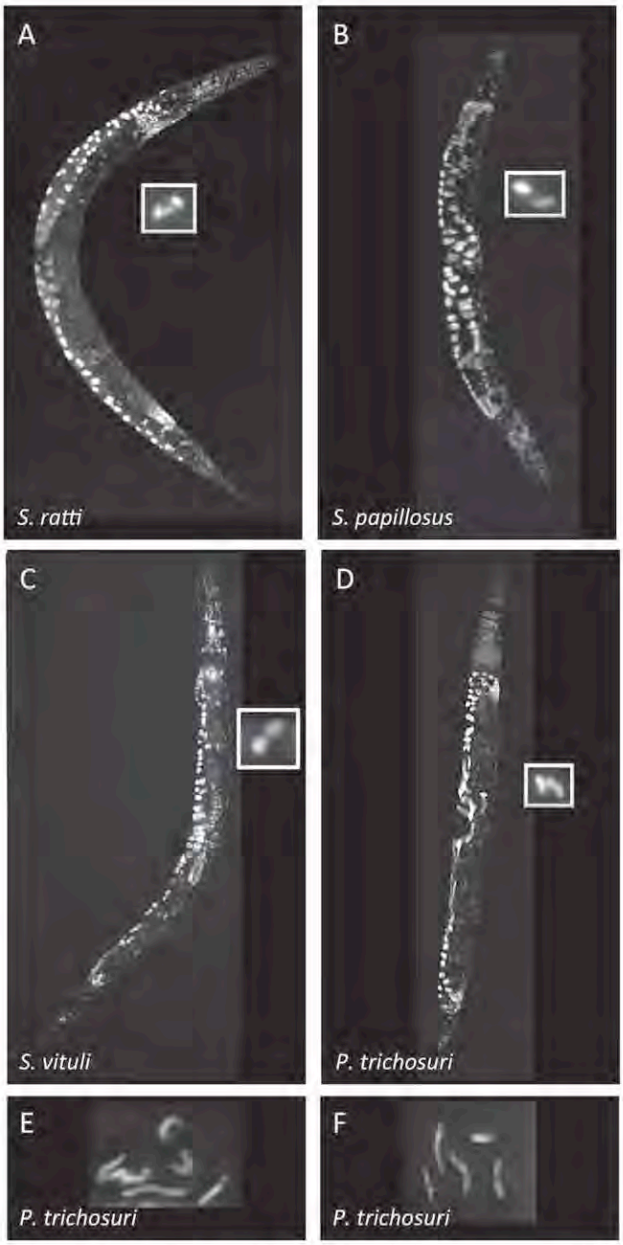

Fig. 3. Chromosome numbers in different species of *Strongyloides* and in *Parastrongyloides trichosuri*. A–D: DAPI stained free-living females of the species indicated are shown at low (10×) magnification. Boxed are meiotic (bivalent) chromosomes in oocytes at high (100×) magnification. E,F: mitotic chromosomes from *P. trichosuri* embryos are shown. Shown is one particular focal plane. Stacks of images were recorded and the chromosomes reconstructed in three dimensions. This allowed unambiguous counting of six chromosomes as in E or five chromosomes as in F.

Table 1. *Strongyloides vituli* males have a hemizygous region

Marker *S. vi.*	Marker *S. ra.*/ chromosome	Marker *S. pa.*/dim?	het. fem/total fem	het. males/ total males
ytP194	*ytP19*/II	*ytP14*/n-dim	8/29	5/14
ytP199	*ytP94*/II	*ytP12*/n-dim	9/11	4/5
ytP200	*ytP33*/I	*ytP129*/n-dim	6/12	2/4
ytP201	*ytP72*/II	*ytP127*/n-dim	11/12	3/4
ytP196	*ytP110*/II	*ytP1*/n-dim	6/6	2/2
ytP189	*ytP71*/X	*ytP83*/dim	16/16	1[b]/13
ytP190	*ytP67*/X	*ytP86*/dim	16/16	0/14
ytP191	*ytP53*/X	*ytP51*/dim	14/14	0/3
ytP192	*ytP104*/X	*ytP135*/dim	13/15	0/3
ytP193	*ytP103*/X	*ytP134*/dim	9/9	0/13
ytP198	*ytP27*/X[a]	*ytP131*[a]/ n-dim	10/10	3/3

Columns: Marker *S. vi.* – marker in *S. vituli* (see Suppl. File 1 – in Online version only); Marker *S. ra.*/chromosome – number of the homologous marker in *S. ratti*/chromosome the marker is located on (Nemetschke *et al.* 2010*a*, *b*); Marker *S. pa.*/dim? – number of the homologous marker in *S. papillosus*/indication if the marker undergoes sex-specific chromatin diminution (dim) or not (n-dim) (Nemetschke *et al.* 2010*a*), het. fem/total fem – number of heterozygous *S. vituli* females found/total number of *S. vituli* females analysed; het. males/total males – number of heterozygous *S. vituli* males found/total number of *S. vituli* males analysed, markers located in a hemizygous region can never be heterozygous.

[a] This is the only marker found by Nemetschke *et al.* (2010*a*) to be X-chromosomal in *S. ratti* but not undergo chromatin diminution in *S. papillosus*.

[b] This single animal may indicate that this particular marker does not undergo diminution in all males or it may be a rare triploid animal, which we do observe occasionally (unpublished) or it may be a PCR artefact.

Table 2. Autosomal and sex-linked loci in free-living *Parastrongyloides trichosuri*

Marker in *P. tr.*	Marker in *S. ra.*/ chromosome	Genotype[a] female	Genotype[a] male	Genotype[a,b] female progeny	Genotype[a,b] male progeny
ytP170	*ytP33*/I	A	G	5×AG	5×AG
ytP188	*ytP107*/X	C	T	22×CT	7×C

As an example the results of one cross for one autosomal and one X-linked marker are given. For more crosses and markers see Suppl. Table 1S – in Online version only. Columns: Marker *P. tr.* – marker in *P. trichosuri* (see Suppl. File 1 – in Online version only); Marker *S. ra.*/chromosome – number of the homologous marker in *S. ratti*/chromosome the marker is located on (Nemetschke *et al.* 2010*b*).

[a] Listed is the result of the sequencing reaction at one of the polymorphic sites of the marker. If two bases are present the individual was heterozygous, if only one base is present the individual was homo- or hemizygous.

[b] Given are [number of progeny with this particular genotype] × [genotype].

the chromosomal locations of putative *S. ratti* orthologues were known (Nemetschke *et al.* 2010*b*) and analysed their inheritance in crosses between single free-living males and one or two females per cross (Table 2, Suppl. Table 1S – in Online version only and data not shown). For the six markers that are X-linked in *S. ratti*, the results of all *P. trichosuri* crosses were consistent with the female progeny receiving two copies, one from either parent, and the male progeny inheriting only one allele from the mother (sex-linked inheritance). All 13 markers whose *S. ratti* homologues are located on an autosome behaved as autosomal markers in *P. trichosuri*. These findings demonstrate that in the free-living generation *P. trichosuri* employs XX/XO sex determination and that the X chromosomes in these two species are homologous.

Reproduction in the parasitic generation of P. trichosuri *is sexual*

In *Strongyloides* sp. the parasitic generation reproduces by mitotic parthenogenesis (genetically demonstrated for *S. ratti* (Viney, 1994; Nemetschke *et al.* 2010*b*) and for *S. papillosus* (Nemetschke *et al.* 2010*a*)) and, at least in most species, parasitic males do not exist (Streit, 2008). In contrast, parasitic males are present in *P. trichosuri* (Mackerras, 1959). Grant *et al.* (2006*b*) have shown that two genetically different strains of *P. trichosuri* do hybridize, when introduced into the same host individual, demonstrating sexual reproduction. To confirm and further characterize sexual reproduction in parasitic *P. trichosuri*, we isolated individual offspring from 17 parasitic females and genotyped them at multiple

Table 3. Genotypes of the progeny of 17 parasitic *P. trichosuri* parasitic females at *ytP1764*

Clutch	Genotypes	Cons. w. clonal r.	Cons. w. self-f.	Cons. w. sing. mat.
1	3×CT, AG, A 1×CT, AG, AG	no	no	yes
2	2×CT, A, A 1×T, A, AG	no	no	yes
3	2×CT, A, G 1×CT, AG, AG 2×C, AG, AG	no	no	yes
4	1×T, A, AG 2×CT, A, G 3×CT, AG, A 1×CT, AG, AG	no	no	no
5	6×T, A, G	yes	yes	yes
6	2×CT, A, G 6×CT, AG, AG	no	no	yes
7	1×T, A, AG 1×CT, A, G	no	no	yes
8	1×T, A, G 5×CT, AG, AG	no	yes	yes
9	1×T, A, G 2×CT, AG, AG	no	yes	yes
10	3×T, A, G 1×CT, AG, G 5×CT, AG, AG	no	no	yes
11	5×T, A, AG	yes*	yes	yes
12	1×T, A, AG	yes	yes	yes
13	2×CT, A, G	yes*	yes	yes
14	7×T, A, AG 3×CT, A, G	no	no	yes
15	1×CT, A, G 1×CT, A, AG 1×CT, AG, A 2×CT, AG, AG	no	no	yes
16	1×C, AG, AG 1×CT, AG, AG	no	no	yes
17	7×T, A, G	yes	yes	yes

Genotypes: [number of progeny with this particular genotype] × [genotype]. *ytP176* contains three polymorphic sites (positions 190, 296, 446). As 'genotype' the sequencing results at these three polymorphic sites are indicated, separated by ','. Two letters indicate that the individual was heterozygous at this position, one letter indicates that the individual was homozygous at this position. From 14 larvae and five males the *ytP176* PCR products were cloned and individual clones sequenced. Seven different combinations of nucleotides at the three polymorphic sites (alleles) were found (190C 296A 446A, 190T 296A 446A, 190T 296A 446G, 190C 296G 446A, 190T 296G 446A, 190C 296A 446G, 190C 296G 446G). Some of the genotypes shown could be explained with multiple allele combinations. The conclusions are independent of this.

Cons. w. clonal r.: consistent with exclusively clonal reproduction: if reproduction is truly clonal all progeny are genetically identical with their mother and consequently with each other; exclusively clonal reproduction can be excluded if in the clutch more than one genotype exists. cons. w. self-f.: consistent with exclusively self-fertilization: if in the mother haploid eggs and sperm are formed and reproduction occurs through self-fertilization, at positions where the mother is heterozygous the offspring can be heterozygous or homozygous for either of the two maternal alleles but no genetic material absent from the mother can be present among the progeny. Exclusive self-fertilization can be excluded if more than two alleles exist within the clutch. cons. w. sing. mat.: consistent with single mating: The progeny can be explained under the assumption that the female mated with a single male. Mating with multiple males must be postulated if it is impossible to derive all genotypes present in the progeny from two parental genotypes.

* For these clutches other markers exclude clonal reproduction (see suppl. Table 2S – in Online version only).

X chromosomal and autosomal loci (Suppl. Table 2S – in Online version only). The results are fully consistent with normal sexual reproduction and not with clonal reproduction. One marker (*ytP176*) was particularly informative because it contains multiple polymorphic nucleotides and occurred in our sample in seven different alleles. In crosses of free-living animals this marker behaved in a clearly autosomal manner. In four different informative crosses (for one example see Suppl. Table 1S – in Online version only) two of the fathers and in total 13 of the male offspring were heterozygous. For this marker, the results of all 17 clutches are given in Table 3. In 10 clutches more than two alleles were present, excluding that the offspring originated entirely from self-fertilization. Further, the genotypes

present in one of the clutches cannot be produced by mating of two individuals, indicating that the corresponding female had mated with multiple males.

The five parasitic males we found we genotyped at two putatively autosomal (*ytP179* and *ytP176*) and at three putatively X-linked (*ytP183*, *ytP184* and *ytP185*) markers. None of the males was heterozygous at any of the three X-linked markers while for the autosomal markers all five were heterozygous at *ytP176* and two were heterozygous at *ytP179*. Although the numbers are rather small these results are also consistent with the hypothesis that the parasitic generation of *P. trichosuri* employs XX/XO sex determination.

DISCUSSION

The finding that *P. trichosuri*, like *S. ratti*, has three chromosomes (haploid, two autosomes and one X) and uses XX/XO sex determination strongly supports the hypothesis that this is the ancestral state for these two genera.

Although it seems likely that sex determination in *P. trichosuri* is genetic, we cannot prove that there is no environmental influence superimposed over the XX/XO system as in *S. ratti*.

The situation with two chromosomes (haploid) in *S. papillosus* and in *S. vituli* is most likely derived and arose through the fusion of the X chromosome with the autosome homologous to the *S. ratti* chromosome I (cf. Nemetschke *et al.* 2010*a*). In the older literature, based solely on cytological studies, different authors disagreed on the number of chromosomes in *S. papillosus* males. While Albertson *et al.* (1979) had observed sex-specific chromatin diminution ($2n = 4$ in females and $2n = 5$ in males) as it was later confirmed genetically (Nemetschke *et al.* 2010*a*), Triantaphyllou and Moncol (1977) found no karyotypic differences between the sexes ($2n = 4$ in both sexes). Distinguishing species within the genus *Strongyloides* based solely on morphological criteria is notoriously difficult (Augustine, 1940; Schad, 1989; Speare, 1989). Therefore frequently the host is used to derive the species of *Strongyloides*. Since we now know that what at the time was considered *S. papillosus* are at least two different species, one possible explanation for the conflicting findings of Albertson *et al.* (1979) and Triantaphyllou and Moncol (1977) might be that in fact different species (e.g. *S. papillosus* and *S. vituli*) were studied. However, our genetic data presented here clearly demonstrate that *S. vituli* males have a hemizygous region and therefore differ genetically from females. We did not have access to early *S. vituli* embryos derived from parasitic mothers, the only stage where in *S. papillosus* the male karyotype of $2n = 5$ is easily observable. Therefore we do not know if in *S. vituli* the region eliminated in males is flanked by retained regions, as in *S. papillosus*. If this region were at the end of a chromosome, chromatin diminution would result in a size reduction of one chromosome but not in an increase of the chromosome number and might therefore be more difficult to detect cytologically.

On the evolutionary path to *Strongyloides* spp., which in the parasitic generation have no males and reproduce clonally, one could imagine an intermediate state where males are still present as an evolutionary relict but no longer or not mandatorily participate in reproduction. Although we cannot technically exclude that females, under certain conditions, can also reproduce without males, either by self-fertilization or parthenogenesis, our data demonstrate that sexual reproduction with outcrossing is the predominant if not the only mode of reproduction in parasitic *P. trichosuri*. This finding is not surprising. Parasitic males appear to be always present in productive *P. trichosuri* infections (Mackerras, 1959; Grant *et al.* 2006*b*) and Grant *et al.* (2006*b*) had demonstrated that outcrossing does occur in the parasitic form of *P. trichosuri*.

Although presented here in the context of an evolutionary question, our results are also interesting in their own right. *Parastrongyloides trichosuri* with its 'choice' between parasitic or free-living lifestyle is a highly attractive study system. Knowing the modes of inheritance in both forms is an important prerequisite for future studies.

Taken together, our findings strongly argue that XX/XO sex determination with two pairs of autosomes is ancestral in the Strongyloididae. More generally, they illustrate the usefulness of *Strongyloides*/*Parastrongyloides* as a genetically traceable system to study how parasitism can evolve and what the developmental and genetic consequences of the transition from a free-living to a parasitic lifestyle might be.

ACKNOWLEDGEMENTS

We thank Dorothee Harbecke for technical assistance and Dr Stewart Bisset for assistance with brushtail possums.

FINANCIAL SUPPORT

This work was funded by the Max Planck Society (A. K., A. D., L. N., A. S.) and La Trobe University School of Molecular Sciences (W. G).

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0031182013001315.

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Sequences of molecular markers

Given are the sequences of the PCR products amplified including the primer sequences (underlined). Markers underlaid yellow are mentioned in the publication. Ambiguity codes in bold color indicate polymorphic sites included in any of the tables or supplementary tables.
Notice: for *S. vituli* the primers designed for *S. papillosus* were used. Therefore the primer sequences are from *S. papillosus*. NNNN... in *S. vituli* markers indicate nucleotides that were not sequenced from *S. vituli* because they are located to close to the primer used for sequencing.

P. trichosuri markers corresponding to markers on *S. ratti* chromosome I:

ytP170:
ATCCATTGATTTGCCCAGTCAAGATGAGTTCTATCACCGTCCTTATTATCTTTT
CTAACTCTGTTGACATAAAACATTGATGCCTCTTGCATATCTTTAACAAAYGG
TAGCTGGCTTTTTTTTRMCWSCTAACCATCCTGTGGATTGGATACCYTCAGCC
ATAGCACTGAGRTGATTAAAAACATTTGATTTTCTGTTGTTGTCTTTAAATTC
AACCATTCCATTCATTARTTCACTGATAGGACCTAGCTTAGAAGCAACGTCTT
CATCASAAAGTTGKGGTTGAGAAGCARCTAACCASATAAAATTTTTCTGATTT
TCAAATATWTCTTTAACTKTATTTCCCTTAAAAATTAATAAAAAAAATTTAAA
AATAATAAAAATAAACTAACAGCTTGACAAAGATCACCACC

ytP171:
CACCTCCTTGTACAGCAGCACCATARGCAACAGCTTCATCAGGATTGATTCCT
CTTGATGGTTCTTTTCCATTGAAAAATTCCTTGATAAGTTGTTGAACCTTTGGA
ATACGTGTAGAACCTCCAACAAGAACAATTTCATGAATTTCATCCTTCTTTAA
TCCAGCATCATCAAGAACCTTTTGRACAGGTTTCATTGTAGCACGGAAAAGA
TCCATATTAAGTTCTTCAAATTTAGAACGAGAAAGAGTTTCTGAAAAGTCGG
CTCCATCAAAAAGAGATTCAACTTCCACGGTAACTTGGTGAGTATTAGAAAG
GGTTCTCTTGGCCTT

ytP172:
GTTGCCAAAACCAGGAAAGATTCAAGTTTTTAAAGCAAATGAAGATTATCTT
CCAAACAATGTAATTTTTWGTTTTAAAAATTATTATTTATAATGTTTAGGAAA
ATGAATTATCAGTGGAGAAAGGACAACTATTGTATGTGAGTGATATTTGTAA
TACTGAAGGTTATATAAATGTATCAATAAATGGCAAAAAAGGATTRTTACCA
AAAAGAATTTTATCTTCAGAAACATCTGAAATCCTTCAATATCCTTTACATGA
AGCATCAAGACGTGGAAATTTAAAATTTGTAAAAGAATGTTTAGATAATGGA
GTTTCTCCTAACAGTTTAGATAAAAGTGGTGCTACACCATTATTTTGGGCATG
TCATTCAGGCTTTGTTGATATCGTAAAATTACTTTTATCATTAAAAAATATTG
ACATAAATAATAAAAATAAAATGGGAGATACTGCGCTTCATGCA

ytP173:

TCGGTATTTTCCTTACTACCGTCATTTTTAAAAGTTTCAACAATTCTTGAAATC
TCCTTCAATGCTAATGATAAGTTGGACTTTTCTAAAAATCTTGCATGTTCATTT
ATCAAAGTTGGATCCAACAAATTTTCCCCAACTAACAATTCACTAAAATATTT
CTTTGTCATTTCCATTCTTTTACTCTTTATTTCATTTGTCTTTCCAGGACATCTA
ATATACATCATTATAAGAGCTTTCTTAGTATTTACATCACTTGGAGCCTCTTTC
AAAACTTTTTCAAAACATTCGATAGCCTTCTCAAATTCTTTATTTTTAACATAA
AGTTGMCCTAAACCAAATATTGGTTGTAAATATGAAGTACCACAACTATTAA
CTGCAATATTGTATTGTCTTAATGCCAAATCTGTTTCTCCC

ytP174:
TCCAGCCAGTAACATGGAAAGAGAATATATCACCTGAAGTACCAACAGTGTA
ACAAACACCGTAATTACTTCCATCAGGACAACAAAAACCTCCTGCTGGCCAA
AATAATTTAGGATCACTATTCAATTTTTTTGCATATTCAACCATTTGATGTTGT
GGTGTTTGACTTGTACTAAGAGCATAGTTCATCTTGAAAACTTTATCTAAAAA
TTCACTTTGAATGTTATAATACTTTGATACAATGTAYAATCCAAATAAATGAC
GATCAACTCCTTTTCCAGTCATTGCATCTCTATAATACTTTTGATGCCCTTCAC
ATGCTTTT

ytP175:
TGCATTGAATTGGTACAGTTTGAATTGAATCTAAAACAGAATAACGTAAGGA
GTCACCATAAGTGTAGTAAGTAACTATTTCTACAGGTATGTAAAATATAATA
ATTATTATGAATCCAGTTATAGCTGAAAATGTAAATTCACTTGGTCTTTTCAT
GTCATGTTGTACATTAGATAATGATGCTCCYCCACCATATGCATAACTTATTA
TTCCAATAGATAAAAATAAGTTTGTATAACTTAAATTTGGAATTGATCTTTCA
ACTGAACACTTAGAATGATCCATTTCCGAGCCAATTATTATAAATAATGAAA
CAAATGCAGTTGATAACATGGCAAATAATGCAATTCCCCAAAAATCA

ytP176:
CCGTGGTTCGGTAGCGTATTTTACATTAACACCATCTGGGATTAATCTTCCTT
CGTCGAAAAGTGGTTGGAAGTCTTCACGAGCAAATTTAGTGAATCCCCATTTC
TTTGAAACAGCAATATATTGTCTACCTGGGAACTTAAACTTGGCACGACGGA
AGGCCTCAATAGCATGATTGACATTACCTTCYTTGACACGGATTGAGAAAAG
AATATCATCAATTCCAACACGAGCTACAAGCCCTTGTGGTTTTCCGTAAGCTC
CTCTCATACCGGTTTGGAGACGATCAGCACCARCACAGGACAACATTTTATT
GATACGTACTACGTGATATGGATGTTTACGAACACGCATATGGAAACCATCT
TTACCACAGTTCTTGACCATATATTTGTTGRCGCAGATACGAGCAGCTTCAAG
AGCCTTAAAAAATGAATAATATTTTRAAGAAAAATTATTTAAAACATACTTCT
GATGAGAGATGTTCTCTTTCATTGGAAATCATGTGAACGCAATGTGGGAATTC
ATCATATTGAGCCTTCTTCTTTCCGAGATCGAAAATTCTGATTTTTGGATCTGG
GACACCACGACAGAAAACGGGATTTT

P. trichosuri markers corresponding to markers on *S. ratti* chromosome II:

ytP177:
ACCGCGGTTTTTATGAATTTTTTGTCCTGGTACAACaGTATTAATACTTTTAAA
TTAATATCAGGTGATCCACAATAATATAAATCACATAAATGTTTTGCATTACC
ATAAGCCTGTTCAATAATATTTTGTATATCACAAGATGGATCTATGCAACCAA
TATAATTTGGATGATCTGGTACCATATCTCCAAATATGGATAAATGTTGATAT
TGAAGCATTCGAATTGATATTCTATTAAGATAGAATCTATTCAAAAAATATTG
AACACCCCAATAATTTGCTGGATTGATACCATCTGCCTCCTCCATCTCAACTA
TTGCATCAGCCATCGTCTGAACGACTGTTGAGTGACGTTTAAGAATATTTTGT
AATGTATCATTAAAACTAAATGATAAAAATAATAATAAYTTTTTAAAAAATA
TAATTACCTCATCAAGGTAGAAGATTCCGCAG

ytP178:
TGTTTGCCTTWAAATCTGTTGAAGGACTATCTAAATCATTGAAAGTCTGTTGG
GAGGAcGGAGCTGCAGCACAATTTTCTTATTTATGGCTAAAAGATACCGCATT
AAGACCTGCATTAATTCATATTGACTTACACACTAAACCAGAGGATTTACAA
ATTAAGAAAGATCTATTAACTGTAACCTGGCCAAGGTATACTGAATCTTCCTT
TACAAGTCAATTCCTTCGTCGGCACATAACACATGGTGATGSAAAGAGTTATG
TAAATAAAAATATTATCAATATAATTGATCATACACCATCCGAGTCATCATAT
TGTCCTAAGATACTTAATGAAAAGGGCTGCCTTATGACTTCGTTAAGTTTAGC
ACCTAAAATTACTCCAATGGGAAGTTTAACATGGACA

ytP179:
CCAGAAATTTGGAACGAAAGCAACAGTCTTTGGATTGTATCCAATCTTCTTGA
TGAAGTTTTGAACTTCATTGGTAACTTCGGTGAAACGAGCTTCACTGTATGGT
GGTTCGGTAGAGTCCATCTTGTTACAGGCGACAATAAGTTGTTTAACTCCAAG
GGTTTGGGCTAAAAGAGCGTGTTCACGGGTTTGTCCATTCTTTGAGATACCAG
CTTCRAATTCTCCAGTACCACAAGCAACGACAAGAACAGCACAATCAGCTTG
GGATGTTCCGGTAATCATATTCTTGATGAAATCTCTGTGACCAGGAGCATCAA
TAATGGTGACATARTATTTAGCTGTTTCGAATTTCCAAAGAGCAATATCGATA
GTGATACCACGTTCTCTTTCAGCCTTAAGTTTATCCAAAACCCAAGCATATTT
GAAAGATCCCTTTCCCATTTCTTGAGCTTCTTTCTCGAATTTTTCGATTGTTCT
CTTGTCAATACCTCCACATTTTGTAGATTAAATGTCCCAGTGGTGGTAGATTT
TCCGGAG

ytP180:
TTTTCTTGCCCATTCCTTTGCCTCTGCACAATCAGGTCTTTCAATAACGAGAGT
AACTTTTCTATTTTCTTGAAGTTGAGCAATAAGTAATTTTCTACAAACATCCTT
ATCAGTAACAGGCATACCCTCAACATCACAAATATGRTCTCCAACTTTAAGTT
GATCATGTGCAATTGATCCTGGATCAGTTTTGGATACCAAAACACGATTTTGA
AAATGGCGAATTCCCAATCCAAGTTTTCTACCCATTTCAAGATTTAAAATTGC
TAATTGGTAAATATATCCTTCTCTTCTCATAATGTTACGTTCTCTTTCATGAGG
AATAGTGATTTTGGCTAACATATCCTGAGATTTCTTGTCATCCCTGATAACAG
TAATGTTAGCCATTGGTGGAGC

ytP181:
CCACACCGGAGCAATATGATACAATAGTTTTTAAACATTTATCATGACAAAC
AAAATTACAAACTAAAAAGAAATATTTGTTAAGAAAAAATAAATATAAAATT
TACCTTCACAAGCATATCCTTGCGATATCATACCCCATATTTTATCACAACAA
TGATGACAATACATAGGTTTACCAAAAGTTTTTTTAACAAAAAAATGYCCAT
GATCTTTTCCAATGGTAGAAGAGGAGAGGAGAGTTGCATCAACATTATGTCC
TTCAGCAGCCATAACTTTG

ytP182:
CCACCATGTGCAGAAACTTGAATAAGAACATTAGCTTCAGGTAAATCAAAGG
ATGTATCAGCAATTTTAGAGACAAATATTGTATTWACTTTAGGATTATTTTGA
AAATTTTTAAGAATTTTCATTCTTTCTTCCTGATTTGTCTCACCATATAAAAAT
GGTTTACCCATCTCAATAGCATATCTCTTAAGAGCATATACATTATCAGAAAA
TACAATAATTTTGTCATTTCTTCTTTCATGAAACTTTATTAAAAAATGACAAA
TYCTATATTTATGTGGATTCATAACAGCAAGAAGAAGTTTYTTTGCTTGAGAA
CTTGTTAAATAATATGCATAAAAATCAGGTTGCATTGGACACCATACTTCCGC
ACATTGAACTTTTGCAATATGTCCTGCTTTCTGAAGTTCCA

P. trichosuri markers corresponding to markers on *S. ratti* chromosome X:

ytP183:
TGGAAACGAGTGTCCAAGAATTGCTAAGCATTTTCTTGCATCTTCTTTTACTC
TAACATCGGTACCTGTGTCTCACTTGTTTTATTAAATAAGGAATAAGTGAYSG
ATTTGAAACTGCTGTCATTCTGCTTGATCTAAATTCTTGAATATGTTTTGTTAA
TGCATTGATTCTTAATAATTTAGAACTTGTTGATTGTGCTATTAACAATTTATC
AACATGCATTTTRGTTAAGTTTACTACTTCRGCACGTGATATACGTTTAATTA
GAATTTTAGATATTTTTGTTGGTGAAACATCACGTGCATGGCTTTTTGTCACTC
CATTTTTATTCAATGGCTGCTTTGTATTTACATTCTTTTCATTAGWGCYAACA
GCATTTTGATCCCARATCGATGTAAGGACACCAGATAAATAACCAAAATATG
TAGGTKGTACAATTTCTACATCTTTATTAGAAAATTCTGCTTTTGTAGATTCTT
TTAATGGTTCATTTGAATTAACWAMAGRATAAATTATTGCATATATTATTAA
ACTTACTTTGCGTTGATGTATTATCTGAAGATAAATCTTTTTTTGCATCTTCAT
TATCTYAAGTTCTATTTTTTCAGTTGTTAATATTTTCTCAGACGTGTCACTAAG
TCCAA

ytP184:
TGGCTGTACGAATCATGAACACATATTTCCTTCTTAATTTATAAGATAGATAT
ATATTAAACAACCAATTTGTTATACGTATACTGAATGTTGTGCTTAGTTGTYA
TAAAAAATGATTAAAGACATCAATGTATAGGGAAATAAGCTCCACCTTTTTG
CATACATTTGAATTCTTAATCGTTAGATAACGTTTTAAAAATAGTATAAATAG
GAATGAATTTCACCATTCTAAACCGTTAACTTTTCTTAATACAACATTCAATG
ATATGTAAGTCTTGCTAAAATTTTTGTATTTATTTAGAGATGCGGTTTGTTTCT
CTTATTGAACAGGATCTYTTATTAAGTACCTCTACAGGAGTTGCTGTAATCTA
ATTACCTTTAATCTTCACAACAATGAAAGCAGAAAGAGGTACATTATTTGTAT
ATATCGAAGACCAGAGTATGACATCAAAGAGTGGATGAAACTTCTATGTGCT

TTAACAACATCAAAAATGATGCAGAGTGTTAGGAGTTACATATAGATAGAAA
AAAGTCATGTTAGTTAGTGAAGGGCAAACTATTCACAAAACAAAAGACYGAT
GCTGACAATTTCAATAAAAAAAAAAACAATTTCAATTAATTCACATTTCCCAT
TGTGCAC

ytP185:
TGACGTGGTCAATTTGCTTTAACGGACAGGGCTGCGGAACCTGCTGTGCCTAT
CAGCTATAACAATGATTATAGAGAATGATATAAATTTAGTTCCAATGTGCAT
AATTGTTCATTTATTAAAAAAACCATTGAACGGTTAAATAATTCTATAGACAT
CATCCTGTATACATCAACATTACTTTATCATAAATTTTTATCAGTAATARAAA
TTTATTTTTATCAAACCATATTATGTAAATAAAATGATAAAATATACATTTTTT
AAGTATACAATTATTTACCAAAATAAAAAAAAACATAATATTTAATTAAAAT
TTAATTCTAAGTATTGTTTATTATATATTTAAAATTGAATGTTATTTTAAATGT
GCTATTGACTACGATAAATTATTAAAAAAAAATCATTTTTATCCTCGAAAGTA
TTATTAAACTTATATCTCTATATCAAATTTTAATAATTAAAACAAAAACAAAA
TTTATGCAAATATTATTTTTAAACACTTTTTCAATGAATATATTTTAAAATAAG
TTACATTAAATCAATTTCTAATTATATATATTTAAACATTGTAAGGTTTGCTTT
ATATTTCTAATTTATTTTTATTTTATATCACGAAAAGTTTATTGTTTATTTACG
CACACAATGCTTGA

ytP186:
ACGCGGTTCCTTGCTAGACCGATAAATCGGTTCTATCTTCTGTGTCTGAGTAT
ACTGCAATGGACACGGTAATTGAACCTTGAGAAAGGCACATTGTCTTAAAAT
ATATATRTATATTTAATTTTATGTAATTTATAAATTACCTAATAATTTCATTTT
CATCTGATACCAATTCAATATCTATTGTATTTGCAACAATCGCTTTATATATC
ATTTCAGACATATCAGGATTTTCAAAGAATAAACATGGATTTATATCTTCATT
AATAATTGTTTTTAAAAATTGACATTTCTCATCCATTAACATTCCTTGACTTCT
CCATTGTATAAAATCACGATGATAGGTCGGATCA

ytP187:
TTTGCAATTATTTGTTTCCATCATGTGAAATAGATAAAACTTCCTCAGCAGTT
GGTCTTAATTCACTTTCATTAATTTTATATGTCAAATTTTGAAATCTTTGAAAC
GTGTAATGTAAAAAWTCACCTTTATCTGGTGGTGCTAATGCTTGAATTTCASA
TTCTTCATGAACTTCTGGAAAAGCCATTTCTGCATCWGGGAAATCAKMARAA
TTTGAAGYATCWKCTATACCACGTACTTCAACACGGATTGG

ytP188:
CATTACGAGCAATTGCAAACAGAGCGCGATAAGATATCTTCAGACGACATAA
TTTGAGTGAACGTARTTTATATGCTGCAGCCCTATTACTGGTACCAAAAAATC
TTTGTAAGTGATAAGAATTTATATATTTRACATTTAATGCCAGATGTGTCATA
TTTGGAATATTATTGGTGATAAGTAATAAAACCTCATCCATTATTCTATTACA
TGAKATATGTAATTTTGTTATCTTCTTACCAAGGGCTCCAATAAAAATRCGTA
AAAATTCATTTGATTGYTCCACTGCTGCATTAGTCATTTCTTCRCAATTTAATT
GTAAATGAATATCAGAAAGGTATGGTAAATTTAATAATCTTGATACAAGATT
TGTTCCATTCTCTTCTCCTGAAACGGATAAACCA

S. vituli markers corresponding to diminished markers in *S. papillosus*:

ytP189:
TGGTTTGACCGTTGGTTTTTNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNCTGATGCTGCCAATCCAGCTCTTTTYGTCA
AAATTCTTATTATTGAAATTTTTGCTTCAGCTATCGGACTTTTTGGAATGATTA
TTGGAATYGTTCAGGTATATTTAACWTATAATTTTATTAATATTTAATTTTTTT
TTCGACAAACCAAAGTCCAAATGGGAAA

ytP190:
TTGGACAATTACTTGGACATTCANNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNATATGGTGTCTTTCCGTATGTAAATAA
TTCCCATAATGTCACGCCAAATGMCCAGACATCTGAGGCTGTTGTATATCTAC
CATGTTGGATTGTCTCTTTACTCATCCATCTTATTGGCATCCAATGACGATGTG
GTGTTTTATAATAATCTTTTTCATACCCTTTTCTTATCATTTGGAAAGCACAAA
TYTTTATTGTTCTTTGATCACTAACGTAAATGTTACGGGTAGCTAAATCTTGA
TGAACATATCCATTTGATGCTAAATAATCCATTCCTTGAGCTATTTGAA

ytP191:
CTGGAGAGGTAAAAGCTGCTGNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNTATCAAAATCAAAGGGAAGTTGGTGAAGCA
TTGAAGGAAGCTTTTGATAAGGAAATTGTTAAGGTAAATCTTTTTATAATATT
ATCTTTATAGTAATATTATTATTCACCCAGAGAGAGGATGTTTTTGTAACAAC
AAAAATATGGAATACCTGCCATTCTAAAAAGAAAGCAAAGGAATGTTTGACT
AGAAGTTTGACTGAACTTGGTCTCAACTATGTTGATCTTGTTCTTATTCATTGG
CCTCATGGATATCTAGAGGATACAAACGAGTTTTTCCCTTTTAATAAAGAAAA
GACCAAGATTTATTACTCCAATATTGATTAYATTGAAACATGGGAAGCTTTAC
AGGATTTGGTWGCTGAAAATATGGCAAAAAGTATTGGACTTTCTAACTTCAA
TATCCAACAGATTCAAAGAGTTTTGGAGGTTGCCAAGAT

ytP192:
CGTTCATTTTGTTGTGATGGANNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNTCRCCAGACTCAAGAGGATAGGTTACAA
TTTTGTTAAAAACAAAAAAAATATCTTGTTCGTTTGTTAAGAATGAAATTAAC
TCCCCAACAGCTGGTTCCAATTCTAATGAGGTAAAATAATCTGCTACAGCAG
CGCTAATACAATGATGTTGACTATCTTTTCTATARTATTCATAATCATCATTCC
CATCAGAGAAATCCTCTTCTGATGTCTCATTATCAAAGTCATCAGAACAATAT
GAATCATAYTCATCAGAGAAACTTTTACTCTTCTTTATAGAACATCCACTAAT
ACCACAAGCACGTGC

ytP193:
TGAAACAGAGGCTATTATGTTTTGANNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNATTGTAAATATTTTTTAGAATTAAATGATGTTG
ATAACAAATATTCACGTGATAACAAACAGCAAAAGTAAGTTKTTTTTTTACTT
RATAACAGTATAATCAAATTCATTTAAGTGATTTTCAAATTCAACATGAATAT

CCTGACAGTCATTCAATGCCACATAATTTATTTGGATCCCAAACTGGTGAGGT
AATAARACCCCCTTCTTCTAATGACAYTGGTATTTATAATGATTATGCTGATT
TTCATATCAGTGCGCATCATAAACAACCTTCTGTTTATCTTAGTARARAAGCT
GTCGTTTGAATGTATATTTTGAACATAGTTATATCATTCAAATTATTGCTTCCG
TTTTGTTTTCCCCTGCATGCCATCAAAATAATCTATAATCGTTTTTTTCTTTCA
TAGTTCCCTTTCATATGTAGTTGTGTCCTTCTACTAATTTTCTTGCATGAAGTC
ATTTTTATTGTATTTGGCACACAGAAATTGACAAATTTAATGTGAGATATATT
TTTAAAACATATGCAATGTATTTTTTACTTCAGGCTCTTTAATATGAAGCAGC
AGTACATGCTGCTTACAACGATTCTCGGATATCTTCA

S. vituli markers corresponding to non-diminished markers in *S. papillosus*:

ytP194:
TTCAACGTGTCTTGGTTTACCNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNATCGCATTCCTTTACCTCCACCACCAGATA
CTGCCTTAAGCATTACTGGAAAACCGATTTTTTTTGCTTCCTCAAATAGTTTAT
CTTTATCCTGACTTTGTCCATSATACCCTTCAATAACAGGAACACCGGCCTTT
CTCATAATGTCTTTTGCAACATTTTTCTGTCCCATATC

ytP196:
GAATAACCATGTTGTCTACACGNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NATGTCTTTTARTAATTTCTCAATATGAAAACGAACACCACGRATGATTTCTG
GTaCAACACCTGTGAACATTACATTCATTTTAGGAAATTGTTCAACTAAAGAA
CCAGCAAGTCTGTTGTCTCCACATCCTAATGTAACCTTGCTTTYCYTTTTTTGG
GAGATTACTTTCTAAAAAGGTAACCAAGTCTGGATGGC

ytP198:
CGCGTGTATTTAGAGGAACGANNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNACTACTAGGACTAAYGGCTCCAGGTAAAC
GTAAAAAATGGTCAGGCATATCTTTTAAAAGAAGAACCTTCAATTAAWAAAA
AAAARGWAATDTTATATMYCYTTTCWCTTAYTTTTTTTCWTATTTCARAAAT
ATCAATAAACCGTTGTTCTTGGAAAAATAATCCAGAGGATGATGAGGGTACA

ytP199:
TCAGCGTTTCCCTCTGTACGNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
ATGTTCTACCAATTTCTTCTCATATAAATAATCTTAAAGAAATTGAAGAGAAA
AGTAACAAGAAGGATAAAGAATTGGAAACRTTAAAAGAATCTATGGAAGAT
ACAAAACCAATTGGTGAATTGTTAAAGAAATGTAAAACTAAATGTCAGGCAA
AAGTTTTGTTACGACTTTTAGATGTTATTACAGAAAAATCCGCTAAAGCTACC
TGTTCAATTACAGCAGCACGTGGTAGAGGAAAGTCAGCTTCACTTGGACTTG
CGATTGCTGGTGCAATAGGTTTTGGGTATTCAAGTATATTTGTCACCTCACCT
AGTCCARA

ytP200:
ATCTCGGCATCCAGTGATTCNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNTTCCCCAAAGTTTGRACTTGAATAGAT
TGACTRTTAATTATTTCAATTTGAGATARTAAATTATCAAAAACTATTGATGT
CTTCTTACATGAATCCATCATTATAGAGTTGACTTTTCCCTTTATTTGAATAAC
ACTTCCTTCGCATTTAAAAATATAAACAGTTTGYTTCATATCATCAATATTAA
CAACAACATTTTTATCATTTTTAAAATATTCAACACACCAAGTTTTACCTTGCT
CCAATTCAAGACGTGGAGGTTTAAYAACTTCAACCTTTGGTGAACTTGATGA
CGTACTTGAYTTTTCTTTATTACCGCCACTAACAATAGATGTAGCGCGAAGAC
TAGGA

ytP201:
CATTATATCGGTGGGGAGGANNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNGGTTGTAAAAATGGAGTTCCATGT
CCTATAGTATCGGGAAAGAATCAAAATGTTACATTTACAATTGATTTCACAA
AATATAGTTTAATTGTTGGTTTACTAAAAAATGATGCACCATATCAATTAATG
TATAAATTAACAGATAGGGTTTCTGGAAAAACTAYCTGCACAATGATTCAAG
CACGA

Kulkarni et al. Supplementary Table 1:
Selected examples of crosses between free-living *P. trichosuri* females and males

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP184 (336)	*ytP53* / X		
		Female 1	CT
		Female 2	CT
		Male	T
		Female progeny	6 x CT
		Male progeny	2 x C

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP183 (227)	*ytP67* / X		
		Female 1	AG
		Female 2	A
		Male	G
		Female progeny	4 x G; 2 x AG
		Male progeny	3 x A

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP185 (208)	*ytP107*[1] / X		
		Female 1	A
		Female 2	A
		Male	G
		Female progeny	7 x AG
		Male progeny	4 x A

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP186 (112)	*ytP102* / X		
		Female 1	A
		Female 2	G
		Male	A
		Female progeny	12 x AG; 2x A
		Male progeny	5 x G; 1x A

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP187 (122)	*ytP104* / X		

		Female	A
		Male	T
		Female progeny	25 x AT
		Male progeny	6 x A

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP188 (281)	*ytP107*[1] / X		
		Female	C
		Male	T
		Female progeny	22 x CT
		Male progeny	7 x C

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP170 (231)	*ytP33*/ I		
		Female	A
		Male	G
		Female progeny	5 x AG
		Male progeny	5 x AG

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP171 (185)	*ytP68*/ I		
		Female	AG
		Male	G
		Female progeny	3 x AG
		Male progeny	3 x G

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP173 (330)	*ytP117*/ I		
		Female	A
		Male	C
		Female progeny	10 x AC; 2 x A[2]
		Male progeny	5 x AC

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP176 (190)	*ytP111*/ I		

		Female	C
		Male	CT
		Female progeny	8 x CT; 13 x C
		Male progeny	6 x CT; 9 x C

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP181 (205)	*ytP89*[1] / II		
		Female 1	C
		Female 2	T
		Male	CT
		Female progeny	1 x C; 7 x CT; 5 x T
		Male progeny	4 x CT; 2x T

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP182 (267)	*ytP89*[1] / II		
		Female 1	CT
		Female 2	C
		Male	T
		Female progeny	11 x CT
		Male progeny	5 x CT; 1 x T

Marker	Marker in *S. ra.* /chromosome	Worms	Genotype
ytP179 (331)	*ytP108* / II		
		Female 1	lost
		Female 2	AG
		Male	AG
		Female progeny	1 x A; 6 x AG; 2 x G
		Male progeny	3 x A; 1 x AG; 1x G

One or two females were crossed with one male. The parents and free-living progeny were genotyped. Listed is the result of the sequencing reaction at the polymorphic position of the marker indicated. If two bases are present the individual was heterozygous, if only one base is present the individual was homo- or hemizygous. The two crosses shown in Table 2 are also included in this table. [1]In some instances two new markers corresponding to the same *S. ratti* marker were made to test for consistancy. [2]These two animals are unexpected and are the only two progenies we found in our

crosses (shown and not shown) that appeared inconsistent with Mendelian inheritance of genetic markers in the free-living generation of *P. trichosuri*. Presumably these cases are due to an earlier mating of the female or to the erroneous transfer of eggs to the mating plates with the female.

Kulkarni et al. Supplementary Table 2:

Mother no.	Progeny genotype at *ytP183* (position 227)	Progeny genotype at *ytP185* (position 208)	Progeny genotype at *ytP179* (position 331)
1	5 x A	3 x A, 1 x AG	3 x AG
2	5 x A	5 x AG	1 x G
3	5 x A	4 x A	3 x A
4	7 x A	6 x A	2 x AG
5	6 x A	5 x A	4 x A
6	10 x A	8 x A	6 x AG
7	2 x A	1 x A	1 x A
8	5 x A	5 x A	4 x A, 1 x AG
9	3 x A	3 x A	2 x AG
10	9 x A	8 x A	8 x AG
11	2 x A, 1 x G, 2 x AG	3 x A, 1 x G, 1 x AG	5 x A
12	1 x A	1 x A	1 x AG
13	2 x A	1 x A	1 x A, 1 x AG
14	9 x A	8 x A	7 x A, 3 x AG
15	1 x A, 3 x G, 1 x AG	2 x A, 2 x G	2 X A, 3 xAG
16	1 x A	1 x A	1 x G, 2 x AG
17	1 x A, 1 x G, 4 x AG	2 x G, 3 x AG	7 x AG

Genotypes of the progeny of *P. trichosuri* parasitic females at *ytP183, ytP185, ytP179*
As "genotype" the sequencing results at the position indicated is given. Two letters indicate that the individual was heterozygous at this position, one letter indicates that the individual was homozygous or hemizygous at this position.

Chromosoma
DOI 10.1007/s00412-015-0532-y

ORIGINAL ARTICLE

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

Arpita Kulkarni[1] · Anja Holz[1] · Christian Rödelsperger[1] · Dorothee Harbecke[1] · Adrian Streit[1]

Received: 7 May 2015 / Revised: 2 July 2015 / Accepted: 6 July 2015

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).

Electronic supplementary material The online version of this article (doi:10.1007/s00412-015-0532-y) contains supplementary material, which is available to authorized users.

✉ Adrian Streit
adrian.streit@tuebingen.mpg.de

[1] Department Evolutionary Biology, Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany

Published online: 24 July 2015

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*

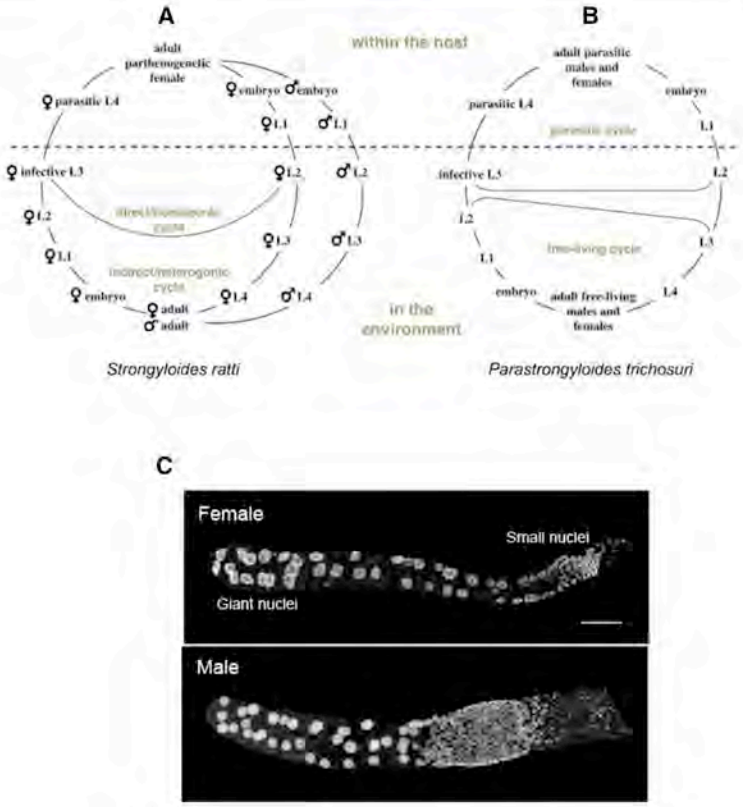


Fig. 1 Life cycle and introduction to the *Strongyloides* gonad **a** The generalized life cycle of *Strongyloides* species. **b** The life cycle of *Parastrongyloides trichosuri*. **c** Representative examples of dissected DAPI-stained gonads from *Strongyloides* spp. females (*top*) and males (*bottom*) 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-

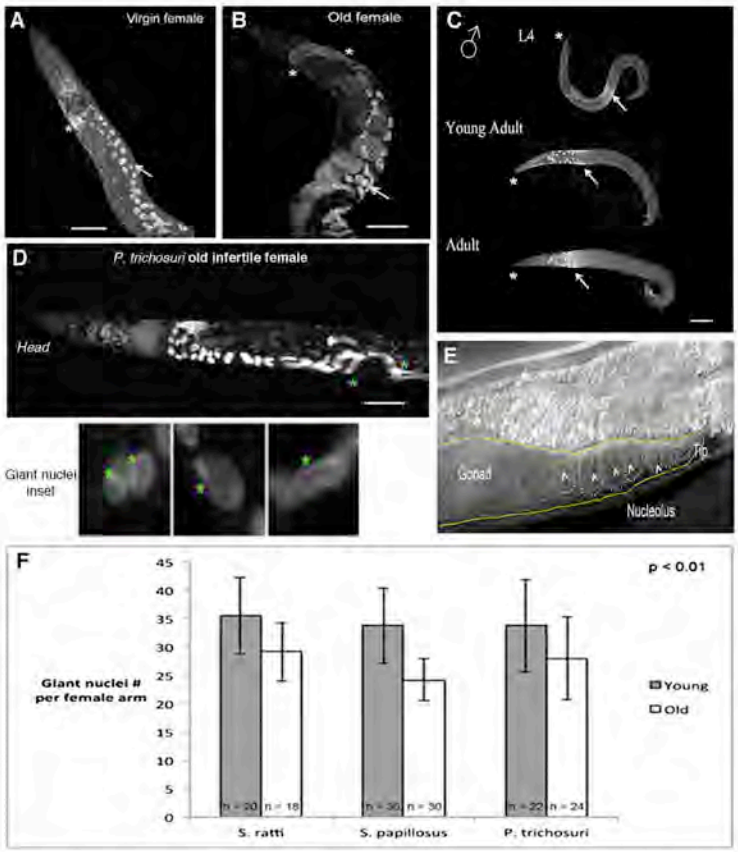


Fig. 2 Morphology and number of germline nuclei. DAPI-stained virgin (**a**) and old *S. papillosus* female (**b**), respectively. *Arrows* point to giant nuclei in the distal gonad arm. Note the change in size, number, and morphology of giant nuclei. The *asterisks* mark the position of the band of 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

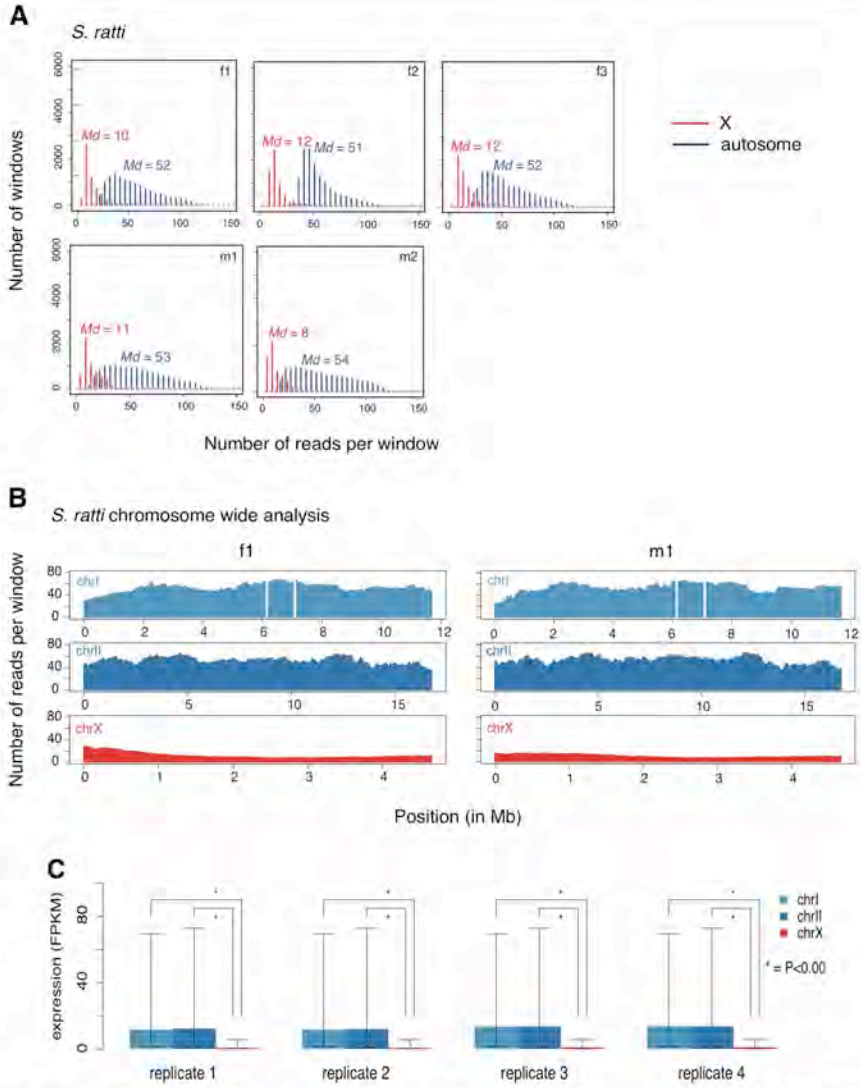


Fig. 3 DNA and RNA sequencing of *S. ratti* giant nuclei **a** Graphs indicate the genome-wide distribution of coverage (non-overlapping 2-kb windows) obtained from sequencing giant nuclei in *S. ratti* females and males, respectively (*f1*, *f2*, and *f3* indicate female biological replicates while *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.

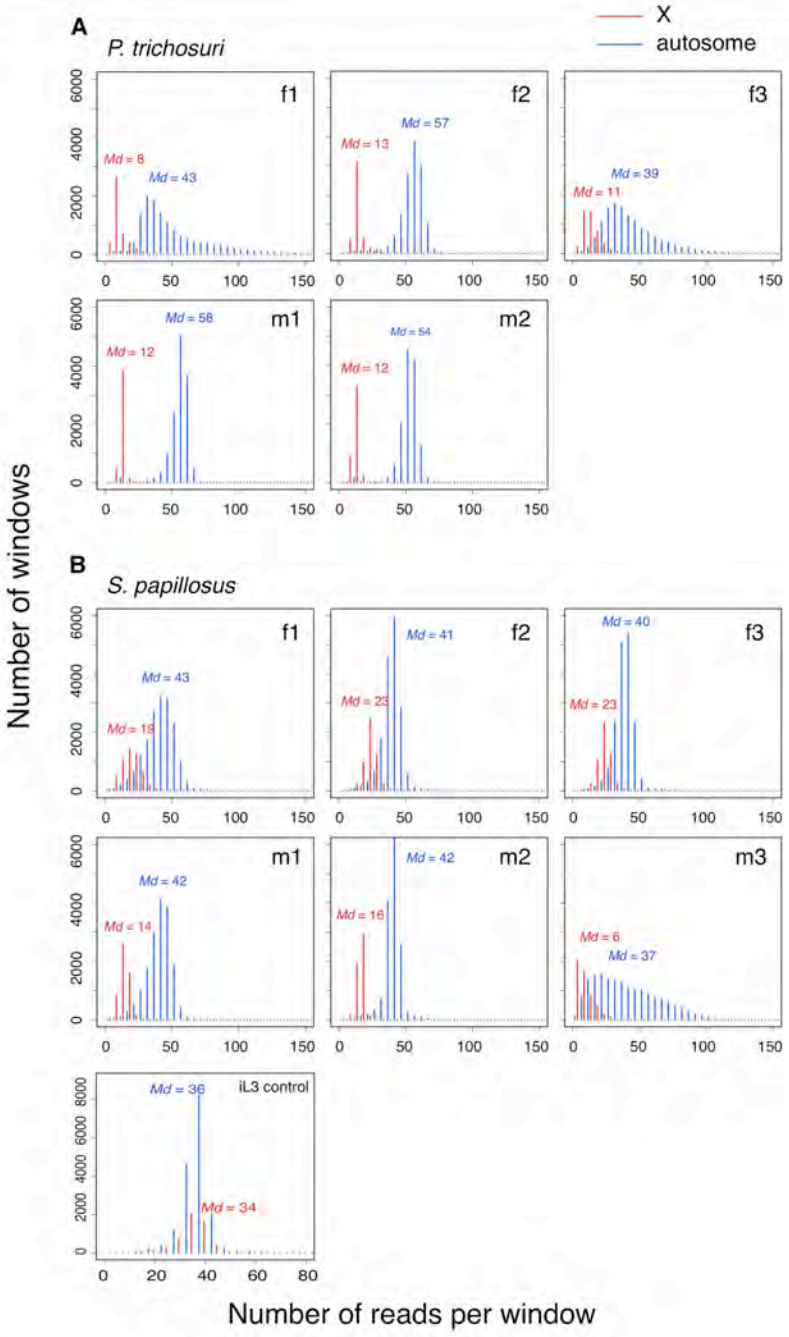
Presence or absence of male determining sperm

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

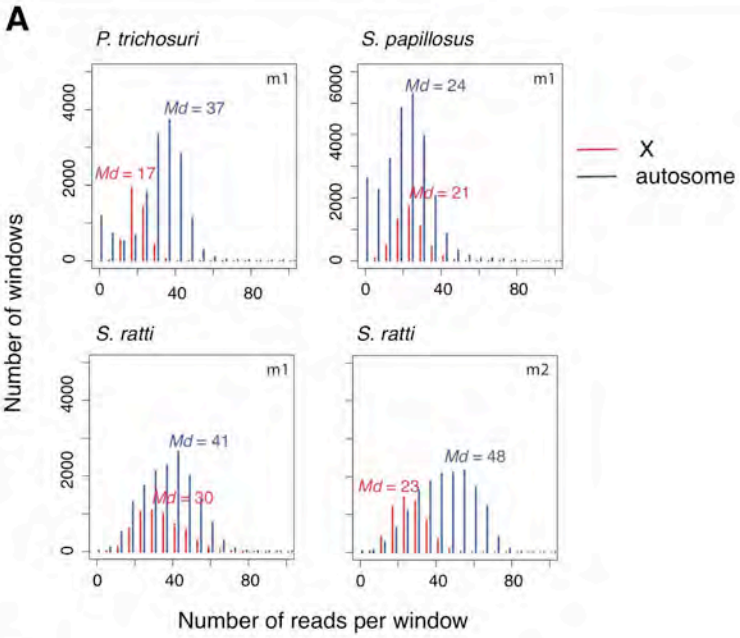

A
P. trichosuri
X
autosome
f1
f2
f3
m1
m2
Md = 8
Md = 43
Md = 13
Md = 57
Md = 39
Md = 11
Md = 12
Md = 58
Md = 12
Md = 54
B
S. papillosus
Md = 19
Md = 43
Md = 23
Md = 41
Md = 23
Md = 40
Md = 14
Md = 42
Md = 16
Md = 42
Md = 6
Md = 37
iL3 control
Md = 36
Md = 34
Number of windows
Number of reads per window

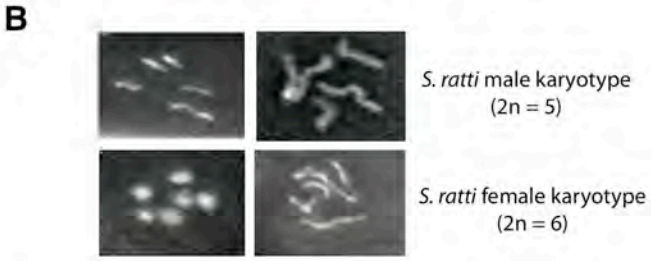
◀ Fig. 4 DNA sequencing from *P. trichosuri* and *S. papillosus* giant nuclei. **a**, **b** Graphs plotting the genome-wide distribution of coverage (non-overlapping 2-kb windows) obtained from giant nuclei sequencing in *P. trichosuri* and *S. papillosus*, respectively (*f1*, *f2*, and *f3* indicate 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

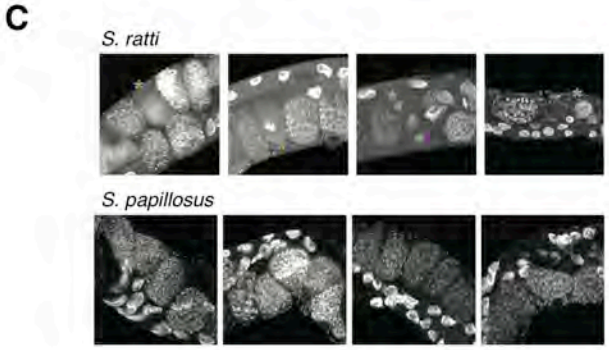
have previously been proposed to act as nurse cells supporting the development of oocytes (Hammond and Robinson 1994), a role that in *C. elegans* is assumed by early meiotic cells in pachytene (Hubbard and Greenstein 2005). In this regard, it is interesting to note that one of the best-characterized 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
0
2000
4000
6000
40
80


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*)

Acknowledgments We thank James Lightfoot for critically reading early versions of this manuscript and anonymous reviewers for the valuable suggestions.

Compliance with ethical standards

Funding This work was funded by the Max Planck Society.

Conflict of interest The authors declare that they have no competing interests.

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

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The Genomic Basis of Parasitism in the *Strongyloides* Clade of Nematodes

Vicky L. Hunt[a], Isheng J. Tsai[a], Avril Coghlan[a], Adam J. Reid[a], Nancy Holroyd, Bernardo J. Foth, Alan Tracey, James A. Cotton, Eleanor J. Stanley, Helen Beasley, Hayley Bennett, Karen Brooks, Bhavana Harsha, Rei Kajitani, Arpita Kulkarni, Dorothee Harbecke, Eiji Nagayasu, Sarah Nichol, Yoshitoshi Ogura, Michael A. Quail, Nadine Randle, Diogo M. Ribeiro, Alejandro Sanchez-Flores, Tetsuya Hayashi, Takehiko Itoh, Dee R. Denver, Warwick Grant, Jonathan D. Stoltzfus, James B. Lok, Haruhiko Murayama, Jonathan Wastling, Adrian Streit, Taisei Kikuchi[b], Mark Viney[b], Matthew Berriman[b]

[a] Equal contributors
[b] Corresponding authors

School of Biological Sciences, Life Sciences Building, University of Bristol, Bristol, BS8 1TQ, UK.
Vicky L. Hunt, Mark Viney

Biodiversity Research Center, Academia Sinica, Taipei 11529, Taiwan.
Isheng J. Tsai

Wellcome Trust Sanger Institute, Wellcome Genome Campus, Hinxton, CB10 1SA, UK.
Isheng J. Tsai, Avril Coghlan, Adam J. Reid, Nancy Holroyd, Bernardo J. Foth, Alan Tracey, James A. Cotton, Eleanor J. Stanley, Helen Beasley, Hayley Bennett, Karen Brooks, Bhavana Harsha, Sarah Nichol, Michael A. Quail, Diogo M. Ribeiro, Alejandro Sanchez-Flores and Matthew Berriman

Division of Parasitology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan.
Isheng J. Tsai, Eiji Nagayasu, Haruhiko Murayama, Taisei Kikuchi

Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Meguro-ku, Tokyo, Japan.
Rei Kajitani, Takehiko Itoh

Department of Infection Biology, Institute of Infection and Global Health and School of Veterinary Science, University of Liverpool, Liverpool, UK.
Nadine Randle, Jonathan Wastling

Max Planck Institute for Developmental Biology, Tübingen, Germany.
Arpita Kulkarni, Dorothee Harbecke, Adrian Streit

Department of Bacteriology, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan.

Department of Bacteriology, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan.

Yoshitoshi Ogura, Tetsuya Hayashi

Department of Zoology, Oregon State University, Corvallis, Oregon, USA.

Dee Denver

Department of Animal, Plant and Soil Sciences, La Trobe University, Melbourne, Victoria, Australia.

Warwick Grant

Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia 19104, PA, USA.

Jonathan D. Stoltzfus, James B. Lok

Key words

Helminth, nematode, genome, *Strongyloides*, *Parastrongyloides,* *Rhabditophanes,* parasitism, transcriptome, proteome, synteny, astacins, SCP/TAPS, gene clusters.

Author contributions

Cultivated and collected parasite material: V.L.H., D.D., W.G., J.B.L., E.N., H.M., A.S., A.K., J.D.S., D.H., T.K., M.V. Prepared DNA, RNA and protein: V.L.H., N.R., T.K., Prepared libraries: M.A.Q., H.B., E.N., Y.O., J.D.S. Assembled the genomes: I.J.T., A.S.F., R.K., T.I. Quality-checked the genomes: A.C. Provided genetic markers and mapping data: A.K., D.H., A.S. Manually improved the genomes: A.T., H.B., K.B., S.N., I.J.T. Predicted the genes: E.S., A.C., B.J.F., I.J.T. Functionally annotated the genome: A.C., D.R., B.H. Curated gene models: A.T., H.B., K.B., S.N., I.J.T., V.L.H. Built a

Compara database: B.H. Analyzed gene structure: I.J.T. Undertook proteomics and initial analysis: N.R., J.W., V.L.H. Analyzed the transcriptome: V.L.H., I.J.T., B.J.F., A.J.R., J.D.S. Analyzed the gene clusters: D.M.R., V.L.H. Analyzed synteny and chromosome alignments: I.J.T., A.J.R. Assembled and analyzed mitochondrial genomes: T.K., I.J.T. Chromatin diminution analysis: B.J.F., I.J.T., A.C. Analyzed gene family clustering: A.J.R., J.A.C., I.J.T. Coordinated the project, managed sequencing, assembly and finishing: N.H., T.H., T.K. Wrote the manuscript: V.L.H., I.J.T., A.C., A.J.R., N.H., T.K., M.V., M.B. Conceived and directed the project: M.V., M.B.

Abstract

Soil transmitted nematodes, including *Strongyloides*, cause the most prevalent of the Neglected Tropical Diseases. Here we compare the genomes of four *Strongyloides* spp., including the human pathogen *S. stercoralis*, and their close relatives that are facultatively parasitic (*Parastrongyloides trichosuri*) and free-living (*Rhabditophanes* sp). A significant paralogous expansion of key gene families – astacin-like metallopeptidase and SCP/TAPS coding gene families – is associated with the evolution of parasitism in this clade. Exploiting the unique *Strongyloides* life cycle we compare the transcriptome of its free-living and parasitic stages, finding that these same genes are upregulated in the parasitic stages, underscoring their role in nematode parasitism.

Introduction

More than a billion people are infected with intestinal nematodes[1,2]. The World Health Organization (WHO) has classified infections with soil transmitted nematodes[3] as one of the 17 most neglected tropical diseases and estimates that worldwide they cause an annual disease burden of 5 million Years Lost due to Disability (YLD), greater than that for malaria (4 million YLD) and HIV/AIDS (4.5 million)[4]. Parasitic nematode infections can impair physical and educational development[1].

Strongyloides spp. are soil-transmitted gastrointestinal parasitic nematodes infecting a wide range of vertebrates[5]. Two species – *S. stercoralis* and *S. fuelleborni* – infect some 100-200 million people worldwide[6,7]. Other *Strongyloides* species infect livestock, such as *S. papillosus* infection in sheep.

Strongyloides spp. are from a clade of nematodes[8,9] that include taxa with diverse lifestyles including free-living (*Rhabditophanes*), parasitism of invertebrates, facultative parasitism of vertebrates (*Parastrongyloides*) and obligate parasitism of vertebrates (*Strongyloides*)[8,9]. Nematodes have independently evolved parasitism of animals several times[10], and thus understanding the genomic adaptations to parasitism in one clade will help in understanding how parasitism has evolved across the phylum more widely.

The *Strongyloides* life cycle alternates between free-living and parasitic generations. The female only, parthenogenetic[11] parasitic stage lives in the small intestine of its host where it produces offspring that develop outside of the host either directly to infective third-stage larvae (iL3s) or via a dioecious, sexually reproducing, adult generation[12], whose progeny are also iL3s. The iL3s penetrate the skin of a host and migrate to its gut[13] where they develop into parasitic adults (**Fig. 1**). Therefore, this life cycle has two genetically identical adult female stages – one obligate and parasitic and one facultative and free-living; we have compared these transcriptomically and proteomically

to reveal the genes and gene products specifically present in the parasitic stage. The closely related genus *Parastrongyloides*[5,14] is similar to *Strongyloides* spp., except that its parasitic generation is dioecious and sexually reproducing, and that it can have apparently unlimited cycles of its free-living adult generation[5,15]**(Fig. 1)**.

Here we report the genome sequences for six nematodes from one superfamily: four species of *Strongyloides* – *S. stercoralis* (a parasite of humans and dogs)*, S. ratti* and *S. venezuelensis* (both parasites of rats, and important laboratory models of nematode infection) and *S. papillosus* (a parasite of sheep); *Parastrongyloides trichosuri* (which infects the brushtail possum *Trichosurus vulpecula*), and the free-living nematode *Rhabditophanes* sp.[8].

To investigate the genomic and molecular basis of parasitism in these nematodes we compared (i) the genomes and gene families of these parasitic (*Strongyloides* and *Parastrongyloides*, the Strongyloididae) and free-living (*Rhabditophanes*) taxa **(Fig. 1)**; (ii) the transcriptomes of parasitic adult females, free-living adult females and iL3s of *S. ratti* and *S. stercoralis*, and (iii) the proteomes of parasitic and free-living females of *S. ratti.* We have identified the genes present in the parasitic species, and the genes and gene products uniquely upregulated in the parasitic stages of *S. stercoralis* and *S. ratti* that, together, are the major genomic and molecular adaptations to the parasitic lifestyle of these nematodes.

Results

Chromosome biology

We have produced a high-quality 43 Mb reference genome assembly for *S. ratti* (**Supplementary Note**), with its two autosomes[16] assembled into single scaffolds and the X chromosome[16] into ten (**Table 1; Fig. 2**). This assembly is

the second most contiguous assembled nematode genome after the *Caenorhabditis elegans* reference genome[17]. We also produced high quality draft assemblies of the 42-60 Mb genomes of *S. stercoralis, S. venezuelensis, S. papillosus, P. trichosuri* and *Rhabditophanes* sp., which are 95.6 – 99.6% complete (**Supplementary Table 1**). With GC contents of 21% and 22% respectively, the *S. ratti* and *S. stercoralis* genomes are the most AT-rich reported to date for nematodes (**Supplementary Table 1**). The ~43 Mb *S. ratti* and *S. stercoralis* genomes are small compared with other nematodes. However, the total protein-coding content of each nematode genome is similar (18-22 Mb versus 14-30 Mb in eight outgroup species; **Supplementary Table 1**). Significant loss of introns as well as shorter intergenic regions account for the smaller genomes from the present study (Spearman's correlation between genome size and intron number $\rho=0.91$, $P < 0.001$ and size of intergenic regions $\rho=0.63$, $P = 0.02$; **Supplementary Table 2**). However, parsimony analysis of intronic positions conserved in two or more species, revealed that substantial intron losses occurred prior to the evolution of the *Rhabditophanes-Parastrongyloides-Strongyloides* clade (**Supplementary Fig. 1**), and are therefore not an adaptation associated with parasitism.

The canonical view of a nematode chromosome, defined nearly twenty years ago using *C. elegans* autosomes (and later confirmed in *C. briggsae*[18]), is of a gene-dense, repeat-poor "center" of conserved genes (based on homology with yeast genes[17]), flanked by two gene-poor, repeat-rich "arms" in which most genes are less strongly conserved. *S. ratti* is the first non-*Caenorhabditis* nematode to have whole chromosomes assembled and presents a strikingly different organisation with relatively little variability in gene density, repeat density or gene conservation to yeast genes along its autosomes (**Supplementary Fig. 2, 3**).

Synteny is highly conserved within the parasitic Strongyloididae, but much less between this family and *Rhabditophanes* (**Fig. 2**). Scaffolds of the parasitic species largely correspond to blocks from a particular *S. ratti*

chromosome, but in a scrambled order. This suggests that intra-chromosomal rearrangement is frequent, but inter-chromosomal rearrangement is rare, a common phenomenon in nematode chromosome evolution[18–20]. The notable exception was for *S. papillosus* and *S. venezuelensis* scaffolds that have many blocks that are syntenic to both *S. ratti* chromosome I and X (**Supplementary Table 3**). This likely reflects the fusion event between chromosomes I and X in these species[21–23]. Associated with this fusion is a change in the chromosome biology of sex determination in these species. *S. papillosus* undergoes chromatin diminution to mimic the XX/XO sex-determining system of *S. ratti*[24] and *S. stercoralis*[21].

By analyzing the differential coverage of mapped sequence data from iL3s (which are all female) and adult males, we were able to identify regions of the *S. papillosus* X-I fusion chromosome that are eliminated from males during diminution (**Supplementary Table 4**). Six scaffolds were identified from the diminished region using existing genetic markers (**Supplementary Table 5**), but our read-depth approach extended this map to 153 scaffolds (18% of the assembly, 10.9 Mb). Interestingly, some genes with orthologs on the X chromosome of *S. ratti* are not diminished in *S. papillosus*, so dosage of these genes in males has changed since the species diverged including three genes on *S. papillosus* chromosome II (confirming earlier work[21]), and 33 that lie in non-diminished regions of the X-I fusion chromosome (**Supplementary Table 6**).

Extensive rearrangement of the mitochondrial gene order

The *S. stercoralis* mitochondrial (mt) genome is highly rearranged compared with nematodes from clades I, III and V[25]. Manual finishing of the mt genomes of the six species revealed that the *Rhabditophanes* mt genome consists of two circular chromosomes, a feature of some other nematode species[26]. Compared with eight outgroup species, *Rhabditophanes* has a conventional gene order but *Strongyloides* spp. and *P. trichosuri* have highly rearranged mt

genomes (**Fig. 2**, **Supplementary Table 7**). Similar observations have been reported in other clade IV parasitic nematodes[26–29] and there is evidence of mt recombination[28,30], which is rarely observed in animals[31]. Consistent with published nematode mt genomes, the gene-based phylogeny of the mt genome (**Fig. 2**) conflicts with phylogenies based on nuclear genes[28,32,33].

Gene families associated with the evolution of parasitism

We predicted the six genomes to each contain 12,451–18,457 genes, numbers comparable to other nematode species (**Table 1, Supplementary Fig. 4**). We then used Ensembl Compara (**Supplementary Note**)[34] to identify orthologs and gene families (**Supplementary Table 8**) in these and eight outgroup species, encompassing four further nematode clades (**Supplementary Fig. 4**). By pinpointing when a new gene family arose, and where a family has expanded or contracted, we could determine which gene families are associated with the evolution of parasitism. The largest acquisition of gene families (1075 families) was found on the branch leading to the parasitic nematodes, *Strongyloides* spp. and *P. trichosuri* (**Fig. 1, Supplementary Fig. 4**). The branches leading to these five parasitic species also showed greater expansion of existing gene families, compared to that in the free-living *Rhabditophanes*. Gain and expansion of gene families in these parasitic species likely reflects the necessary adaptations required by these species to be able to parasitize vertebrate hosts while maintaining a free-living phase.

The most expanded *Strongyloides* spp. gene families encode astacin-like metallopeptidases and SCP/TAPS (SCP/Tpx-l/Ag5/PR-1/Sc7[33-35], also known as CAP-domain) proteins, present in multiple subfamilies (based on Ensembl Compara analysis, **Supplementary Table 8,** and protein domain combinations, **Supplementary Table 9**). The astacin family was the most expanded with 184-387 copies in *Strongyloides*/*Parastrongyloides*, compared with *Rhabditophanes* and with eight outgroup species, showing that this

212 expansion accompanies the evolution of parasitism (**Fig. 1; Supplementary**
213 **Table 10**). Among the outgroup species the hookworm *Necator americanus*
214 has 82 astacin coding genes, and the free-living *C. elegans* 40[35].

215

216 SCP/TAPS proteins are often immunomodulatory molecules in parasitic
217 nematodes[36] and have been investigated as potential vaccine candidates
218 against *N. americanus*[37,38]. We found 89-206 SCP/TAPS coding genes in the
219 *Strongyloides* spp. genomes, comprising a subfamily not present in *P.*
220 *trichosuri*, *Rhabditophanes* or the eight outgroup species (**Fig. 1;**
221 **Supplementary Tables 8 - 10**). In *N. americanus* there are 137 SCP/TAPS
222 coding genes[39], suggesting that this gene family has independently expanded
223 twice: in nematode clades IVa and V.

224

225 Receptor-type protein tyrosine phosphatases have a putative role in
226 signaling[40] and are expanded in *Strongyloides* and *Parastrongyloides* (52-75
227 genes), compared with *Rhabditophanes* (13), and the eight outgroup species
228 (up to 39 genes). Acetylcholinesterase coding genes were expanded in
229 *Strongyloides* and *Parastrongyloides* (30-126 genes), compared to
230 *Rhabditophanes* (1) and 1-5 genes in our outgroup species. Some families
231 show sub-clade specific expansion; for instance, *S. papillosus / S.*
232 *venezuelensis* have a paralogous expansion of genes encoding Speckle-type
233 POZ domains (92-130 genes), compared with *S. ratti / S. stercoralis* (9-10
234 genes) (**Fig. 1; Supplementary Table 8**).

235

236 No function or annotation could be assigned to approximately one third (26-
237 37%) of the genes present in the six species, but 50% of these could be
238 assigned to novel gene families. The six largest of these families occurred
239 only in *Strongyloides* and *Parastrongyloides*, comprising a total of 630 genes.
240 We have named these *Strongyloides genome project families (sgpf) 1-6*
241 (**Supplementary Table 11**). Most of the protein sequences of *Sgpf-1, -5 and -*
242 *6* are predicted to have signal peptides and transmembrane helices

suggesting they encode cell surface proteins. *Sgpf-3* and *-4* predicted protein families include leucine-rich repeat domains, which are often involved in protein-protein interactions.

Gene families expanded in parasitic species are upregulated in parasitic life stages

We identified genes and gene families that are likely to play a key role in the parasitic lifestyle of *S. ratti* and *S. stercoralis*, by comparing the transcriptomes of parasitic and free-living female stages. We generated *S. ratti* transcriptome data and used previously published *S. stercoralis* data[41]. A total of 909 *S. ratti* and 1,188 *S. stercoralis* genes were upregulated in parasitic females compared with free-living females (edgeR, fold change>2, FDR<0.01; **Supplementary Tables 12, 13**), of which 204 were one-to-one orthologs**.** This small overlap between closely related species suggests rapid evolution at the transcriptional level, perhaps reflecting adaptation to different host species.

The two most expanded *Strongyloides* gene families – astacin-like metallopeptidases[42–45] and SCP/TAPS[36] – dominated the list of genes differentially expressed in the parasitic female. In *S. ratti* and *S. stercoralis,* respectively, 58 and 62% of astacins and 57 and 71% SCP/TAPS genes were differentially expressed in parasitic vs. free-living females (**Fig. 3; Supplementary Tables 10, 13**). However, other paralogously expanded genes were not enriched among the upregulated genes suggesting they may not be important for parasitism. Both *Strongyloides* and *Parastrongyloides* infect their hosts by skin penetration; the larvae then migrate through the host, and adult females in the host live in the mucosa of the small intestine[46,47] where they feed on the host. Astacins have previously been associated with a role in tissue migration by nematode infective larvae[43,48]. The astacins of the *Strongyloides* parasitic females likely have a role in breaking down the host mucosa in which they live. Teasing apart the role of different astacin gene family members in the migration and gut-dwelling phases of this life cycle

could provide insights to allow new therapeutic interventions to be developed. For *S. ratti* and *S. stercoralis* respectively, 63 and 53 % of the SCP/TAPS genes upregulated in the parasitic female encode a signal peptide suggesting that they may be secreted from the worm into the host. An immunomodulatory role for SCP/TAPS proteins has also been suggested based on the inhibitory effect that these proteins have on neutrophil and platelet activity in hookworm infections[36,49,50].

Other gene families commonly upregulated in the parasitic females of both species, compared with free-living females and iL3s, included those coding for transthyretin-like proteins, prolyl endopeptidases, acetylcholinesterases, trypsin-inhibitors, and aspartic peptidases (**Fig. 3, Supplementary Table 14**). The transthyretin-like genes had some of the highest fold changes of genes upregulated in the parasitic females (**Supplementary Table 13**). Transthyretin-like genes are a large, nematode-specific gene family[51], expressed in adult parasitic stages[52–54], and are distant relatives of vertebrate transthyretins that are involved in transporting thyroid hormones[55]. While some aspartic peptidases are essential for the digestion of host hemoglobin in blood-borne parasites[56,57], it has been proposed that others are involved in digesting other host macromolecules[58].

Hypothetical protein-coding genes accounted for a large proportion (20-37%) of the differentially expressed genes from pairwise comparisons of parasitic females, free-living females and iL3s, and included genes with the highest relative expression levels (**Supplementary Table 13**). These novel genes are likely to be important to these distinctive phases of the life cycle, including in parasitism. Three small novel gene families (*sgpf-7-9*) were predominantly upregulated in *S. ratti* parasitic females, two of which are predominantly secretory or membrane-targeted (**Supplementary Table 11)**. In contrast, the largest hypothetical protein-coding gene families, *sgpf-1-6,* accounted for only a small proportion (1% in both *S. ratti* and *S. stercoralis*) of all differentially

expressed hypothetical protein-coding genes suggesting they do not have roles involved in parasitism.

Using gene ontology annotations to summarize the putative functions of upregulated genes revealed distinct differences between the life cycle stages of both species (**Fig. 3, Supplementary Table 15**). The genes upregulated in iL3s appear to be associated with sensing the environment, and with signal transduction, and were the most consistent between *S. ratti* and *S. stercoralis.* The products of free-living female expressed genes have core metabolic and growth-related roles (such as in cytoskeleton and chromatin). In parasitic stages, the dominant functional categories were proteases, consistent with the abundant astacins (**Fig. 3, Supplementary Table 15**).

Much of the parasitic proteome may be secreted

We compared the proteome of the *S. ratti* parasitic and free-living females in parallel to the transcriptome analysis. A total of 569 proteins were upregulated in parasitic females, compared with free-living females (and 409 were upregulated in free-living females) among the 1,266 proteins that we detected in total (**Supplementary Tables 12, 16**).

In parasitic females just 9% of proteins upregulated in the parasitic female proteome overlapped with the transcriptome, compared with 37% for free-living females (**Supplementary Fig. 5**). This may reflect a more dynamic transcriptome in parasitic females with greater turnover of the transcriptome and/or proteome; genes transcribed in one life cycle stage but translated in another have been previously observed in *S. ratti*[59]. However, the small overlap likely reflects, at least in part, an increased proportion of secreted proteins in the parasitic female compared with free-living stages, which would not appear in our whole-worm proteomic data. Indeed there is a greater discrepancy in the number of protein-coding genes identified in the

transcriptome with signal peptide domains versus those found in the proteome of parasitic females (43% of transcriptome, 21% of proteome; χ^2 = 38.119, df = 1, *P* = < 0.0001) than free-living females (26% of transcriptome, 22% of proteome, χ^2 = 2.2018, df = 1, *P* = 0.1379). There was, however, a significant positive relationship between the upregulation of genes and proteins in the same life cycle stage (**Supplementary Fig. 5**).

In the parasitic females, the most commonly upregulated protein families were aspartic peptidases, prolyl endopeptidases and acetylcholinesterases (**Supplementary Table 16**). Parasitic females expressed proteins mainly with electron carrier and isomerase activity involved in catabolic, metabolic and homeostatic processes (**Fig. 3; Supplementary Table 16**). Only five astacin-like metallopeptidases and no SCP/TAPS were upregulated in the parasitic proteome, but these dominated the parasitic transcriptome (**Supplementary Fig. 5**). Astacin-like metallopeptidases have been reported in the secretome of *S. ratti* parasitic stages[60], but it remains to be seen to what extent the large genomic and transcriptomic presence of astacins and SCP/TAPS is reflected in the excretory/secretory proteome.

Parasitism-associated genes are found in co-expressed clusters

We observed that genes upregulated in the parasitic females and iL3s were often physically clustered in the genome, more so than compared to genes upregulated in the free-living female (see **Supplementary Table 17**). To test whether this clustering was significant we asked whether clusters of three or more adjacent genes, upregulated in the same life cycle stage, occurred more often than would be expected by chance. We found that 31%, 4% and 26% of upregulated genes were in such clusters in *S. ratti* parasitic females, free-living females and iL3s, respectively, while in *S. stercoralis* this was 34%, 2% and 34% (**Supplementary Table 17**)). This clustering is more than would be expected by chance (**Supplementary Fig. 6; Supplementary Table 17**). The parasitic female clusters were larger (19 and 16 genes in the largest *S. ratti*

366 and *S. stercoralis* clusters, respectively) compared with those of the iL3s (9
367 and 14 genes) and free-living female stages (3 genes) (**Supplementary**
368 **Table 17**). Although nematodes, including *S. ratti*[61], have operons these
369 clusters are unlikely to be operons because (i) the average intergenic distance
370 among clustered genes does not differ from the genome-wide average
371 (**Supplementary Fig. 6**) and (ii) cluster members include genes on both
372 strands.

373

374 Many of the parasitic female and iL3 clusters comprised genes from multiple
375 gene families. However, clusters of genes upregulated in the parasitic female
376 were more likely to comprise genes from the same gene family. The majority
377 (88-73 % for *S. ratti* and *S. stercoralis*, respectively) of these parasitic female
378 clusters were of genes belonging to the same Compara gene family; this is
379 greater than for iL3s (8-10%) (**Supplementary Table 17-19**). Two gene
380 families dominated parasitic female clusters: astacins (24 and 23% of
381 parasitic female clusters for *S. ratti* and *S. stercoralis*) and SCP/TAPS (15 and
382 11%). Tandem expansions of astacin and SCP/TAPS genes could provide a
383 plausible explanation for the preponderance of these gene families in the
384 parasitic female expression clusters. However, even with the exclusion of the
385 astacin and SCP/TAPS families, most remaining parasitic female clusters still
386 comprised genes from the same gene family (85 and 65% for *S. ratti* and *S.*
387 *stercoralis*, respectively); fewer clusters from the same gene family occurred
388 for iL3s (7 and 9%) compared to parasitic females (**Supplementary Table**
389 **18**).

390

391 Phylogenetic analysis of astacins, including the eight outgroup species,
392 showed that 139 *S. ratti* genes form one distinct clade (**Fig. 4**), presumably
393 derived from a single ancestral astacin gene. Similarly the *S. ratti* SCP/TAPS
394 gene family has almost exclusively expanded from one ancestral gene (**Fig.**
395 **4**). These gene clusters likely arose by tandem duplication of genes, as has
396 occurred for other large gene families, for example in *C. elegans*[17]. However,
397 in contrast to *C. elegans*, physical adjacency of the duplicated genes has

been maintained in *Strongyloides,* perhaps due to the expansions being recent and therefore not having yet been broken-up by recombination. Alternatively the adjacency may be functional, for example there being pressure to maintain a common regulatory environment. Clustering of gene families was relatively rare among *Rhabditophanes* and eight outgroup species (**Supplementary Table 18)**, meaning that this clustering is specific to the *Strongyloides*/*Parastrongyloides* lineage and thus to the parasitic lifestyle in this clade.

The clusters of genes upregulated in the parasitic females were themselves chromosomally clustered forming 'parasitism regions' (**Fig. 4**). In *S. ratti* a third of genes upregulated in the parasitic female are concentrated in three regions of chromosome II, most notably a 3.6 Mb region at one end of chromosome II, comprising 171 genes that were upregulated in the parasitic female transcriptome (**Supplementary Fig. 2**). A similar pattern is evident in *S. stercoralis* where seven scaffolds and contigs with a high density of genes upregulated in the parasitic female also belong to chromosome II; 46% of the 171 *S. ratti* genes belong to just eight different gene families including those coding for aspartic peptidases, astacin-like metallopeptidases, SCP/TAPS, transthyretin-like and trypsin inhibitor-like proteins. This is the first report of chromosomal clustering of genes important to nematode parasitism and hints at possible regulatory mechanisms for parasite development.

Discussion

Understanding the molecular and genetic differences between parasitic and free-living organisms is of fundamental biological interest, but also essential to identify novel drug targets, and other methods to control nematode parasitism and the diseases that it causes. Comparative genomics of six taxa from an evolutionary clade that transitions from a free-living to parasitic lifestyle, combined with transcriptomic and proteomic analysis of parasitic and free-living female stages of *Strongyloides* spp. is a powerful way to discover the

molecular adaptations to parasitism. We find that a preponderance of genes expanded in parasitic species are specifically used in the parasitic stages and are within genomic clusters, concentrated in regions of chromosome II. Astacin-like metallopeptidase and SCP/TAPS coding genes are prominent amongst parasitism-associated genes. We have found genomic clustering of these and other parasitism-associated genes, which is likely to have been initiated during the adaptation to parasitism, followed by subsequent repeated gene duplication, associated with adaptation to different hosts. This genomic arrangement may facilitate expression of a parasitic transcriptional program by these parasites. In addition to providing a compelling model of the evolution of parasitism, transgenesis of *Strongyloides* and *Parastrongyloides* is possible[62–67], uniquely among parasitic nematodes, which will allow functional genomic studies, directed by our findings, to further explore the genetic basis of nematode parasitism.

Acknowledgements

We thank from the WTSI: Coline Griffiths, David Willey, Richard Rance and DNA Pipelines; Jacqueline Keane and Daria Gordon for bioinformatics support; Matthew Dunn for the *S. venezuelensis* optical map; Anne Babbage for laboratory support; Magdalena Zarowiecki for gene finding and functional annotation advice. We thank for technical help Louise Hughes and Laura Weldon (University of Bristol); Holman Massey, Jr., Xinshe Li and Hongguang Shao (University of Pennsylvania); Dana. K. Howe and Riana I. Wernick (Oregon State University); Hubert Denise (European Bioinformatics Institute); Mitsuru Yabana (Tokyo Institute of Technology); Akina Hino and Ryusei Tanaka (University of Miyazaki) and Atsushi Toyoda (National Institute of Genetics) for sequencing; Dong Xia (University of Liverpool) for proteomics support and technical help.

Funding

The *S. ratti* transcriptome and proteome work was funded by Wellcome Trust grant 094462/Z/10/Z awarded to M.V., J.W. and M.B. The *S. ratti, S. stercoralis, S. papillosus, P. trichosuri* and *Rhabditophanes* sp. genome sequencing and the *S. venezuelensis* optical mapping was funded by Wellcome Trust grant 098051. The *S. venezuelensis* work was supported by JSPS KAKENHI (Nos. 24310142, 21590466 and 24780044), KAKENHI for Innovative Areas “Genome Science” (No. 221S0002) and the Integrated Research Project for Human and Veterinary Medicine of the University of Miyazaki. I.J.T. was supported by JSPS Postdoctoral Fellowship Program for Foreign Researchers. Work was funded by grants AI050668 and AI105856 from the US National Institutes of Health (NIH) to J.B.L., and by Resource-related Research Grant RR02512 from N.I.H. to Dr. Mark Haskins, which provided research materials for the study. J.D.S. received support from NIH training grant AI060516. AK was supported by a pre-doctoral stipend from the

Max Planck society. Work by AK, DH and AS was funded by the Max Planck Society.

Online Methods

Parasite material, sequencing and assembly

Larval material of *S. ratti*, *S. stercoralis*, *S. venezuelensis* and *S. papillosus* was obtained from fecal cultures of infected laboratory animals; for *Parastrongyloides trichosuri* and *Rhabditophanes* sp. KR3021 material was obtained from stages grown on agar plates. To produce the *S. ratti* reference genome a combination of Sanger capillary, 454 and Illumina-derived sequence data was used, while data for the other species were generated using Illumina technology. The *S. ratti* genome was initially assembled using Newbler v.2.3[68] (for the capillary and 454 sequence data) and AbySS v.1.3.1[69] (for the Illumina data); Illumina paired-end reads were mapped to this with SMALT (Hannes Ponstingl, pers. comm.; www.sanger.ac.uk/resources/software/smalt/). The genomes of the other species except *S. venezuelensis* were assembled using a combination of SGA assembler[70] and Velvet[71], from 100 bp paired-end Illumina reads, produced from short (~500 bp) fragment[72] and 3 kb mate-pair libraries[73]. Illumina reads were used in the IMAGE[74] and Gapfiller[75] software to fill gaps, and in ICORN[76] to correct base errors. Gap5[77] was used to manually extend and link scaffolds using Illumina read pairs. Genetic markers[78] were mapped to the *S. ratti* assembly to order and orient scaffolds, and in *S. papillosus* to assign scaffolds to chromosomes and regions of putative chromosomal diminution. The *S. venezuelensis* genome was assembled using the Platanus assembler[79] and improved as described above for other species. The resulting v2 *S. venezuelensis* assembly was further scaffolded using an optical map produced using an Argus optical mapping platform (Opgen). CEGMA v2[80] was used to assess the completeness of each assemby.

Assembled sequences were scanned for contamination from other species, using a series of BLASTX and BLASTP[81] searches against vertebrate and invertebrate sequence databases. Repeat sequences in the assemblies were characterized using RepeatModeler

(http://www.repeatmasker.org/RepeatModeler.html) and TransposonPSI (http://transposonpsi.sourceforge.net).

Mitochondrial genomes were assembled using MITObim assembler[82] with the *C. elegans* mitochondrial genes as seeds. The gene order of each assembly was confirmed by PCR. A mitochondrial protein-coding gene sequence phylogeny was constructed using RaxML v7.2.8[83].

Identifying regions that undergo chromatin diminution or belong to the X chromosome

To identify chromosomal regions that undergo chromatin diminution in *S. papillosus*, and scaffolds that belong to the X chromosome in *S. ratti*, *S. stercoralis*, and *P. trichosuri*, DNA of males and females from each species was sequenced and mapped to the appropriate reference genome using smalt v0.7.4 (Hannes Ponstingl, pers. comm.; www.sanger.ac.uk/resources/software/smalt/). The read depth was calculated for each scaffold using the BedTools function genomecov[84] and all scaffolds were classified as diminished/X or non-diminished/autosomal based on differences in read coverage. Since males are hemizygous for the diminished region in *S. papillosus*, and for the X chromosome in the other species, a male: female read-depth ratio of 0.5:1 was expected in diminished or X scaffolds relative to autosomes, whereas in non-diminished/autosomal region the ratio would be expected to be close to 1:1

Gene prediction and functional annotation

Genes were predicted using Augustus[85] – with a training set of approximately 200-400 manually curated genes per species, aligned transcript data and *S. ratti* protein sequences as hints – supplemented with non-overlapping predictions from MAKER[86]. If there was more than one alternative splice pattern for a gene prediction in the combined Augustus/MAKER gene set we only kept the transcript corresponding to the longest predicted protein. Astacin gene models and a subset of SCP/TAPS gene models from *S. ratti*, *S. venezuelensis* and *S. stercoralis* were manually curated prior to phylogenetic analyses.

A protein name was assigned to each predicted protein based on manually curated orthologs in UniProt[87] from selected species (human, zebrafish, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Schistosoma mansoni* orthologs) where possible. If a predicted protein was not assigned a protein name based on its orthologs, then a protein name was assigned based on InterPro[88] domains in the protein.

Gene Ontology (GO) terms were assigned by transferring GO terms from human, zebrafish, *C. elegans*, and *D. melanogaster* orthologs using an approach based on the Ensembl Compara approach for transferring GO terms to orthologs in vertebrate species[34], but modified for improved accuracy in transferring GO terms across phyla. Manually curated GO annotations were downloaded from the GO Consortium website[89], and for a particular predicted protein in the present study, the manually curated GO terms were obtained for all its human, zebrafish, *C. elegans*, and *D. melanogaster* orthologs. From this set the last common ancestor term (in the GO hierarchy) was found for each pair of GO terms from orthologs of two different species (*e.g.* a *C. elegans* ortholog and a zebrafish ortholog) and then transferred to our predicted protein. GO terms of the three possible types (molecular function, cellular component and biological process) were assigned to predicted proteins in this way. Additional GO terms were identified using InterproScan[90].

Gene orthology and species tree reconstruction

Eight outgroup species were used, encompassing four previously defined nematode clades[10] (clade I, *Trichinella spiralis, Trichuris muris;* clade III, *Ascaris suum, Brugia malayi*; clade IV, *Bursaphelenchus xylophilus, Meloidogyne hapla*; clade V, *Necator americanus, C. elegans*), together with the six species from the present study to construct a Compara database using the Ensembl Compara pipeline[34]. The database was used to identify orthologs and paralogs; gene duplications and gene losses; as well as gene families shared among the species, or sub-sets of the species, or specific to one species.

4,437 gene families were identified that contained just one gene from each species and that were present in at least ten species out of the six species and the eight outgroups. An alignment for the proteins in each family was built using MAFFT version v6.857[91], poorly-aligning regions were trimmed using GBlocks v0.91b, and the remaining columns were concatenated. For each alignment, the best-fitting amino acid substitution model was identified as that minimising the Akaike Information Criterion from the set of models available in RAxML v8.0.24[83], testing models with both pre-defined amino acid frequencies and observed frequencies in the data, and all with the CAT model of rate variation across sites. A maximum likelihood phylogenetic tree was constructed based on the concatenated alignment, with each protein alignment an independent partition of these data, applying the best-fitting substitution model identified above to each partition. This inference used RAxML v8.0.24 with ten random addition-sequence replicates and 100 bootstrap replicates, and otherwise default heuristic search settings.

Analysis of intron-exon structure, and synteny analysis

Introns that were present in two or more species were identified from gene structures and full gene nucleotide alignments of 208 single-copy orthologs using ScipPio[92] and GenePainter[93]. The output from GenePainter was parsed into DOLLOP (PHYLIP package; Felsenstein, J.) to infer intron gain and loss on every node of the species tree using maximum parsimony.

Whole-assembly nucleotide alignments were produced between *S. ratti* and the other five species using nucmer[94]. Each scaffold from the other species was assigned a chromosome based on its nucmer alignment to a *S. ratti* chromosome. To identify syntenic regions, conserved blocks of three consecutive orthologous genes or more in the same order and orientation were defined by DAGchainer[95], between the *S. ratti* reference and each of the other five species. To gain a high-level view of synteny, PROmer[96] was used to identify very highly conserved sequence matches, based on translated sequence, after which scaffolds from a particular species were ordered by matching to *S. ratti* chromosome and position in that chromosome, and the matches plotted using Circos[97].

612

613 ***Transcriptome and proteome analyses***

614 For *S. ratti* and *S. stercoralis* the transcriptomes were compared from the
615 parasitic female, free-living female and third stage infective larvae (iL3s). For
616 *S. ratti*, free-living females were picked individually from cultures of *S. ratti*-
617 infected rat faeces, from where iL3s were also collected; parasitic females
618 were collected by dissection of *S. ratti*-infected rats[98]. Two biological
619 replicates were collected for parasitic and free-living females. To minimise
620 experimental noise these samples were divided approximately equally and
621 used for both transcriptomic and proteomic analysis. A single biological
622 sample was used for iL3 transcriptomic analysis. RNA was prepared from
623 Trizol, and poly(A)RNA selected with Dynabeads, acoustically sheared and
624 reverse transcribed to construct Illumina libraries that were sequenced. For *S.*
625 *stercoralis* we used previously published data[41]. RNA-seq data were analyzed
626 using R v.3.0.2 and the bioconductor package edgeR[99] to identify genes
627 differentially expressed between all pairwise combinations of the three life-
628 cycle stages.

629

630 For *S. ratti* the proteome was also compared between the parasitic and free-
631 living females. Equivalent samples of the material collected for the
632 transcriptome analyses were used. Protein was extracted by freeze / thawing,
633 mechanical grinding and chemical extraction and digested with trypsin. The
634 resulting peptide mixture was analyzed by liquid chromatography-mass
635 spectrometry. Proteins were identified and quantified using Progenesis. For
636 downstream analyses at least two unique peptides were required to identify
637 proteins . Protein abundance (iBAQ) was calculated from Progenesis.

638

639 For both the transcriptome and proteome data, GO analysis was performed in
640 R using TopGo v.2.16.0 and Fisher's exact test.

641

642 ***Astacins and SCP/TAPS***

643 Genes encoding astacins and SCP/TAPS were identified using Interproscan.
644 For these gene families we aligned amino acid sequences of all *S. ratti* and
645 eight outgroup species' members using MAFFT[91]. The alignments were edited

with TCS[100] using the weighted option and the distance matrix of the new alignment was calculated using ProtTest[101]. The phylogenetic tree was constructed by maximum likelihood using RAxML[83] with 100 bootstrap replicates.

Gene clusters

Clusters of genes were identified as three or more adjacent genes upregulated in the same stage of the life cycle. The members of a cluster were considered to share a common gene family where ≥50 % of genes belonged to the same Compara gene family. To investigate the number of clusters expected by chance for a particular life cycle stage, for *n* genes upregulated in a particular stage, we randomly selected *n* genes from the genome, and calculated the number of clusters seen for the *n* random genes; this was repeated 1000 times and the mean value calculated.

Accession Codes

The *S. ratti, S. stercoralis, S. papillosus, S. venezuelensis, P. trichosuri* and *Rhabditophanes* genome assemblies, predicted transcripts, protein and annotation (*.GFF) files are available from the WormBase Parasite resource and are registered under BioProject accessions PRJEB125 (S_ratti_ED321_v5_0_4), PRJEB528 (S_stercoralis_PV0001_v2_0_4), PRJEB525 (S_papillosus_LIN_v2_1_4), PRJEB530 (S_venezuelensis_HH1_v2_0_4), PRJEB515 (P_trichosuri_KNP_v2_0_4) and PRJEB1297 (Rhabditophanes_sp_KR3021_v2_0_4) (http://parasite.wormbase.org/). The raw genomic data are available from the ENA via accession numbers detailed in **Supplementary Table 20**.

The transcriptomic data are available from ArrayExpress under accession numbers E-ERAD-151 and E-ERAD-92 (*S. ratti)* and the DRA under accession number PRJDB3457 *(S. venezuelensis*) (**Supplementary Table 21**).

677

678

679

680 **Fig. 1. Evolution and comparative genomics of *Strongyloides* and**
681 **relatives.** The life cycles of six clade IVa nematodes showing the transition
682 from a free-living lifestyle (in *Rhabditophanes*), through facultative parasitism
683 (*P. trichosuri*), to obligate parasitism (*Strongyloides* spp.), and the phylogeny
684 of these species (maximum-likelihood phylogeny based on a concatenated
685 alignment of 841,529 amino acid sites from 4,437 conserved single-copy
686 orthologous genes). Values on nodes (all 100) are the number of bootstrap
687 replicate trees showing the split induced by the node, out of 100 bootstrap
688 replicates. The phylogeny is annotated with the numbers of gene families
689 appearing along each branch of the phylogeny (+values on each branch) the
690 numbers of gene duplications and gene loss events within gene families (bar
691 charts on each branch), and the number of gene origins and gene losses in
692 18 astacin families (upper numbers in boxes) and ten SCP/TAPS families
693 (lower numbers in boxes) as estimated by the Ensembl Compara pipeline.
694 The pie charts summarize the evolutionary history of the genome of each
695 species, defining genes shared among all six species, the five parasitic
696 species (Strongyloididae, which includes all except *Rhabditophanes*), the four
697 *Strongyloides* species, and species-specific genes. The host species of the
698 parasites are shown: for *P. trichosuri* the brushtail possum, for *S. ratti* and *S.*
699 *venezuelensis* the rat, for *S. stercoralis* humans, and for *S. papillosus* sheep.

700

701 **Fig. 2. Nuclear genomic synteny and mitochondrial genomes of four**
702 ***Strongyloides* spp., *P. trichosuri,* and *Rhabditophanes* sp.** (a) The *S. ratti*
703 genome, our best assembled genome, is used as the reference sequence;
704 synteny is based on sequence matches. Graduation of color across the *S.*
705 *ratti* chromosomes represents position along the chromosome for
706 chromosome I (yellow-red), chromosome II (blue-purple) and chromosome X
707 (green). Black boxes represent scaffolds >1Mb; scaffolds <1Mb are grouped
708 together and shown in grey. (b) The mitochondrial gene order and phylogeny
709 for our six species and seven outgroup species that encompass four
710 nematode clades. Our eighth outgroup species, *Meloidogyne hapla,* was

711 excluded due to insufficient mitochondrial genome data. Inverted sequences
712 are shown by gene boxes with inverted text. The maximum likelihood tree
713 (left) was constructed using 12 mitochondrial proteins. Amino acid sequences
714 were aligned before concatenation (**Supplementary Note**).

715

716 **Fig. 3. The parasitic female, free-living female and infective third-stage**
717 **larvae transcriptomes of *Strongyloides* spp.** The progeny of the parasitic
718 female pass out of the host (as larvae for *S. stercoralis*, or eggs and larvae for
719 *S. ratti*) where infective third stage larvae (iL3s) can develop directly, or free-
720 living males and females develop, whose progeny develop into iL3s; iL3s then
721 infect hosts. The human parasite, *S. stercoralis*, can undergo internal auto-
722 infection (grey dashed line) where iL3s develop and internally reinfect the
723 same host. The transcriptome of the parasitic females, free-living females and
724 iL3s were compared for *S. ratti* and *S. stercoralis.* Representative GO terms
725 that were significantly enriched (light grey areas, left) and Ensembl Compara
726 gene families significantly upregulated (dark grey areas, right) for each of
727 these three stages of the lifecycle is summarized. The pie charts show the
728 proportion of the GO terms common to *S. ratti* and *S. stercoralis*, or unique to
729 either. Numbers in the dark grey boxes represent the number of genes
730 upregulated in each gene family for *S. ratti* and *S. stercoralis.*

731

732 **Fig. 4. *Strongyloides*-specific expansions and chromosomal clustering**
733 **of gene families.** (a) Astacin-like metallopeptidases and (b) SCP/TAPS are
734 the two major *Strongyloides ratti* gene families upregulated in the
735 transcriptome of parasitic females. Left shows the phylogeny of each of these
736 for *S. ratti* and our eight outgroup species and the crayfish *Astacus astacus*.
737 *S. ratti* genes are in light blue. Right shows the distribution of these genes in
738 the genome, plotted as clusters of physically adjacent genes in the genome.
739 Numbers above the peaks are the number of genes in a cluster of physically
740 neighboring genes; ticks below the axis denote scaffold boundaries for
741 chromosome X. The transcriptomic expression of these genes (in RPKM,
742 reads per kilobase per million mapped reads) for parasitic females, free-living

743 females and iL3s are shown on a grey scale, and the results of pairwise
744 edgeR analysis of the gene expression among these lifecycle stages is shown
745 in red or blue where a gene is upregulated. The color representing
746 upregulation (red or blue) in a given stage of the life cycle relates to the color
747 of the name of that stage for each pairwise comparison (fold change > 2, FDR
748 < 0.01); no differential expression is shown as a white block.

749

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Supplementary Table 5. The use of genetic markers to identify regions of chromatin diminution in *S. papillosus*. For each *S. papillosus* genetic marker (a sequence tagged site), we experimentally determined whether the marker was diminished in *S. papillosus*[6,12] (and unpublished data). For some markers we also determined on which *S. papillosus* chromosome the marker was located. The markers were subsequently computationally mapped to scaffolds in the *S. papillosus* assembly. Six were identified as diminished and 19 as non-diminished. The scaffolds identified as diminished or non-diminished based on read depth analysis (Supplementary Table 4; column F) agreed with those identified based on genetic markers, providing validation of the read depth approach.

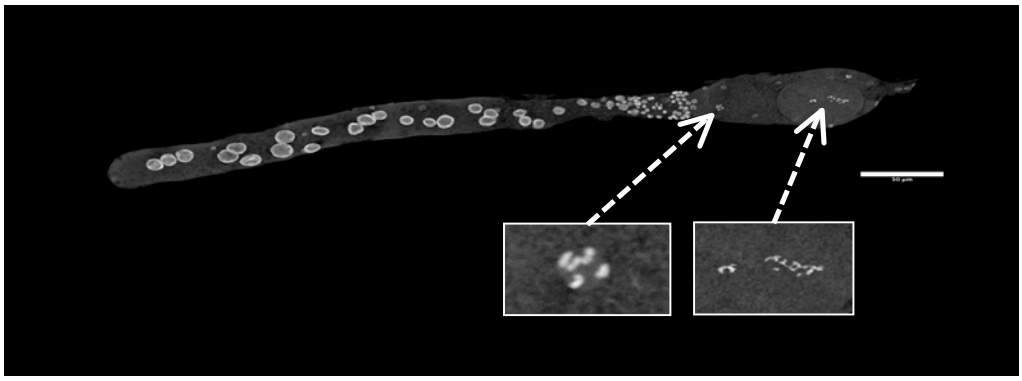
Scaffold to which the marker was computationally mapped	Genetic marker	Chromosome carrying marker[a]	Marker diminished? – genetic analysis	Scaffold diminished? – read depth analysis[b]
SPAL_scaffold0000018	ytp1	II	No	No
SPAL_scaffold0000001	ytp2	II	No	No
	ytp46	II	No	
SPAL_scaffold0000015	ytp3	I	No	No
SPAL_contig0000008	ytp4	Not Done	No	No
SPAL_scaffold0000043	ytp5	Not Done	No	No
SPAL_scaffold0000003	ytp14	II	No	No
	ytp12	II	No	
SPAL_scaffold0000056	ytp8	Not Done	No	No
SPAL_scaffold0000044	ytp9	Not Done	No	No
SPAL_scaffold0000065	ytp10	I	No	No
SPAL_contig0000002	ytp48	Not Done	No	No
	ytp11	Not Done	No	

SPAL_scaffold0000041	ytp13	Not Done	No	No
SPAL_scaffold0000011	ytp15	I	No	No
SPAL_contig0000011	ytp44	I	No	No
SPAL_scaffold0000005	ytp49	Not Done	No	No
SPAL_contig0000043	ytp126	II	No	No
SPAL_scaffold0000047	ytp127	II	No	No
SPAL_contig0000006	ytp128	I	No	No
SPAL_contig0000184	ytp129	I	No	No
SPAL_scaffold0000059	ytp131	II	No	No
SPAL_scaffold0000019	ytp50	I	Yes	Yes
	ytp83	I	Yes	
SPAL_scaffold0000035	ytp85	Not Done	Yes	Yes
	ytp84	I	Yes	
SPAL_scaffold0000009	ytp86	I	Yes	Yes
SPAL_scaffold0000028	ytp133	I	Yes	Yes
SPAL_contig0000141	ytp134	I	Yes	Yes
SPAL_scaffold0000013	ytp135	I	Yes	Yes

a. *S. papillosus* chromosome carrying the marker, determined genetically

b. Read depth analysis from Supplementary Table 4.

Supplementary Figure 7. The chromosome number of *Rhabditophanes* sp.



Dissected *Rhabditophanes* sp. (KR 3021) gonad. Distal is to the left, proximal to the right. The left inset shows condensed chromosomes in an oocyte, the right inset condensed chromosomes in an early embryo. The gonads were dissected and stained with DAPI as described[9]. Condensed chromosomes in oocytes and early embryos of seven different worms were counted using 3D reconstructions from confocal optical sections. Shown is a projection; the size bar is 50 µm. The chromosome number is 5 (meiotic bivalents) in oocytes and 10 in embryos. Although the 10 embryonic chromosomes cannot be counted in this particular overview projection, it illustrates that there are more than in the oocyte. KR3021 had been described as parthenogenetic[102] or as gonochoristic[103]. We did not observe any males. Females maintained individually from early larvae stages successfully reproduced; sperm were not observed in the females. Together these observations are consistent with *Rhabditophanes* sp. (KR 3021) reproducing by meiotic parthenogenesis (as Félix *et al*[102]) with n=5 chromosomes.

Germline organization in *Strongyloides* nematodes reveals alternative differentiation and regulation mechanisms

Arpita Kulkarni, James W. Lightfoot* and Adrian Streit*

Department Evolutionary Biology, Max Planck Institute for Developmental Biology, D-72076 Tübingen, Germany

*Contributed equally as principle investigators

Corresponding Author:

Adrian Streit

Max-Planck-Institut für Entwicklungsbiologie

Department IV

Spemannstrasse 35

D-72076 Tübingen, Germany

adrian.streit@tuebingen.mpg.de

Phone: 0049 7071 601 403

Fax: 0049 7071 601 498

Running title: *Strongyloides* spp. germline

ABSTRACT

Nematodes of the genus *Strongyloides* are important parasites of vertebrates including man with infections causing severe morbidity. Despite the medical and economic significance of this genus, surprisingly little is known of their germline organization and how this influences their parasitic life strategies, as well as the evolutionary significance of their differing mechanisms of reproduction. Here we analyze the germline structure in several *Strongyloides* and closely related species. We uncover striking differences in development; germline organization and fluid dynamics compared to the model organism *Caenorhabditis elegans*. With a focus on *Strongyloides ratti*, we reveal that the proliferation of germ cells is restricted to early and mid larval development, thus limiting the number of progeny. In order to understand key germline events (specifically germ cell progression and the transcriptional status of the germline), we employ the use of conserved histone modifications, in particular H3Pser10 and H3K4me3. The evolutionary significance of these events is subsequently highlighted through comparisons with six other nematode species, revealing underlying complexities and variations in the development of the germline among nematodes.

Keywords: germline, nematodes, *Strongyloides,* histone modification, germline chromatin

INTRODUCTION

The round worm *Strongyloides stercoralis* is among the most prevalent parasitic nematodes in humans causing disease and even morbidity. However Strongyloidiasis is still considered a neglected tropical disease (Olsen et al., 2009). Other members of this genus are being developed as model organisms, given their abundance and ease in experimental manipulation over *S. stercoralis*. These species are particularly useful for parasitological research of medical and veterinary interest, and also for the study of basic biological questions such as 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). Additionally, the genus of *Strongyloides* is in a very interesting position phylogenetically, having close relatives representing extremely divergent modes of reproduction and life styles, ranging from free-living (as either facultative or obligate animal parasites) to entomopathogenic and even plant parasitic (Fig. 1A) (Blaxter et al., 1998; Holterman et al., 2006). Therefore, *Strongyloides* spp. and their relatives have a great potential for further development as highly informative models for comparative evolutionary studies. Our current understanding of such evolutionary aspects comes from other nematode species like the well-established (but phylogenetically very distantly related) nematode models *Caenorhabditis* and *Pristionchus* spp. (Sommer and Bumbarger, 2012). The rat parasite *Strongyloides ratti* (*S. ratti*) and the sheep parasite *Strongyloides papillosus* (*S. papillosus*) have been developed as model representatives of the genus (Eberhardt et al., 2007; Viney, 1999; Viney and Lok, 2007). Whilst *S. ratti* can be conveniently maintained in their natural host, *S. papillosus* is maintained in rabbits, which can act as permissive laboratory hosts. The ease in accessing free-living adults (see below) in addition to a number of recently developed resources for working with *Strongyloides* spp. and their close relative *Parastrongyloides trichosuri* (*P.*

trichosuri), a facultative parasite of Australian possums (Eberhardt et al., 2007; Grant et al., 2006a; Grant et al., 2006b; Nemetschke et al., 2010b; Shao et al., 2012; Viney et al., 2002), now render this group of parasites more experimentally utilizable. The genera *Strongyloides* and *Parastrongyloides* together constitute the nematode family of Strongyloididae (Dorris et al., 2002).

Life histories of *S. ratti*, *S. papillosus* and *P. trichosuri*

The life cycle of *Strongyloides* spp. (Fig. 1B) has been reviewed recently {Streit, 2008 #645; (Viney and Lok, 2015). The parasitic worms are all female and live in the small intestines of their respective hosts. They reproduce by mitotic parthenogenesis but nevertheless give rise to female and male offspring. These young female offspring have two life-style choices: either they develop into filariform third-stage infective larvae (L3i) and upon infection of a new host develop into parasitic adults (termed direct or homogonic development), or they develop by a rhabditiform L3 stage, along with all the males, to finally give rise to a facultative sexually reproducing free-living generation (termed indirect or heterogonic development). Offspring of the free-living adults are all female and bound to develop into parasites, with very few known exceptions (Streit, 2008; Yamada et al., 1991). *P. trichosuri* is the best-studied representative of *Parastrongyloides,* a genus closely related to *Strongyloides* (Dorris et al., 2002). These species also form parasitic and free-living generations of reproducing adults. Nevertheless, their life history (Fig. 1C) and reproductive modes differ from those of *Strongyloides* spp. in interesting ways (Grant et al., 2006b). Firstly, parasitic males exists in *Parastrongyloides* (Mackerras, 1959) and reproduction in this generation is sexual (Grant et al., 2006b; Kulkarni et al., 2013). Secondly, free-living *Parastrongyloides* spp. produce progeny of both sexes and lastly, members of this genus (*P. trichosuri* in particular) have been shown to undergo an unlimited number of consecutive free-living generations (Grant et al., 2006b),

making it a facultative parasite. The life cycle of the free-living generation in both *Strongyloides* and *Parastrongyloides* is rather short, even in comparison to *C. elegans* (Fig. 1D).

Sex determination

In all species of *Strongyloides* investigated thus far, the sex ratio of the progeny produced by parasitic females is under the influence of the host's immune system (reviewed in (Streit, 2008)), with an increasing immune response against the worms leading to a higher proportion of males. Male and female *Strongyloides* worms normally differ in their chromosomes (Streit, 2008). However, the finer details of the sex chromosomes may differ amongst species. For example, *S. ratti* females have two X-chromosomes, but males have only one, in addition to the two pairs of autosomes in both sexes. Hence, *S. ratti* employs an environmentally influenced XX/X0 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 *S. ratti* chromosomes X and I is combined into one large chromosome (Nemetschke et al., 2010a). In the males of this species, sex specific chromatin diminution creates a hemizygous region largely corresponding in sequence to the X chromosome in *S. ratti.* Presumably this chromatin diminution event helps to functionally restore the ancestral XX/X0 sex determining system (Albertson et al., 1979; Kulkarni et al., 2013; Nemetschke et al., 2010a). During this process, an internal portion of one chromosome is eliminated but both ends are retained as separate chromosomes, leading to the 2n = 5 in males and 2n = 4 in females karyotype. By contrast, *P. trichosuri* employs chromosomal XX/X0 sex determination with 2n = 6 in females and 2n = 5 in males. In this species there is no indication for an environmental influence on sex determination (Grant et al., 2006b; Kulkarni et al., 2013).

The germline in the nematode family of Strongyloididae

The gonads in both sexes of the model nematode *C. elegans* are essentially tubular (for an introduction to the *C. elegans* gonad and germ line see (Hubbard and Greenstein, 2005). The hermaphroditic gonad has two arms, one extending anteriorly and one posteriorly, with both arms terminating in a central vulva. In the males, the gonad has just one arm with a posterior opening. A somatic cell, termed the distal tip cell (DTC) sits at the very tip of each gonad arm and signals the nearby germ cells to proliferate mitotically via Delta/Notch signaling (Kimble and Crittenden, 2005). Once cells move out of the reach of the DTC signal, they exit the mitotic cell cycle and initiate meiosis, thereby beginning their differentiation into gametes. Hence *C. elegans*, like many other nematodes (Rudel et al., 2005; Rudel and Sommer, 2003) maintains a stem cell population at the distal end of each gonad arm and this creates a constant flow of increasingly differentiated germ cells from the distal tip to the proximal end (Fig. 2, (top) *C. elegans* germline and insets 1-4). In addition, all cells in the distal portion of the gonad, which is situated at the dorsal side of the adult worm, open into a common central rachis and form a large syncytium.

While the overall morphology of the gonad is very similar to that of *C. elegans,* the appearance and organization of the germ cells differs greatly in *S. ratti* and other members of the Strongyloididae (Fig. 2, *S. ratti* germlines and insets 1-4), to that of *C. elegans* (Hammond and Robinson, 1994; Triantaphyllou and Moncol, 1977). For *S. ratti*, the distal arm was shown to contain giant nuclei, which have been reported to have a DNA content of up to several hundred C (Fig. 2, *S. ratti* inset 1), with 1C being the DNA content of a haploid set of chromosomes (Hammond, 1994 #1007). The distal arm (i. e the region with the giant nuclei) is followed by a band of very small, compact nuclei at the gonadal loop (Fig. 2, *S. ratti* inset 2), proximal to which presumably meiotic nuclei with condensed chromosomes can be observed

(Fig. 2, *S. ratti* insets 3 and 4). Further along the gonad, differentiated oocytes or sperm (depending on the sex) are present, very similar to *C. elegans*.

Other than these basic morphological aspects, information regarding germline development and gene expression for Strongyloididae members is currently lacking. For *C. elegans*, many aspects of the control of germline development and gene expression have been shown to act through chromatin modifications, with these thus being extensively studied and well characterized (Kimble and Crittenden, 2005; Schaner and Kelly, 2006). Histone modifications have also been shown to be important determinants for establishing transcriptionally active and inactive domains in different organisms, (Rando, 2012) and therefore are an integral part of germline regulation not only in *C. elegans*.

Here, we present the first detailed comparative characterization of the germlines of free-living males and females of Strongyloididae (*S. ratti*, *S. papillosus* and *P. trichosuri*) and compare this with *C. elegans*. We confirm that in the adult gonad, the distal arm is occupied by giant non-dividing polyploid nuclei, and that there are no mitotically proliferating stem cells here, despite the presence of a DTC-like cell in these species. Additionally, it appears that proliferation of the germ nuclei is generally restricted to early and mid larval development. Further, we describe differences in the germline chromatin of Strongyloididae members (especially in *S. ratti*) and *C. elegans,* by using conserved histone modifications with a particular emphasis on Histone3 phosphorylated at serine10 (H3Pser10) and Histone3 tri-methylated at lysine4 (H3K4me3). The variations in these histone modifications are further explored through comparisons with three other nematode species (*Rhabditophanes KR3021*, *Panagrolaimus PS1159* and *Panagrellus PS1163*), each of which are at an increasing phylogenetic distance to *S. ratti*, unveiling further complexities in germline development amongst nematodes. Finally we discuss the possible implications this has on regulating cell cycles and active transcription for Strongyloididae members.

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 local authorities. *P. trichosuri* was cultured in continuous free-living cycles (Grant et al., 2006b) at 20°C on NGM plates seeded with *Escherichia coli* (*E. coli)* OP 50 bacteria (Stiernagle, 1999) supplemented with a piece of autoclaved rabbit feces. *C. elegans* N2, *Panagroliamus PS 1159, Panagrellus PS1163* and *Rhabditophanes KR3021* were all maintained on NGM plates seeded with *E. coli* OP 50 bacteria at 20°C, with the exception of *Rhabditophanes KR3021* which was kept at 15°C.

DAPI staining/Microscopy

Adult worms (of the desired age) were fixed with ice cold 100% Methanol (Roth GmbH and Co. KG) and directly mounted (without a rehydration series) on polylysine coated glass slides in 10 µl of Vectashield (Vector Laboratories Inc., Burlingame CA 94010) containing 1µgmL^{-1} DAPI (4',6-diamidino-2-phenylindole from Roche). Confocal stacks of the entire gonad were taken for manual counting of nuclei and processed in Image J (Fiji). For dissected gonad DAPI imaging, the same protocol was followed after gonad dissection.

Gonad staging

Worms were collected by the Baermann funnel method every two hours after hatching as described by (Basir, 1950). Each worm sample was split into two, one was directly viewed under the Differencial interference contrast (DIC) microscope and the other was used for DAPI staining and confocal imaging as described above.

Transmission Electron Microscopy of S. papillosus gonads

Samples were cryo-fixed with a Baltec HPM-010 high pressure freezer and were freeze substituted in a Leica AFS-2 according to following protocol: 56 hours at -90°C in acetone with 5% gallic acid monohydrate (Roth, Karlsruhe, Germany), 3 hours warmed up to -60°C, 5 x washed with pre-chilled acetone, 24 hours at -60°C with 2%OsO4, 0.5%UA, 0.5 GA, 2%H20, warmed up to 0°C at 4°C/hour, 5x washed with pre-chilled acetone and infiltrated over a total period of 29 hours with increasing concentrations of epoxy resin (EMbed-812-kit, Science Services, Munich, Germany) finally the samples were cured at 60°C for 48 hours in flat embedding molds. Longitudinal semi-thin sections were stained with osmium tetraoxide and viewed in a FEI Tecnai G^2 Spirit transmission electron microscope operating at 120 kV. Images were taken with a Gatan Ultrascan 4000 camera at maximum resolution using the manufacturer's software.

BrdU labeling of C. elegans, S. ratti and P. trichosuri germlines

5-Bromo-2'-deoxyuridine (BrdU) labeling of germlines was done as described by Crittenden et al., 2006 and dissected germlines were visualized at different time points (3 hours, 6 hours, 12 hours and 16 hours) by staining against an anti-BrdU-FITC labeled antibody (BD Biosciences).

Immunostaining of nematode species

Gonads from adult worms were dissected in egg buffer (118 mM NaCl, 48 mM KCl2, 2mM CaCl2, 2mM MgCl2, 5mM HEPES) containing 0.1 % Tween and immediately fixed in 1 % paraformaldehyde for 5 minutes. Slides were frozen in liquid nitrogen, freeze cracked and then immersed for 1 minute in methanol at –20°C and transferred to PBS-Triton X 100 (1x PBS, 1.5 % Triton X 100). Blocking in 0.7 % BSA in PBS-Triton X 100 was then carried out for 1 hour. Primary antibodies were incubated over night at room temperature, slides were then washed 3 times for 10 minutes in PBS-Triton X 100 and secondary antibodies were added and incubated for 4-6 hours at room temperature. Following 3 washes for 5 minutes in PBS-Triton X 100, the slides were counterstained in 10 µl of Vectashield (Vector Laboratories Inc.) containing 1µgmL^{-1} DAPI (Roche). The following primary antibodies were used at the indicated dilutions: Anti-BrdU-FITC labeled (1:2.5, BD Biosciences), rabbit H3K9/K14ac (1:500, Diagenode), rabbit H4K20me1 (1:500, Diagenode), mouse H3K27ac (1:500, Diagenode), mouse H3K27me3 (1:500, Diagenode), rabbit H3K9me1 (1:500, Diagenode), rabbit H3Pser10 (1:200, Millipore), mouse H3K4me3 (1:500, Diagenode), rabbit α –Tubulin (1:200), rabbit α-Actin (1:200). The following secondary antibodies were used for visualization: Goat anti-rabbit labeled with Alexa Flor 488 (1:200), Goat anti-mouse labeled with Cy3 (1:200) and mouse anti-Biotin labeled with Cy3 (1:200).

All images were acquired as a stack of optical sections with an interval of 0.65µm using an Olympus confocal FV1000 microscope and processed in Image J (Fiji) and Adobe Photoshop CS.5.

Florescent insitu hybridization (FISH) *in S. ratti*

Gonads from adult worms were dissected in egg buffer (118 mM NaCl, 48 mM KCl2, 2mM CaCl2, 2mM MgCl2, 5mM HEPES) containing 0.1 % Tween and immediately fixed in 1 % paraformaldehyde for 5 minutes. Slides were frozen in liquid nitrogen, freeze cracked and then immersed for 1 minute in methanol at –20°C and then transferred to 2x SSCT thrice for 5 minutes each. Then the slides were dehydrated for 3 minutes each in 70%, 90% and 100% ethanol after

which the slides were left to air-dry. After air- drying, the slides were ready for adding the hybridization mix containing the FISH probes. The amount of probe per slide was between 150-200ng. The final concentration of the hybridization mix after adding the probe was 2x SSCT, 50% Formamide, 10% w/v dextran sulfate. 15µl of this mix was added per slide followed by heating the slides at 93°C for 2 minutes in a heated block. The slides were then removed and incubated over night at 37°C in a humid chamber. For post hybridization washes, the slides were rinsed in 2x SSCT 50% Formamide at 37°C for 30 minutes. This was followed by 3 washes in 2x SSCT for 5 minutes each. Then the slides were blocked in 1% BSA in 2x SSCT for 30 minutes. Then 50µl of the primary H3Pser10 antibody was added per slide and incubated over night at room temperature in a humid chamber. Then slides were washed in 2x SSCT, 3 times for 10 minutes each and incubated with 50µl of the labeled secondary antibody (1:200) for 4-6 hours. Finally, the slides were washed 3 times in 2x SSCT for 10 minutes each and were counterstained in 10 µl of Vectashield (Vector Lab. Inc.) containing 1µgmL^{-1} DAPI (Roche) and ready for imaging using the Olympus confocal FV1000 microscope.

Preparation of FISH probes

22-25bp probes were ordered from Eurofins MWG Operon (after ensuring preferably a single hit in the genome on the required chromosome). The ready-made probes were labeled internally with Biotin along their entire length. They were visualized using a mouse monoclonal anti-Biotin Cy3 antibody from Sigma-Aldrich.

S. ratti FISH probe	Sequence	Chromosome and position	*ytP* marker/contig information
FISH-1	CGATCCATTCAAAAAGAAAGCT GAA	Autosome II at -4.1cM	ytP91

FISH-2	CTGAACTTCAAGCAGAATTACGTGAAG	Autosome II at -4.1cM	ytP91
FISH-3	CTTACTTTGGATAAATCATTTAA	Autosome II at 0.0 cM	ytP113
FISH-4	GAATATTGACCGTTGCTGGATCTTTA	Autosome I at 0.0cM	ytP37
FISH-5	TTGCCGGAGTTCCGACAATGGGAG	Autosome I at 0.0cM	ytP37
FISH-6	TGCTATGAAAGCTGGTTGGAAACAA	Autosome I at 2.2cM	ytP117
FISH-7	ATTAAAATTACTGATAAATAACTC	Chromosome X	S.ratti_chrx_000001
FISH-8	AGCAGAATATAAAAGGAAGAACAAACTG	Chromosome X	S.ratti_chrx_000001
FISH-9	GTTATTTTCTATTAAAGACGGTGAAGA	Chromosome X	S.ratti_chrx_000001

RESULTS

Timing of germ cell proliferation in Strongyloididae

Simple cytological observation has revealed no obvious mitotic cell divisions in the germline of the adult free-living *Strongyloides* spp., implying that these organisms do not maintain a population of proliferating stem cells in their adult germlines. In order to test this hypothesis, we first performed a detailed electron and light microscopic characterization of the distal gonad of *S. papillosus* adult females (Fig. 3) and males (data not shown). Given the high degree of similarity in the organization of the gonads between *S. papillosus, S. ratti* and *P. trichosuri*, we assume that this morphological description holds true for the latter species as well. Detailed cytological examination using the Transmission Electron Microscope (TEM) found no indication for mitotic activity in any part of the germline, for either *S. papillosus* males or females. Nevertheless, a DTC was observed, which assumed a somewhat different position than in *C. elegans* (Fig. 3B and C). Most notably, it appears that this DTC-like cell, which is noticeably smaller may sit lop-sided in these species, and does not necessarily cap the whole distal tip as it does in *C. elegans* or *Pristionchus pacificus* (Fig. 3D). We also observed processes of the DTC-like cell making contact internally with the gonad, at regular intervals along the entire distal arm (Fig. 3E and F). Furthermore, we found the cytoplasm of the distal arm to be very rich in ribosomes and mitochondria (Fig. 3G).

In order to then determine when germ cell proliferation occurs and to ascertain how germline development coincides with the larval stages, we stained different developmental stages of *S. papillosus*, *S. ratti* and *P. trichosuri* with DAPI (binds nucleic acids) and additionally performed Bromo-deoxyuridine (BrdU) incorporation experiments (BrdU uptake indicates actively cycling cells in S phase) for these stages in *S. ratti* and *P. trichosuri* with *C. elegans* as a

control (Fig. 4A and B, see Material and Methods). We first came up with a schematic for how the germline grows in these species, by correlating the development of the gonad at different larval molts under the Differential Interference Contrast (DIC) microscope and with DAPI staining. In both species of *Strongyloides* and in *P. trichosuri*, we observed mitotic figures only in the germlines of larvae L3 and younger under DAPI staining (SupplFig. 1A). While such an in depth analysis was not done before, our results nevertheless corroborate earlier reports (Triantaphyllou and Moncol, 1977) confirming no cellular divisions in the adult germlines of either sex. In addition, we observed that the giant nuclei first appeared in L3 larvae (Fig. 4A), coinciding with the arrest of all detectable mitotic germline activity in the distal arm. This is consistent with our observation of no BrdU in the adult giant or small nuclei, for animals that had been exposed to BrdU only after reaching the fourth larval stage (Fig. 4B). However, BrdU was incorporated into all the germline nuclei for animals that were exposed to it as younger larvae (SupplFig. 1B). BrdU was also readily detected in the germ nuclei of *C. elegans* control animals that were treated with it as L4 larvae and adults.

Position of the central rachis and germline fluid dynamics in *S. papillosus* and *P. trichosuri*

As the central rachis and syncytium is so prominent in the structure of the *C. elegans* germline, we were interested in looking at the presence, position and possible function of the central rachis in *Strongyloides* species. Using a combination of TEM and staining against α-Tubulin (Fig. 5A), we found that the central rachis in these species begins just prior to the band of small compact nuclei before the gonadal loop region (in females), instead of running all along the distal arm as in *C. elegans*. In males, the rachis is slightly less pronounced, with it being detected at the base of the band of small compact nuclei (data not shown).

Due to the gonadal structure and function in *C. elegans*, there is a constant flow of cytoplasm from the distal tip towards the proximal end of the gonad, indicating active transport. If small lipid droplets are injected at any place in the distal gonad, they are transported proximally via the central rachis in the gonad, where-upon they are incorporated into growing oocytes (Wolke et al., 2007). As the central rachis in *Strongyloides* species was distinctively different in position, we performed the same experiment in *S. papillosus* and in *P. trichosuri* (Fig 5B), obtaining in part similar results to *C. elegans*. While droplets placed just before the gonadal loop or at the loop moved proximally and were rapidly incorporated into growing oocytes, droplets injected along the distal arm prior to the gonad bend were not transported at all, for either species. This supports our earlier microscopic observations and confirms that a functional central rachis starts just prior to the gonad loop in these species (Fig. 5A).

Meiotic progression and histone modifications in the germlines of *S. ratti*

Until now, nuclei in *Strongyloides* spp. were only recognized as meiotic once they had cytologically observable condensed bivalent chromosomes (Nigon and Roman, 1952; Triantaphyllou and Moncol, 1977). In an attempt to determine the actual position of the onset of meiosis in the *S. ratti* germline, we used antibodies that had in other systems (particularly in *C. elegans*) been shown to be meiosis specific. We stained dissected gonads of *S. papillosus* and *S. ratti* (both sexes) with anti-RAD-51 (Rinaldo et al, 2002) and anti-REC-8 (Pasierbek et al, 2002) antibodies, along with anti-SMC-3, which localizes to both meiotic and mitotic cells (Chan et al, 2003). In order to test if these commercially available antibodies might recognize the corresponding *Strongyloides* proteins we also performed western blot analyses. Although all three antibodies did recognize single defined bands of the expected size in Western blots (SupplFig. 2A), no specific immuno-staining patterns were obtained with any of these

antibodies in the *Strongyloides* germline (SupplFig. 2B**,** data shown for only *S. ratti* RAD-51 stainings). For the moment it must remain open if RAD-51, REC-8 and SMC-3 are indeed not present in *Strongyloides* gonads or if the heterologous antibodies we used are not suitable to detect these proteins *in situ* for these species. In order to overcome this problem we focused on antibodies against conserved histone modifications (SupplFig. 3A). We chose two histone modifications for comparison, one which in *C. elegans* marks mitotically dividing germ nuclei (H3 phosphorylated at serine 10, H3PSer10) and one marking transcriptionally active chromatin regions (H3 tri-methylated at lysine 4, H3K4me3) respectively (Hsu et al., 2000). Interestingly, while we did detect the published pattern in our *C. elegans* control (Hsu et al., 2000), H3Pser10 was completely absent from the distal gonad arm in *S. ratti* females (Fig. 6, *S. ratti* and *C. elegans* panels). Nevertheless, it was detected in the band of small compact nuclei, in a diffused and weak pattern (Fig. 7, *S. ratti* and *C. elegans* panels). In comparison, a very strong H3K4me3 signal was seen in the giant nuclei along the entire distal arm and in the small compact nuclei in female *S. ratti*. Additionally, it appears that H3K4me3 assumes a sub-nuclear localization that was mutually exclusive to the H3Pser10 signal in the small compact nuclei (Fig. 7, *S. ratti* panel and inset). By contrast in *C. elegans*, H3K4me3 was weak but detectable at the distal tip and fairly strong in pachytene nuclei in accordance to previous literature (Schaner and Kelly, 2006).

Evolutionary significance of histone modifications

The dramatic differences in the germline histone modifications observed between *S. ratti* and *C. elegans* may be either due to the structural and functional differences in the germlines of these two species, or may just reflect the large phylogenetic distance between these two. In order to ascertain the evolutionary significance of these differences, we performed similar stainings using anti-H3Pser10 and anti-H3K4me3 on dissected gonads of

five other clade IV nematode species (*S. papillosus, P. trichosuri, Rhabditophanes KR3021, Panagrolaimus PS1159* and *Panagrellus PS1163*), each of varying phylogenetic distance to *S. ratti* (Fig. 6 and 7), and then compared these to the clade V model nematode *C. elegans* (For nematode phylogenies see (Blaxter et al., 1998; Holterman et al., 2006)). We limited our analysis to females because some of the species involved were parthenogens. Although we observed striking differences between the species we studied, we noticed some general trends in staining patterns (Fig. 6, 7 and Table 1). If species shared a gonad organization resembling that of *S. ratti* (i.e *S. papillosus*, *P. trichosuri* and *Rhabditophanes KR3021*), then they did not show any H3Pser10 staining in the distal gonad (Fig. 6, top 4 panels). Conversely the free-living clade IV members including *Panagrolaimus PS1159* and *Panagrellus PS1163*, which have an overall germline organization more comparable with *C. elegans*, showed H3Pser10 staining in what appeared to be dividing germ cells in the distal gonad, just like in *C. elegans* (Fig. 6, bottom 3 panels). On the other hand, strong H3K4me3 staining extended up to the distal tip of the gonads of all clade IV members analyzed, with the notable exception of *P. trichosur*i, which did not show either H3K4me3 or H3Pser10 staining in the distal gonad (the few signals obtained for both antibodies are from nuclei of the somatic gonad; note that all images shown are projections and not individual focal planes). Three (*S. papillosus*, *Panagrolaimus* and *Panagrellus*) of the five clade IV species showed H3Pser10 staining in the gonadal loop region, just like in *S. ratti* (Fig. 7). Additionally, whilst in *S. ratti* and *Panagrellus PS1163*, the H3Pser10 signal was concentrated in a rather small spot and the H3K4me3 and H3Pser10 signals appeared mutually exclusive, in *S. papillosus* and *Panagrolaimus PS1159*, the areas occupied by H3Pser10 were larger and partially overlapping with H3K4me3 (Fig. 7, insets). We also noted that in *S. papillosus* H3Pser10 stains strongly only in the proximal part of the band of small nuclei (Fig. 7, *S. papillosus* panel and inset). Even though we used roughly age-matched worms (adult females) for these analyses, it must be

noted that we did find indications in *S. papillosus* that staining patterns with H3Pser10 depended at least partially on age (SupplFig. 3B).

Sex-specific histone modifications in Strongyloididae

In parallel, we investigated sex-specific histone modification differences within the Strongyloididae, by analyzing male gonads in *S. ratti*, *S. papillosus* and *P. trichosuri* with H3Pser10 and H3K4me3 (Fig. 8, Table 2). We found that the staining patterns in the distal gonads were indistinguishable between males and females of the same species (Fig. 6 and Fig. 8A). However, the small nuclei in males are far more condensed than in free-living females, allowing clearer visualization of H3Pser10 and H3K4me3 localization within the nucleus (Fig. 8B, insets). In *S. ratti* male small nuclei, the H3Pser10 signal occupied nearly a third of the nucleus and was distinct from H3K4me3, which occupied the remaining portion (see Fig. 8B *S. ratti* inset). In *S. papillosus* males, H3Pser10 stained the entire nucleus evenly, with this staining being much stronger in the proximal part of the band of small nuclei (Fig. 8B, *S. papillosus* inset), just like our observation in *S. papillosus* females. In *P. trichosuri*, condensed spermatogenetic chromosomes proximal to the small nuclei stained strongly for H3Pser10, which was never observed in either of the two species of *Strongyloides* (Fig. 8B, *P. trichosuri* inset). Interestingly, in parasitic *S. ratti* and *S. papillosus* females, we found H3K4me3 and H3Pser10 staining patterns very similar to the ones in free-living males (SupplFig. 4 and insets). This is remarkable, given that parasitic females of *Strongyloides* are known to reproduce asexually by mitotic parthenogenesis and not undergo anything resembling meiosis.

H3Pser10 is an X-chromosome specific histone modification in *S. ratti* males

In order to determine if the mutually exclusive regions staining for H3Pser10 and H3K4me3 represent different chromosomes, we performed fluorescent in situ hybridization (FISH) experiments with X-chromosome specific and autosome-specific probes on dissected male *S. ratti* gonads (Fig. 9). All our X-chromosome specific FISH probes co-localized with the region of the nucleus displaying H3Pser10 staining (Fig 9A). On the contrary, all autosome-specific probes did not co-localize with H3Pser10 staining (Fig 9B). Therefore, at least in males, H3Pser10 appears to be an X-chromosome specific histone modification in the small nuclei, indicating different chromatin modification states of the X chromosome and the autosomes.

Germline chromatin in *S. ratti*

Finally, in order to gain a better understanding of germline chromatin, and in particular chromosome specific histone modifications, we analyzed five other histone modifications in the *S. ratti* germline, in both sexes (Fig. 10 and 11, Table. 3). The five-histone modifications selected are associated with either active transcription, e.g. H3K9/K14ac and H3K27ac, or chromatin silencing, e.g. H3K9me1, H3K27me3 and H4K20me1 (Kouzarides, 2007; Schaner and Kelly, 2006). For evidence of chromosomal-specificity, we always co-stained with one of the two modifications (H3K4me3 or H3Pser10) described above. Antibodies against H3K9/K14ac and H3K27ac (Fig. 10) stained both the giant and the small nuclei in both sexes strongly, potentially indicating active transcription. This is consistent with our H3K4me3 stainings. In addition, we did find indications for sex-specific staining differences for both these markers. In females, these two histone modifications stained the different gonad compartments evenly, while in the males certain regions stained more brightly than others. In particular, H3K27ac stained giant nuclei at the distal tip more intensely than over the gonad arm (Fig. 10, H3K27ac male and female distal panels).

Furthermore, H3K27ac stained small nuclei in the distal part of the band in males more strongly than towards the proximal (Fig. 10, male and female gonadal loop panels), whereas H3K9/K14ac stained small nuclei at the proximal part of the band more intensely than towards the distal (Fig. 10, male and female gonadal loop panels).

Conversely, germlines of *S. ratti* stained against histone modifications known to be involved in silencing (H3K9me1, H3K27me3 and H4K20me1) showed only weak stainings (Fig. 11). In the giant nuclei of both males and females, H3K27me3 and H4K20me1 stainings could sometimes only be detected as bright puncta at the nuclear periphery, which is presumably stained heterochromatin (Fig. 11, distal gonad panels). Intriguingly, the only exception was H4K20me1, which stained the band region containing small nuclei in males very strongly (Fig. 11, male and female gonadal loop panels). Overall, we observed similar staining patterns for all transcription activation markers used (namely H3K4me3, H3K9/K14ac and H3K27ac), with all these modifications consistently staining both the giant and small nuclei. On the other hand, the silencing markers were absent or only weakly detected in these nuclei. The exact roles and functions of each of these histone modifications and their potential effect on germline development and regulation now need to be investigated further. Based on our results, H3Pser10 remains the only clearly X-chromosome specific histone modification identified in the *S. ratti* male germline. Consequently, it appears that *S. ratti* not only demonstrates an altered germline structure, but also altered chromatin regulation through differing histone modifications in comparison to *C. elegans.*

DISCUSSION

Nematodes are powerful organisms in which to study the development and evolution of multiple organ systems. Phylogenetically, Clade IV species occupy an important position amongst nematodes (Blaxter et al., 1998). The genus *Strongyloides* consists of fairly close relatives displaying a wide range of life styles, with many species documented as economically relevant parasites of plants and animals (Blaxter et al., 1998; Holterman et al., 2006). However, a comparative approach for studying the evolution of the reproductive systems in such species has been lacking, compared to the well-studied non-parasitic clade V nematodes, *C. elegans* and *P. pacificus* (Sommer and Bumbarger, 2012). This work is therefore, a first step towards bridging this gap using members of the genus *Strongyloidies*.

Although *C. elegans* and *Strongyloides* nematodes share superficial morphological similarity, leading to them being classified as close relatives in the pre-molecular age (Blaxter et al., 1998), they differ greatly in many respects. One major difference based on cytological observations is that the entire distal gonad of *Strongyloides* spp. consists of a population of just endoduplicated giant nuclei, whereas the same region in *C. elegans* contains both proliferating germline stem cells and early meiotic nuclei. We, in agreement with earlier investigators (Hammond and Robinson, 1994), have hypothesized that the polyploid giant nuclei in these parasitic nematodes have nurse cell activity, that helps provide a rapid supply of material during oogenesis, potentially allowing germ cells to move quickly through meiosis and early embryonic development. This is important given the nematodes' short reproductive window (Gardner et al., 2004, 2006). In *C. elegans*, there appears to be no specialized population of cells fulfilling this sole function. Instead, meiotic cells in pachytene (which go on to give rise to oocytes) perform this role (Wolke et al., 2007). All our results presented here are consistent with this proposed nurse cell function for giant nuclei in Strongyloididae. The richness in ribosomes and mitochondria in the distal arm we found in our TEM studies, along with the strong immuno-stainings against histone modifications generally associated with

high transcriptional activity, strongly argue for high gene expression in the distal gonad of *S. ratti* and other members of the Strongyloididae. This is supported by a recent publication (Kulkarni et al. 2015) based on quantitative DNA and RNA sequencing from the distal gonad arm, which found evidence for strong gene expression in the giant nuclei of Strongyloididae. We showed that highly expressed genes are predominantly autosomal. This is achieved partially by differential DNA amplification in the giant nuclei, resulting in autosomal portions of the genome being present in higher copy numbers than X chromosomal portions.

A second striking difference to *C. elegans* appears to be the lack of proliferating germline stem cells in the Strongyloididae adult gonad. None of our observations presented here (i.e. our detailed light and electron microscopic analyses and the BrdU incorporation experiments) yielded any indication of DNA replication or mitotic divisions in the adult germline. Therefore, these results strongly support the idea that Strongyloididae members build up a stock of germ cells during larval development, and then draw from this stock once they have matured. Indeed, free-living adults of these species are very short lived (one to two days of reproductive activity) and produce only a few dozen progeny (Gardner et al., 2004, 2006). For comparison, the longer-lived *C. elegans* can reproduce for several days and produces several hundred progeny (Wegewitz et al., 2008).

Based upon its position at the distal tip, we have identified a somatic cell that may be the distal tip cell (we call this the DTC-like cell) in these species. The DTC in *C. elegans* has been implicated in maintaining a stem cell population in the distal gonad along with guiding the growth of the gonad arms during larval development (Kimble and Crittenden, 2005). In this regard it will be interesting to evaluate the lineage of this DTC-like cell in *Strongyloides* spp. along with studying its role in gonad development, organization and function. To this

end, DTC ablation experiments will prove most useful in uncovering novel cell control mechanisms at play in these species.

Until the recent discovery of DNA methylation on N6-Adinine in *C. elegans* (Greer et al., 2015), it was thought that *C. elegans* lacks all DNA methylation (Simpson et al., 1986) and thus depends mainly on histone modifications to dictate chromatin structure. Histone modifications are thought to directly control function of local genomic regions, by increasing or decreasing the accessibility of DNA for transcription (Schaner and Kelly, 2006). Additionally, the X-chromosome has been shown to be under-expressed in the germlines of *C. elegans* and *S. ratti* (Kelly et al., 2002; Kulkarni et al., 2015). Since little is currently known about histone modifications in *Strongyloides* spp., we first characterized different chromatin modifications of germ cells in *S. ratti*, and then compared these with other clade IV nematodes and with *C. elegans*. We began by concentrating on two modifications, H3K4me3 and H3Pser10, which in *C. elegans* have very distinct staining patterns. Strong H3K4me3 stainings extended up to the distal tip in both species of *Strongyloides*, as well as all other clade IV nematodes, with the notable exception of *P. trichosuri.* In *C. elegans*, H3K4me3 staining coincides with cells known to be highly active transcriptionally (Schaner and Kelly, 2006). Therefore, our H3K4me3 results might hint that in many clade IV nematodes the distal gonad tip is a place of high transcriptional activity, irrespective of whether the cells in this region are mitotically dividing or not. All the other histone modifications marking active transcription (Peterson and Laniel, 2004; Rando, 2012) (H3K9/K14ac and H3K27ac) were high in the same places as H3K4me3 in the *S. ratti* gonad, which is reassuring.

The second modification we analyzed was H3Pser10. In the distal gonad of some clade IV nematodes (*Panagrellus* and *Panagrolaimus*) and *C. elegans*, this modification appears to be specific for dividing nuclei, meaning this role in the distal gonad may be conserved. In species without dividing cells in the distal gonad; we consistently did not observe any H3Pser10

staining. However in *S. ratti* males, H3Pser10 specifically marks the X chromosome in spermatogenic nuclei. This is interesting, because in this species the males sire only female progeny. Although some sperm without an X chromosome (nullo-X sperm) exists in *S. ratti*, it is likely that X-bearing sperm are preferentially made over nullo-X sperm(Kulkarni et al., 2015). While H3Pser10 has been implicated in multiple gonad related processes such as mitosis, apoptosis and gametogenesis(Kouzarides, 2007), it has also been implicated in dosage compensation, another process that requires distinguishing the X chromosome from the autosomes. For example, elevated H3Pser10 levels are detected on male X-chromosomes in Drosophila (Wang et al., 2001). We found two additional histone modifications, namely H3K27me3 and H4K20me1 that are known to play a role in dosage compensation in other animals, to be abundant in the small compact nuclei of *S. ratti* males. H3K27me3 levels are found to be elevated on the inactivated X-chromosome, resulting in mammalian dosage compensation (Heard and Disteche, 2006; Leeb and Wutz, 2010; Payer and Lee, 2008). Likewise, mono-methylation of H4K20me1 is sometimes implicated in transcription elongation (Vakoc et al., 2006; Wang et al., 2008), but has also been linked to mitotic chromosome condensation in mammals and other animals (Oda et al., 2009). The presence of H4K20me1 opens up the question as to whether this modification is important for spermatogenesis in *S. ratti*. Interestingly for *C. elegans*, the dosage compensation complex (DCC) is known to maintain high levels of H4K20me1 on the hermaphrodite X-chromosomes relative to the autosomes, suggesting that increased H4K20me1 levels regulate X-chromosomal gene expression via chromatin compaction (Vielle et al., 2012). Since little is currently understood on how dosage compensation is achieved in *Strongyloides* nematodes or if it occurs at all, our characterization of these histone modifications in *S. ratti* males therefore represents a first overview. This clearly indicates that further research is required to elucidate the functional consequences and evolutionary implications of these patterns in Strongyloididae.

Interestingly in *S. papillosus,* which lacks an independent X-chromosome, H3Pser10 was found to stain the entire nucleus evenly. But surprisingly in *P. trichosuri*, which is known to produce male progeny, H3Pser10 marks the condensed meiotic X chromosomes and autosomes alike. We never noted this modification on condensed meiotic chromosomes in either *S. ratti* or *S. papillosus* males. These striking and varied differences in H3K4me3 and H3Pser10 stainings within the three closely related representatives of the Strongyloididae suggests that this group of nematodes has either recently undergone, or is still in the process of undergoing rapid evolutionary changes. This may be a response to becoming parasitic and/or parthenogenetic (in the parasitic generation).

Due to the current limitation in knowledge and tools for *Strongyloides* species, we must assume that the differences between *Strongyloides* spp. and *C. elegans* with regards to the different histone modifications, are probably a combination of their large phylogenetic distance and completely different germline organizations (Fig. 12). However, it must be noted, that the Strongloididae gonad is probably a highly derived structure, and therefore care must be taken while drawing direct conclusions based on other systems. When attempting to make such comparisons, one has to keep in mind that the gonads of clade IV nematodes are not as well characterized as in *C. elegans,* and it is therefore not easy to define corresponding populations of cells. Another issue with *Strongyloides* spp. is that they need to be grown in fecal cultures, where the development of individual worms cannot be easily followed. The age of individual worms can therefore only be estimated.

From our comparative observations of the staining patterns with *C. elegans*, it appears likely that the small compact nuclei at the gonadal loop in *Strongyloides* spp. represents nuclei in some stage of meiotic development. We would therefore expect that this small region would be equivalent to the extended meiotic zone in *C. elegans* (occupying nearly two thirds of the distal arm length). This conclusion is supported by two observations: firstly, there is no

other region in the gonad which could be meiotic (given that this region is preceded by highly endoduplicated nuclei, and followed directly by oocytes or sperm) and secondly, the *Strongyloides* species we studied are known for their normal diploid genetics (Eberhardt et al., 2007; Nemetschke et al., 2010b; Viney et al., 1993), meaning that haploid gametes must necessarily be formed. It is of interest then cytologically, that the small nuclei look very different from meiotic cells of *C. elegans*. In particular, no condensed chromosomes reminiscent of the ones in the different meiotic zones from *C. elegans* appear in this region in members of Strongyloididae.

Overall, our findings presented here enhance our understanding of the biology of a group of fascinating but poorly understood parasites. Additionally, they also illustrate the usefulness of the *Rhabditophanes* spp. (free-living) / *Parastrongyloides* spp. (facultative parasite) / *Strongyloides* spp. (obligate parasite with a single free-living generation) in comparative studies. Given their multiple advantages, such systems could be used in the future to address questions about the emergence of parasitic life styles and the evolutionary consequences of becoming parasitic. Finally, novel insights into germline regulation during development may only be uncovered by looking into atypical species.

Acknowledgements

We thank Anja Holz for help with gonad dissections, Dorothee Harbecke for technical support, Dee Denver for *Rhabditophanes* sp., Ralf J. Sommer for *Panagrolaimus* and *Panagrellus* sp., Metta Riebesell for TEM images of *P. pacificus* and Alex Dulovic for proof reading the manuscript. This work was funded by the Max Planck Society.

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Figure legends

Fig. 1. Introduction to *Stronygloides* nematodes

A. A schematic cladogram used to illustrate the phylogenetic position and inter-relationships of *Strongyloides* with other nematode species based on {Holterman, 2006 #626}. In brackets are the life styles for each species. Species discussed further in the text are marked with red asterisks. Branch lengths are meaningless. **B**. The generalized life cycle of *Strongyloides* species (left) compared to the life cycle of *Parastrongyloides trichosuri* (right). **C**. A comparison of the developmental timing between *C. elegans* (according to WormAtlas) and *Strongyloides* spp. from hatching to adulthood. For *Strongyloides* species, molt timings are in a wider range because males develop and molt faster in comparison to females. The times for *Strongyloides* spp. are post start of culture (essentially post deposition with the feces).

Fig. 2. Comparisons between the *C. elegans* and *Strongyloides* gonads

(Top) A DAPI stained dissected *C. elegans* hermaphroditic (adult) gonad, showing progression of germ cells in the germline (distal tip is to the left). The numbers 1-4 indicate the immediate insets below, with each inset showing the characteristic morphology (mitotically dividing cells at distal tip, crescent-shaped nuclei at transition zone, 'bowl of spaghetti' in the pachytene zone and condensed chromosomes at diakinesis respectively) of germ nuclei for those regions. **(Bottom)** DAPI stained dissected gonads from *S. ratti* adult females (top) and males (bottom) showing the completely different gonadal organization in comparison to *C. elegans*. Note the shorter but broad nature of the *S. ratti* male gonad in comparison to the female (adult males are smaller in size to adult females, adults are approximately 28-30 hours post culturing). Here the entire distal arm is occupied by intensely staining giant nuclei, followed by a band of small compact nuclei at the gonadal loop (asterisk). Except for even more strongly condensed small nuclei in males, the organization is identical in both sexes. Insets 1 and 2 are derived from female gonads. The band of small nuclei is followed proximally in females by nuclei, which might be in diakinesis (shown in inset 3) and in males with condensed presumably meiotic chromosomes (inset 4). Scale bar 50µm.

Fig. 3 Transmission Electron Microscopy (TEM) of the *S. papillosus* free-living female gonad

A. Semi-thin longitudinal TEM sections of a *S. papillosus* female showing the entire gonad (outlined in red) in the body of the adult worm, with the vulva (top, central). Adults are approximately 28-30 hours post culturing. **B**. TEM section showing the distal tip of the gonad, DG, with giant nuclei, GN (outlined in blue) and the distal tip cell, DTC (in yellow). **C**. Zoom in of the DTC (yellow) showing its nucleus, DTCN (in red) in addition to the giant nuclei, GN (in blue) in the distal gonad, DG. **D**. TEM section from *Pristionchus pacificus* showing the DTC (outlined in

yellow) sitting as a cap on the distal tip (outlined in grey), with germ cells GC, around a central rachis 'rc' (in red). This organization is similar to what is found in *C. elegans* (image to the curtsey of Metta Riebesell. **E**. The distal tip cell and its processes (outlined in yellow) making contact with the distal gonad at regular intervals. **F**. Zoom in showing a DTC process (in yellow) innervating the distal gonad, close to two giant nuclei GN (in blue). **G**. Zoom in at the distal tip (with DTC outlined in yellow) showing the high density of ribosomes (electron dense regions) and mitochondria, M (outlined in white) in the distal gonad, DG.

Fig. 4 Staging for *Strongyloides* species and germline proliferation

A. The schematic showing germline growth post hatching (indicated in hours to the left, with suitable approximations made for molt timings for the two *Strongyloides* species) at different larval molts (indicated to the right). The giant nuclei are first observed at the L3 stages, which marks an end of mitosis in these cells (and therefore in the distal arm). **B**. Bromo-deoxyuridine (BrdU) incorporation assays in *C. elegans*, *S. ratti* and *P. trichosuri* germlines. Panels 1 and 2 indicate position, 1 for the distal gonad (with giant nuclei in *S. ratti* and *P. trichosuri*) and 2 for the gonadal loop region (with small nuclei in *S. ratti* and *P. trichosuri*) respectively in females (top 2 rows) and males (bottom rows) at 3h post BrdU exposure. For both females and males, the BrdU channel is shown as separate in grey (top) or as merged with DNA (BrdU in green, DNA in blue). In *C.elegans* hermaphrodites, BrdU is seen incorporated into mitotically active cells in the proliferative zone at the distal tip of the germline 3h post BrdU exposure (*C. elegans*, panel 1). In comparison, no detectable BrdU uptake is seen within giant nuclei or the small compact nuclei in *S. ratti* and *P. trichosuri* (in either sex) indicating no active cell divisions in these regions. Similar results were obtained at 6, 12 and 16h post exposure to BrdU in these two species, although a cytoplasmic signal was often seen. Scale bar 20μm.

Fig. 5. Central core and fluid dynamics

A. The central core in *S. papillosus* dissected female gonads visualized by staining against α-Tubulin (green) and DAPI (blue). Panel 1 shows no visible central canal in the distal gonad (with the giant nuclei) when stained with α- Tubulin (top), but a clearly visible canal in Panel 2 (outlined in white) starting just before the gonadal loop (beginning of the band of small nuclei). Bottom panels show merge with DAPI. **B**. Differencial interference contrast (DIC) timelapse images of an injection experiment using mineral oil in a *P. trichosuri* adult female gonad, showing the rapid movement and incorporation of an oil droplet (if injected into the central core) proximally into growing oocytes. The gonad arm is outlined in yellow, the oil drop in red, developing oocytes are labeled and outlined in white dashes, the spermatheca (Sp.) is in blue, embryos labeled in the uterus outlined with green. Soon after injection, the single large oil drop is broken into smaller droplets, which quickly move proximally past the gonadal loop (seen in the left most image marked as the oil drop, at 5 minutes post injection). Once proximal, the oil droplets move further down into developing oocytes (seen in the middle image at 20 minutes post injection) to finally become incorporated within them (seen in the right most image, 1 hour post injection).

Fig. 6 H3K4me3 and H3Pser10 staining patterns in females of different nematode species

A. A comparative image showing H3K4me3 (in red) and H3Pser10 (in green) staining patterns (individual channels are separated according to color and labeled on top) obtained in 7 different (adult) nematode species in the distal part of their gonads (distal tip is to the left for each). This region consists of giant nuclei in *Strongyloides* species. Note the similarity in gonad organization in *S. ratti*, *S. papillosus*, *P. trichosuri* and *Rhabditophanes* KR3021; and between *Panagrolaimus PS1159, Panagrellus PS1163* and *C. elegans.* For nematodes with a gonad organization similar to *S. ratti,* note the lack of H3Pser10 staining (in green) in the distal gonad, whereas its presence in species with a gonad organization similar to *C. elegans*.

Scale bar 10µm (Note: adults are approximately 28-30 hours post culturing for *Strongyloides* species, but for other nematodes, were morphologically young females carrying eggs in their uteri).

Fig. 7. A comparative image showing H3K4me3 (in red) and H3Pser10 (in green) staining patterns (individual channels are separated according to color and labeled on top) obtained in the same 7 nematode species in the gonadal loop region (for orientation, distal gonad arm is to the left for each). This region consists of the small compact nuclei in *Strongyloides* species. Insets (right most) are zoom ins' of the respective areas marked in white in the merge panels, showing co-localization patterns of H3K4me3 and H3Pser10 in each species. Note the localized dot-like H3Pser10 staining in the nuclei of *S. ratti* and *Panagrellus PS1163*, but its even distribution in the nuclei in *S. papillosus* and *Panagrolaimus PS1159*. H3Pser10 is absent in *P. trichosuri* (signal is from somatic sheath cells), *Rhabditophanes KR3021* and *C. elegans*. Scale bar 10µm.

Fig. 8. H3K4me3 and H3Pser10 staining patterns in the males of *S. ratti*, *S. papillosus* and *P. trichosuri*

A. A comparison of H3K4me3 and H3Pser10 stainings in the distal gonads (region containing giant nuclei) in the adult males of all 3 species (individual channels are separated according to color and labeled on top). Note the lack of H3Pser10 staining in *S. ratti* and *S. papillosus*, but the presence of H3K4me3. For *P. trichosuri*, note the complete lack of both H3K4me3 and H3Pser10 in this region. This pattern is similar to the stainings obtained in the respective females for each species. Scale bar 10µm. **B**. A comparison of H3K4me3 and H3Pser10 in the gonadal loop, the region containing the small nuclei in the males of all 3 species. Insets (far

right) are zoom ins' of the areas marked in white in the merge panels, showing co-localization patterns for H3K4me3 and H3Pser10 in each species. Note the H3Pser10 staining to be localized to a part of the nucleus in *S. ratti*, to be evenly distributed in *S. papillosus* and to be only on condensed meiotic chromosomes in *P. trichosuri*. Scale bar 10μm.

Fig. 9 H3Pser10 is an X-chromosome specific histone modification in *S. ratti* males

A. The band of small compact nuclei in adult males (for orientation, distal arm is to the top) showing co-localization of H3Pser10 (in green) and the X-chromosomal FISH probe (in red) within the nuclei. Inset (far right) is a zoom in of the region marked in white in the merge panel. **B**. The band of small nuclei in males showing no co-localization (inset, far right) of H3Pser10 (in green) with an autosomal FISH probe (in red) within these nuclei. Scale bar 15μm.

Fig. 10 Histone modifications generally marking active transcription in the *S. ratti* germ line

A. Distal gonad panels (with distal tip to the left) from dissected male and female gonads of *S. ratti* adults stained against transcription activation markers H3K9/K14ac (top 2 panels) and H3K27ac (bottom 2 panels) in combination with H3K4me3 and H3Pser10 respectively. **B**. Gonadal loop panels for males and females from (**10A**) stained against the same antibodies. Scale bar 10μm.

Fig. 11 Histone modifications generally marking silencing in the *S. ratti* germ line

A. Distal gonad panels from dissected male and female gonads stained against H3K27me3 (top 2 panels), H3K9me1 (middle 2 panels) and H4K20me1 (bottom 2 panels) in combination

with H3Pser10 or H3K4me3 respectively. **B**. Gonadal loop panels in males and females (from **11A**) for these same antibodies. Scale bar 10μm.

Fig. 12 Figure summarizing phylogenetic relationships, gonadal organization, life styles and histone modifications in the nematodes species studied. Note that species with similar gonad organizations (*Strongyloides, Parastrongyloides* and *Rhabditophanes*) lack H3Pser10 staining in their distal arms, while species (*Panagrellus* and *Panagrolaimus*) with a similar gonad organization to *C. elegans* show H3Pser10 in this region.

Table. 1 H3Pser10 and H3K4me3 staining patterns in seven different nematode species.

Table. 2 H3Pser10 and H3K4me3 staining patterns in Strongyloididae males.

Table. 3

A. The patterns of seven different histone modifications in *S. ratti* female germlines. **B**. The patterns of the same seven different histone modifications in *S. ratti* male germlines.

SupplFig. 1

A. DIC image (left) showing a *S. ratti* larva undergoing the L1 – L2 molt. The gonad is outlined in white in the body of the larva. The Inset (to the right) shows the corresponding DAPI staining (zoom in of the gonad) at L1 – L2 molt showing mitotic figures and hence active cell

division in the gonad. **B.** Dissected gonad from *S. ratti* L3-stage larva as control showing incorporation of BrdU in both giant and small nuclei (distal tip is to the left). Scale bar 10µm.

SupplFig. 2

A. Western blots for SMC-3, REC-8 and RAD-51 in *P. trichosuri*, *S. ratti* and *S. papillosus* adult worms (whole worm lysates) showing a single band at expected positions for each, with alpha-tubulin used as loading control. **B**. RAD-51 staining (in green) in combination with H3K4me3 (in red) in the *S. ratti* male germline showing no RAD-51 in the distal gonad, the region with the giant nuclei (top) and no meaningful pattern in the band of small nuclei at the gonad loop (bottom). Much of the RAD-51 signal received here (shown in the inset, bottom right) is probably background noise, as it was not in the same focal plane as the germ line nuclei (all images shown are projections of stacks from multiple focal planes).

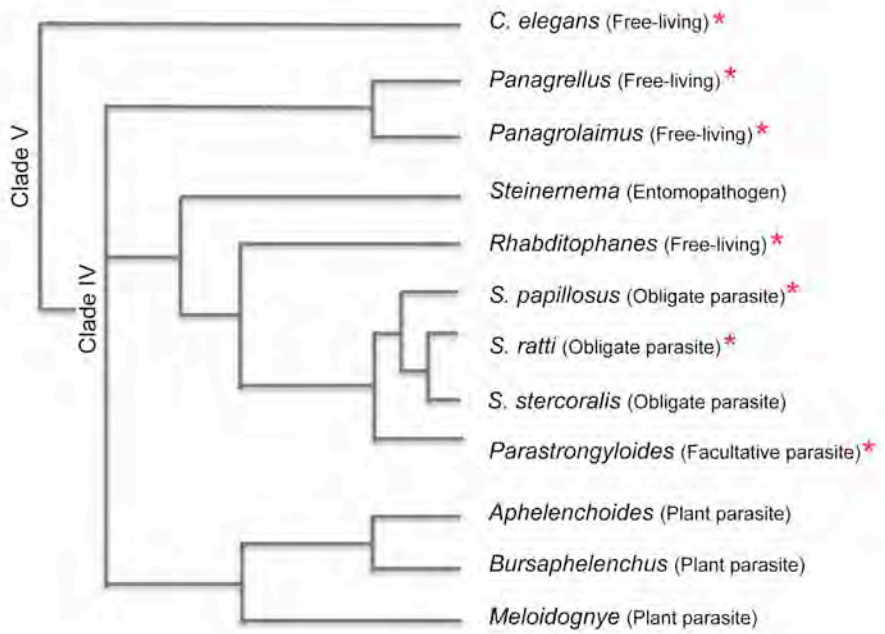
SupplFig. 3

A. Western blots for H3Pser10 and H3K4me3 in *P. trichosuri*, *S. ratti* and *S. papillosus* adult worms (whole worm lysates) showing a single band at expected positions, with alpha-tubulin used as loading control. **B.** H3Pser10 antibody staining of dissected young (bottom) and old (top) *S. papillosus* female gonads. The term 'old' is used here to indicate a mated female (or a female that has begun active oogenesis), whereas young is before the L4-adult molt. H3Pser10 is briefly seen on condensed chromosomes at the onset of mating, and from then on in the small nuclei, illustrating possible age related staining patterns in this species.

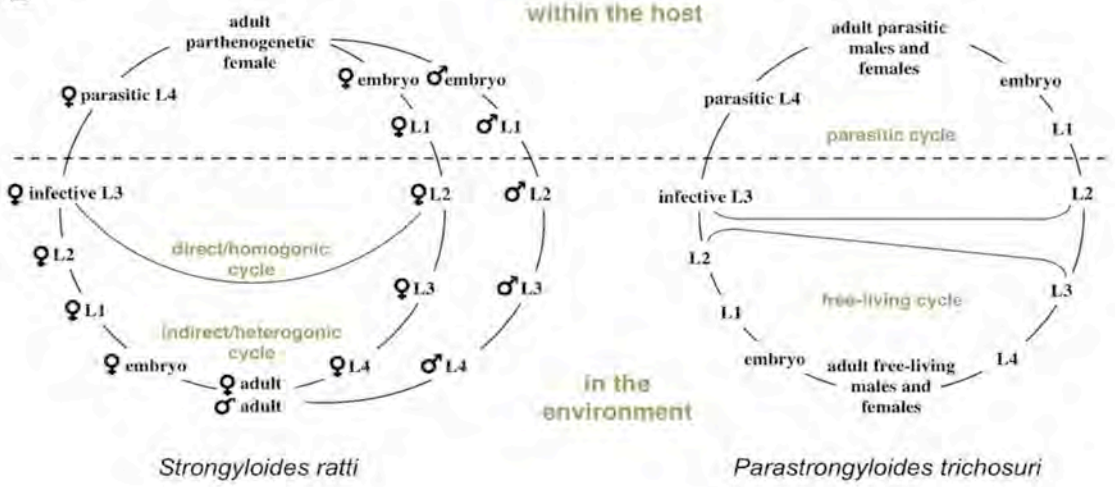
SupplFig. 4 H3Pser10 and H3K4me3 staining in adult parasitic *S. ratti* and *S. papillosus* females

(**Top**) Distal and gonadal loop panels for *S. ratti* parasitic female. Inset (bottom) shows a zoom in marked in white in the merge panel. Note the mutually exclusive localization of H3Pser10 and H3K4me3. (**Bottom**) Distal and gonadal loop panels for *S. papillosus* parasitic female. Inset (bottom) shows a zoom in marked in white in the merge panel. Note the even localization of H3Pser10 and H3K4me3.

A



B

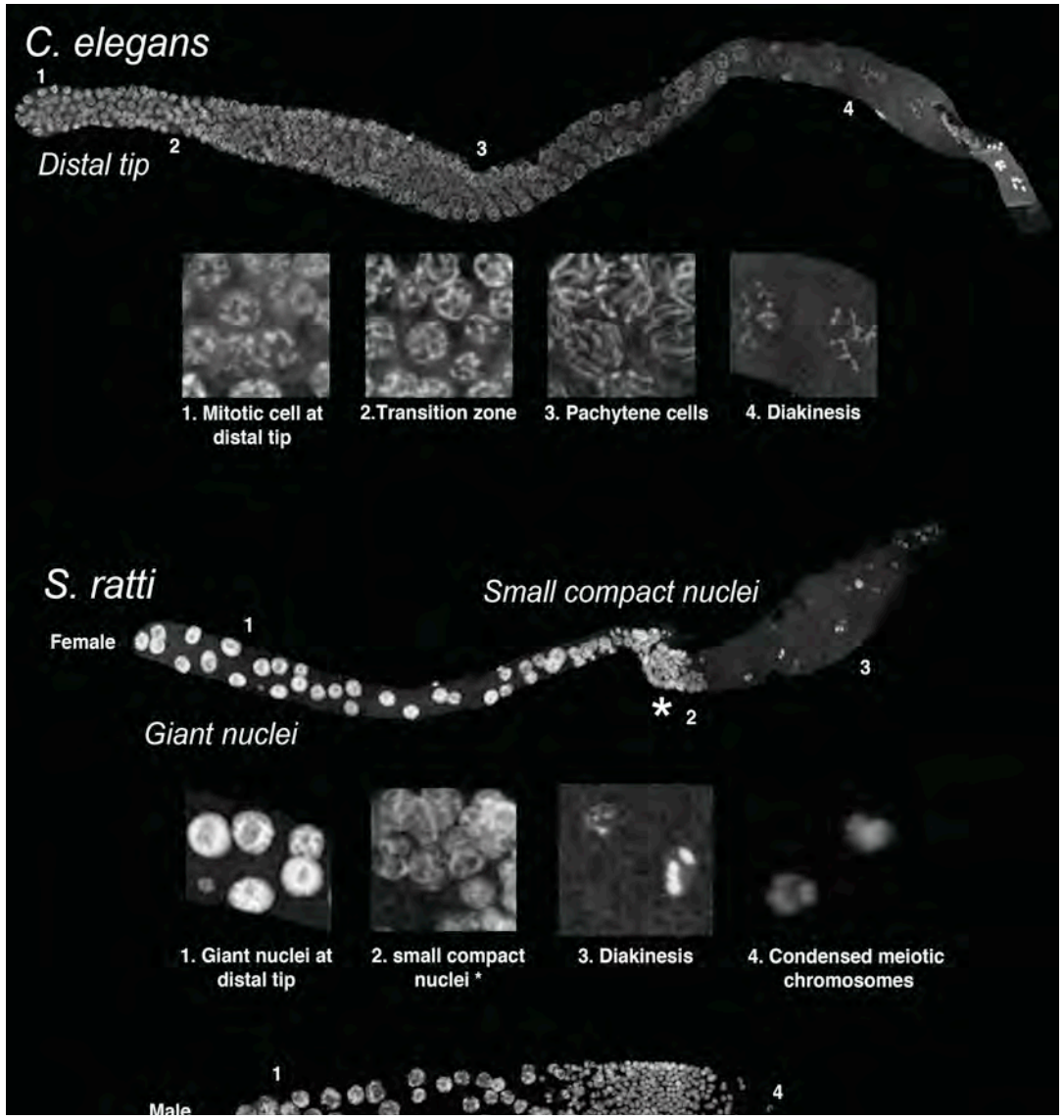


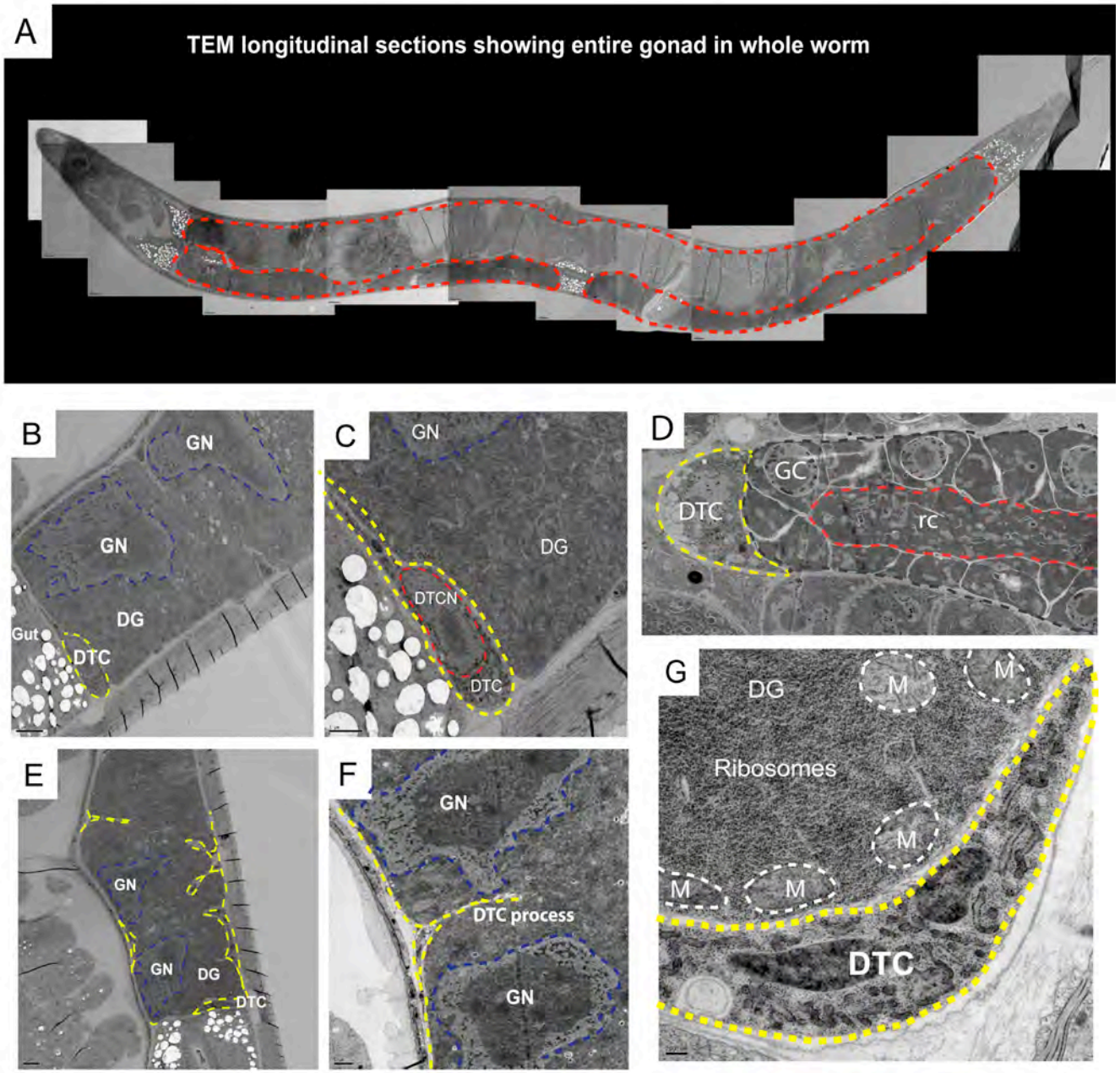
Strongyloides ratti *Parastrongyloides trichosuri*

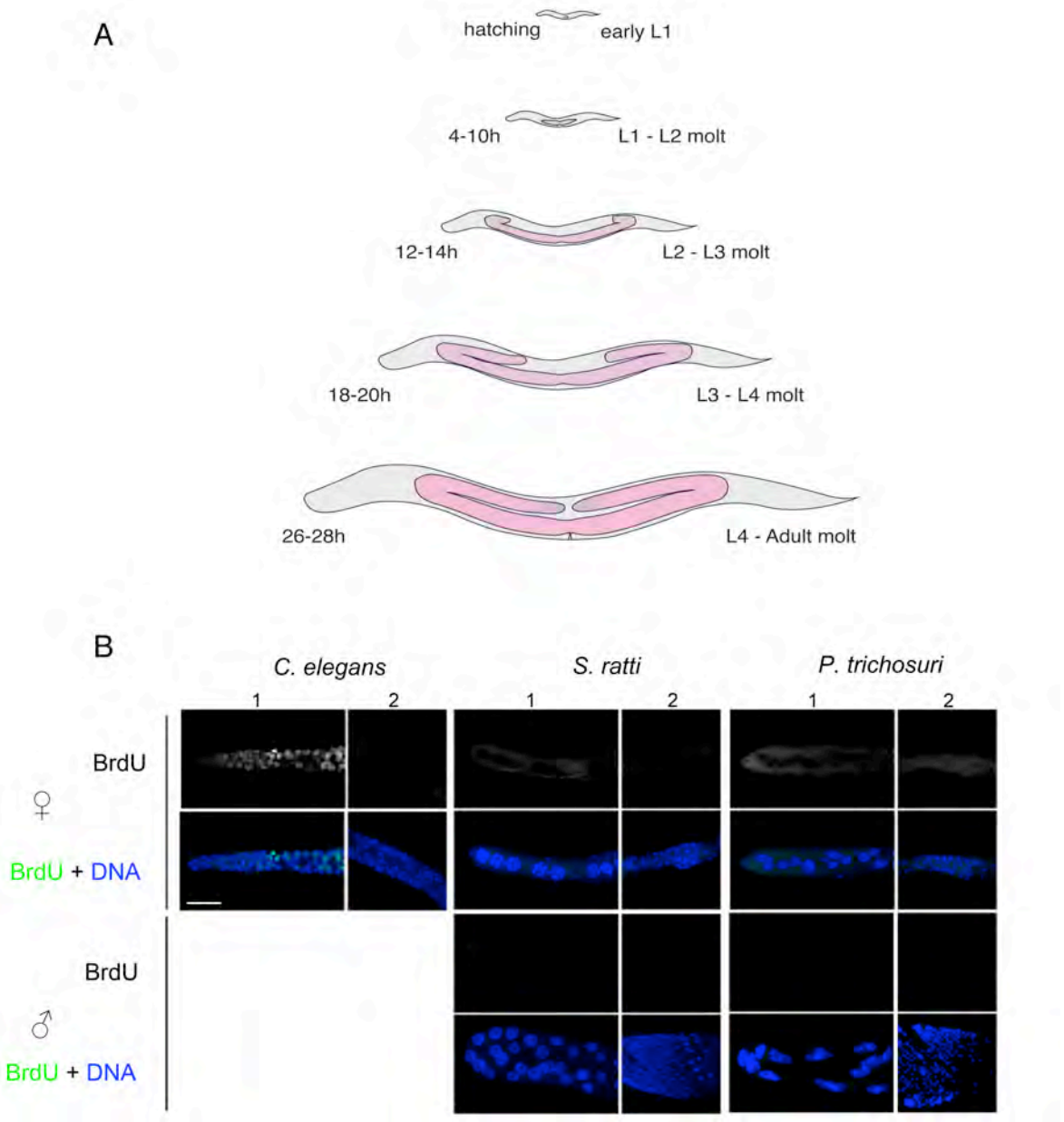
C

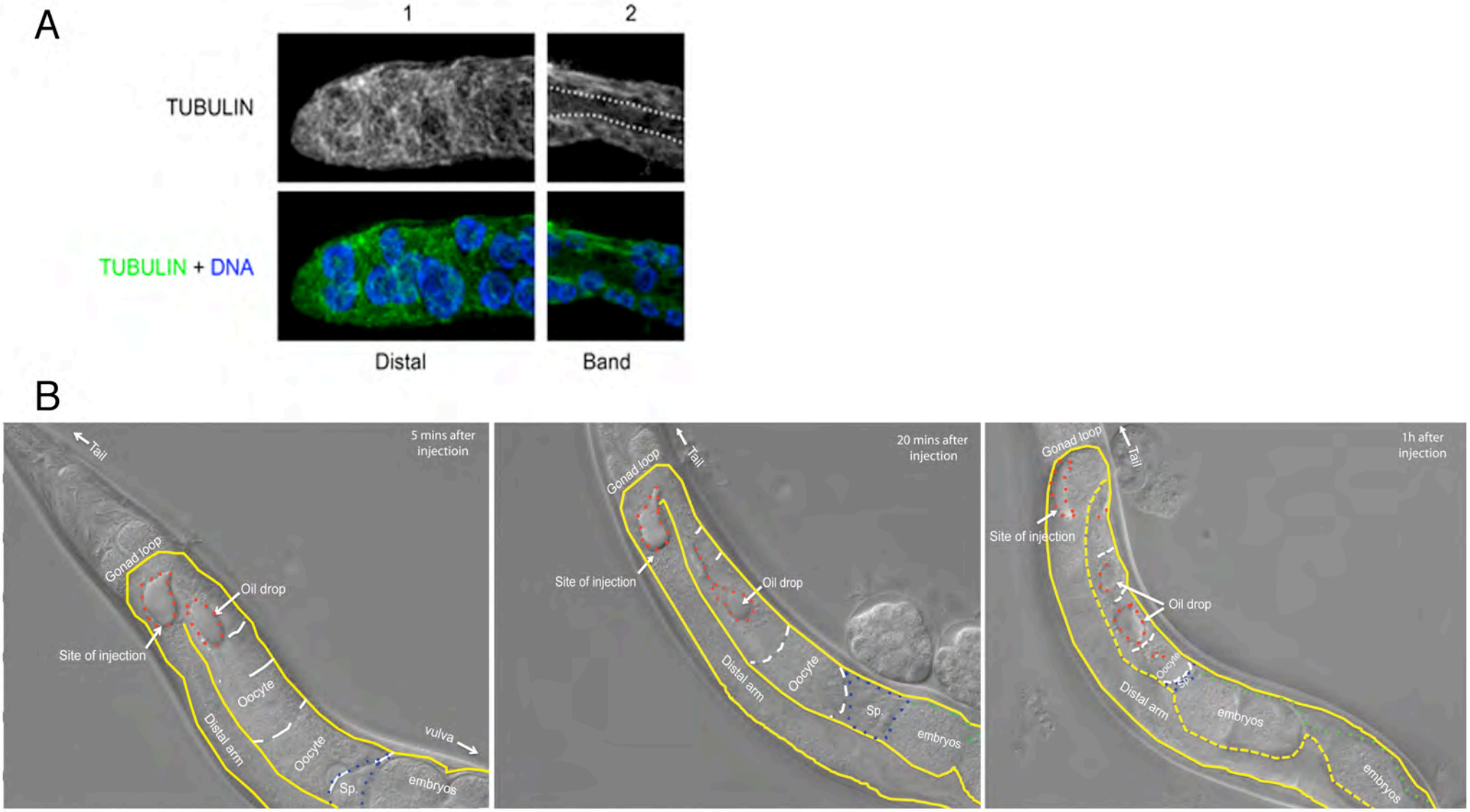
Molt	*C. elegans* (22°C)	For *Strongyloides* species	
		S. ratti (23°C)	*S. papillosus* (26°C)
L1 - L2*	~12h	~4h	10-12h
L2 - L3*	~20h	8-12h	~14h
L3 - L4*	~28h	14-18h	18-20h
L4 – Adult*	~38h	24-28h	24-26h
Adult capable of laying eggs	~46h	~28h	~28h

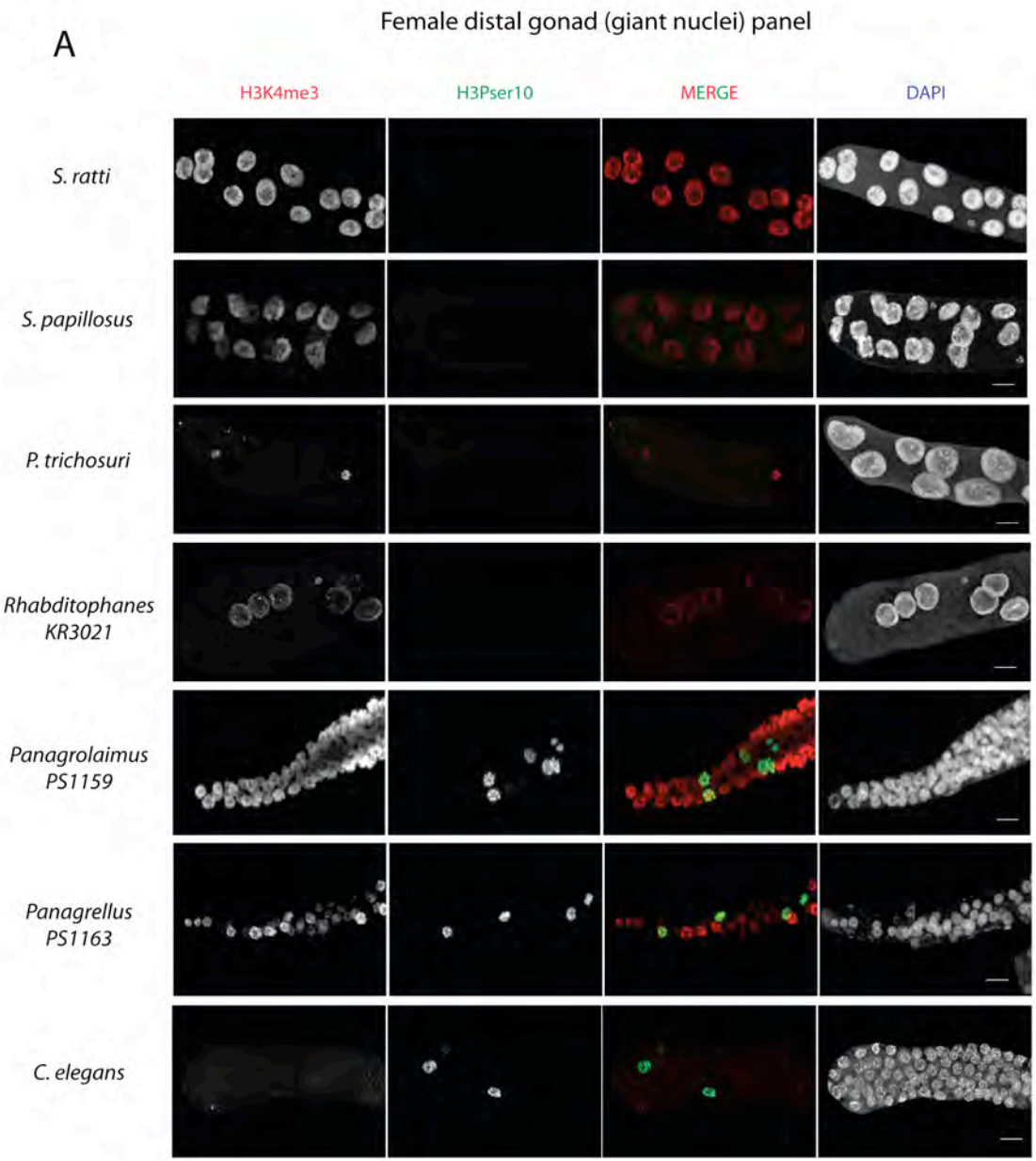
*Time estimates are post culturing for *Strongyloides* species

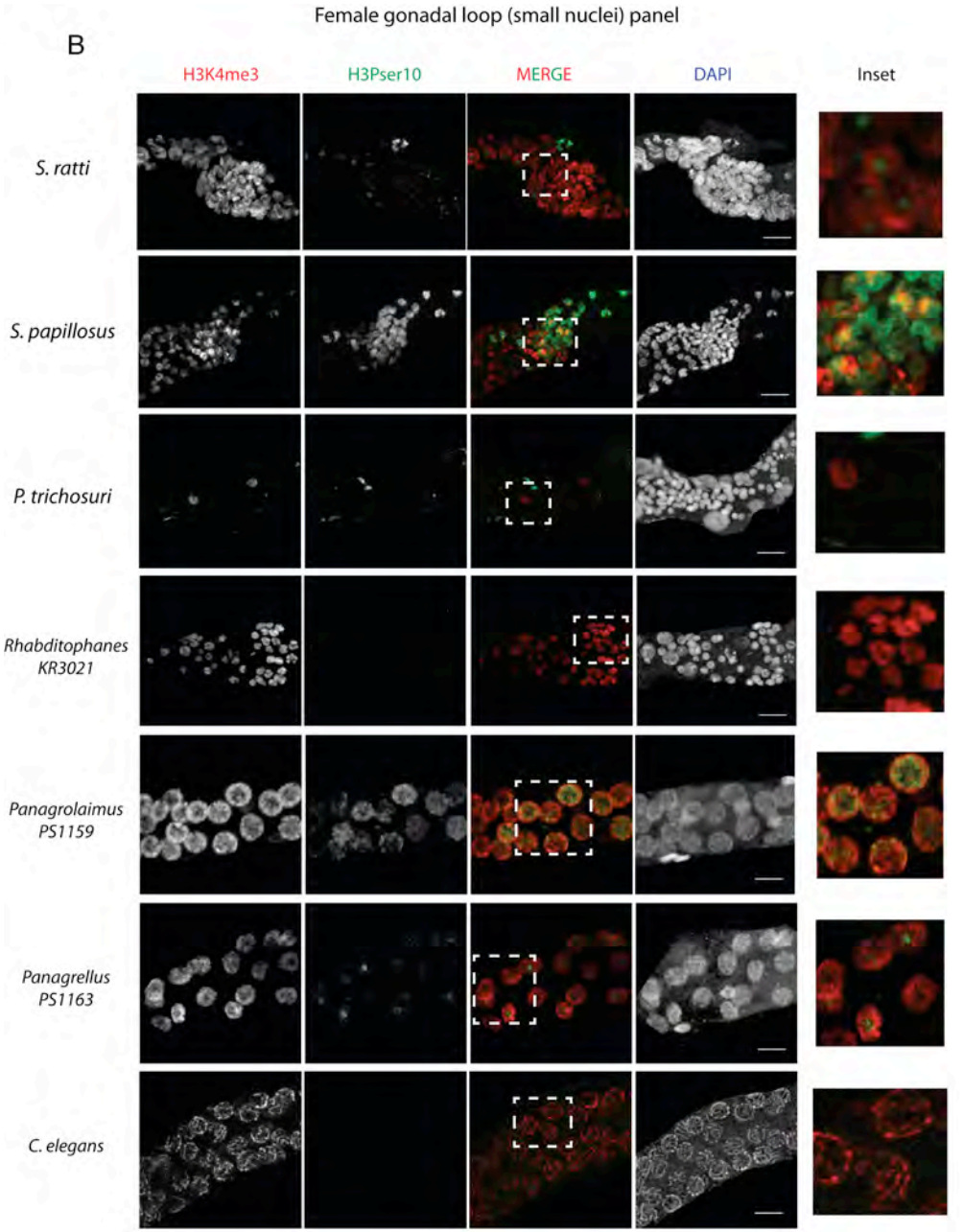

C. elegans
1
2
3
4
Distal tip
1. Mitotic cell at distal tip
2.Transition zone
3. Pachytene cells
4. Diakinesis
S. ratti
Small compact nuclei
Female
1
2
3
*
Giant nuclei
1. Giant nuclei at distal tip
2. small compact nuclei *
3. Diakinesis
4. Condensed meiotic chromosomes
1
4
Male

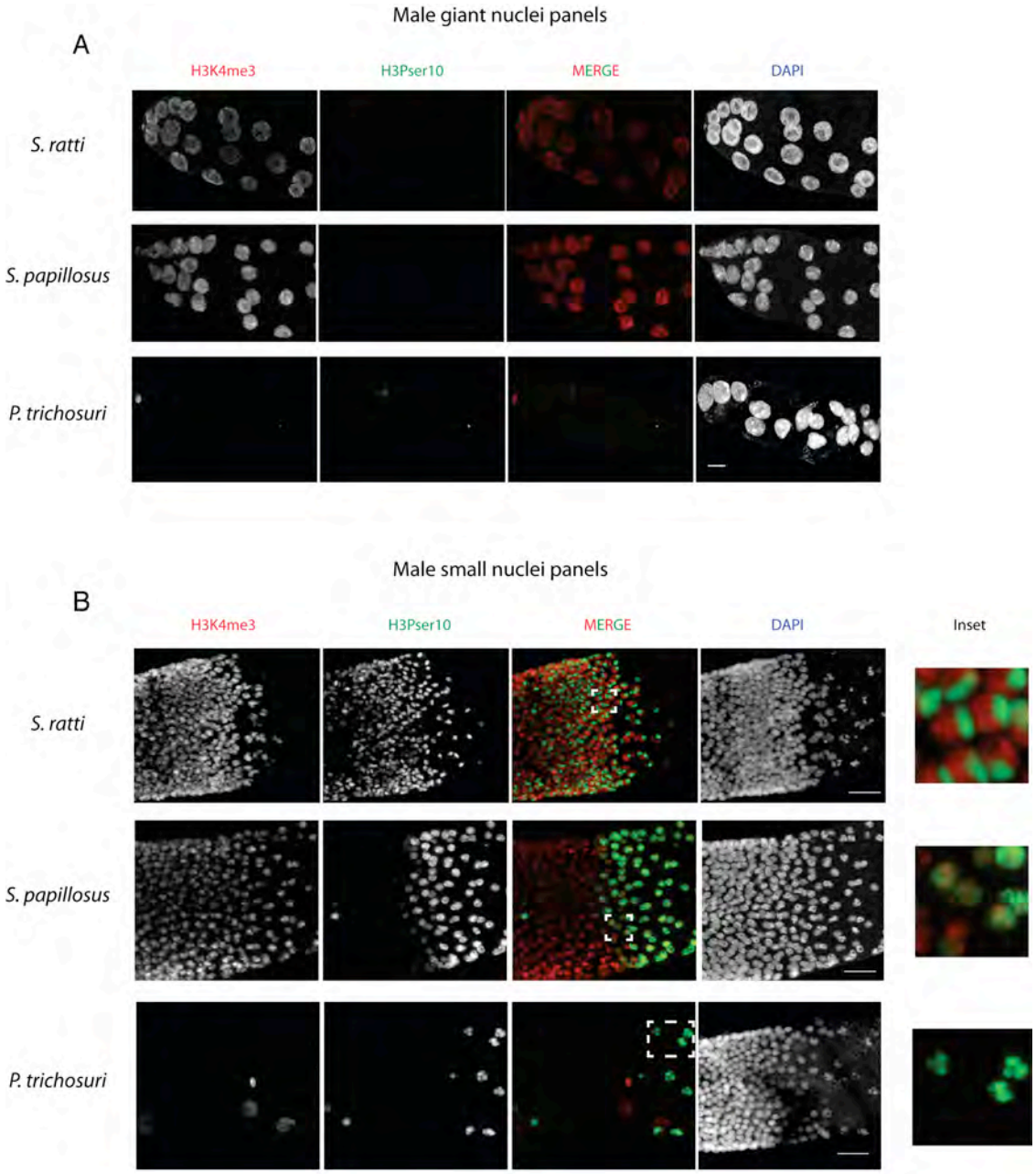

A
TEM longitudinal sections showing entire gonad in whole worm
B
GN
GN
DG
Gut
DTC
C
GN
DG
DTCN
DTC
D
GC
DTC
rc
E
GN
GN
DG
DTC
F
GN
DTC process
GN
G
DG
M
M
Ribosomes
M
M
M
DTC

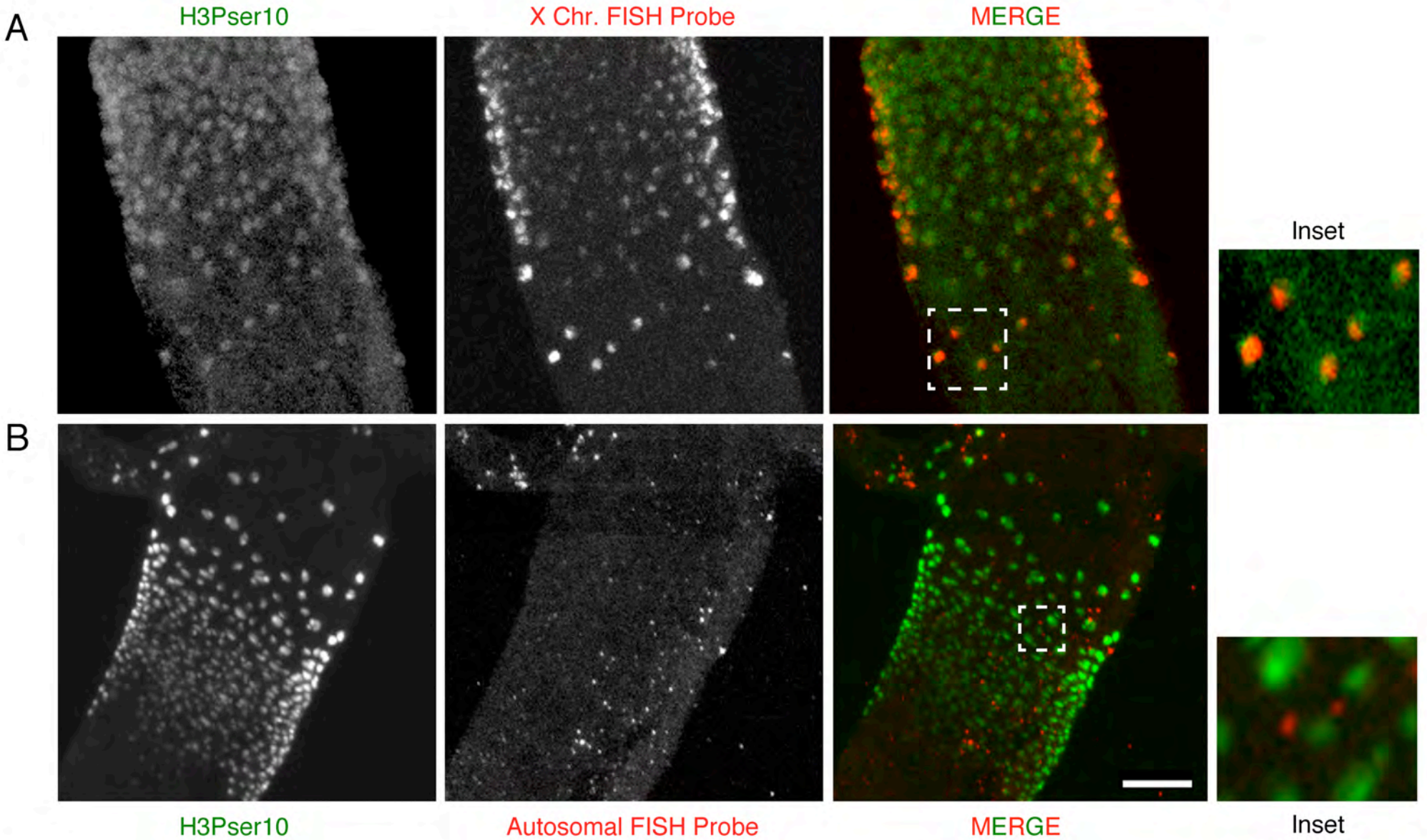

A
hatching
early L1
4-10h
L1 - L2 molt
12-14h
L2 - L3 molt
18-20h
L3 - L4 molt
26-28h
L4 - Adult molt
B
C. elegans
S. ratti
P. trichosuri
1
2
1
2
1
2
BrdU
♀
BrdU + DNA
BrdU
♂
BrdU + DNA


A
1
2
TUBULIN
TUBULIN + DNA
Distal
Band
B
5 mins after injectioin
Tail
Gonad loop
Oil drop
Site of injection
Oocyte
Oocyte
Distal arm
vulva
Sp.
embryos
20 mins after injection
Tail
Gonad loop
Site of injection
Oil drop
Distal arm
Oocyte
Sp.
embryos
1h after injection
Gonad loop
Tail
Site of injection
Oil drop
Oocyte
Sp.
Distal arm
embryos
embryos

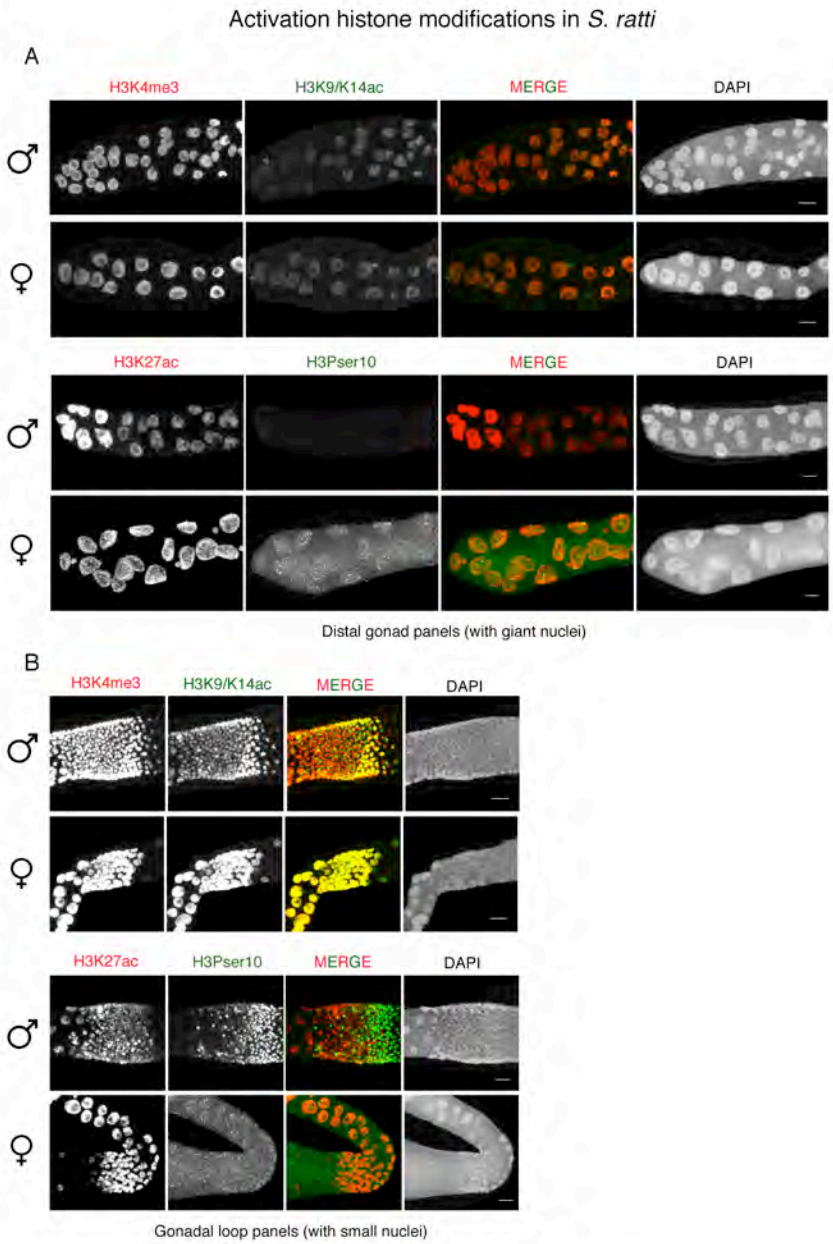

A
Female distal gonad (giant nuclei) panel
H3K4me3
H3Pser10
MERGE
DAPI
S. ratti
S. papillosus
P. trichosuri
Rhabditophanes KR3021
Panagrolaimus PS1159
Panagrellus PS1163
C. elegans

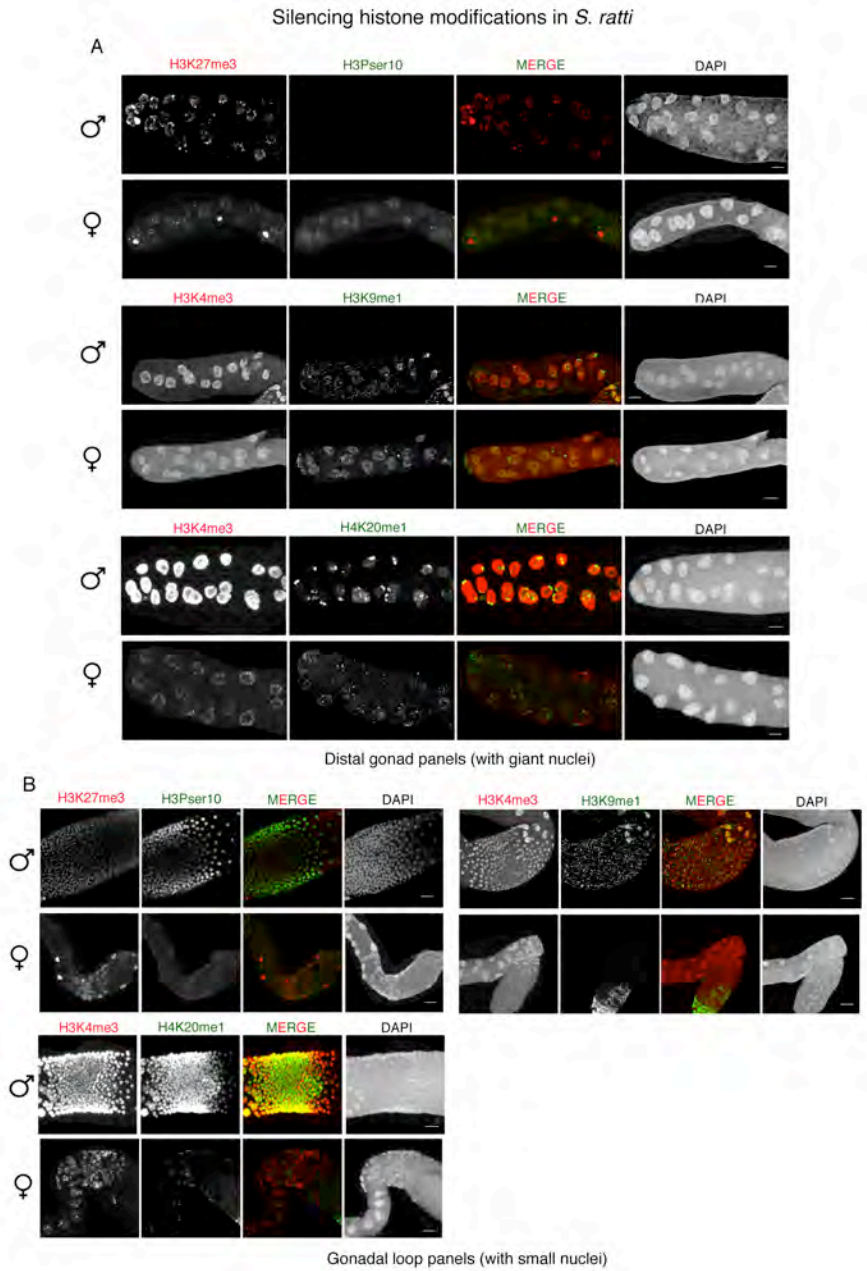

Female gonadal loop (small nuclei) panel
B
H3K4me3
H3Pser10
MERGE
DAPI
Inset
S. ratti
S. papillosus
P. trichosuri
Rhabditophanes KR3021
Panagrolaimus PS1159
Panagrellus PS1163
C. elegans

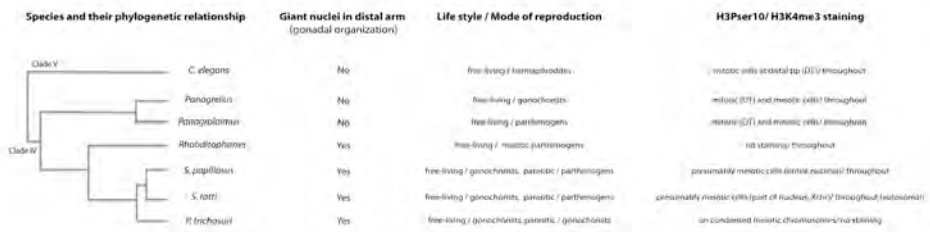

Male giant nuclei panels
A
H3K4me3
H3Pser10
MERGE
DAPI
S. ratti
S. papillosus
P. trichosuri
Male small nuclei panels
B
H3K4me3
H3Pser10
MERGE
DAPI
Inset
S. ratti
S. papillosus
P. trichosuri


A
H3Pser10
X Chr. FISH Probe
MERGE
Inset
B
H3Pser10
Autosomal FISH Probe
MERGE
Inset

9


Activation histone modifications in S. ratti
A
H3K4me3
H3K9/K14ac
MERGE
DAPI
♂
♀
H3K27ac
H3Pser10
MERGE
DAPI
♂
♀
Distal gonad panels (with giant nuclei)
B
H3K4me3
H3K9/K14ac
MERGE
DAPI
♂
♀
H3K27ac
H3Pser10
MERGE
DAPI
♂
♀
Gonadal loop panels (with small nuclei)


Silencing histone modifications in S. ratti
A
H3K27me3
H3Pser10
MERGE
DAPI
H3K4me3
H3K9me1
MERGE
DAPI
H3K4me3
H4K20me1
MERGE
DAPI
Distal gonad panels (with giant nuclei)
B
H3K27me3
H3Pser10
MERGE
DAPI
H3K4me3
H3K9me1
MERGE
DAPI
H3K4me3
H4K20me1
MERGE
DAPI
Gonadal loop panels (with small nuclei)



12

Nematode (female)	Phylogenetic distance to *S. ratti*	Gonadal organization similar to *Stongyloides*	Nematode clade	Life style/ Mode of reproduction	Distal arm		Gonadal loop	
					H3Pser10	H3K4me3	H3Pser10	H3K4me3
S. ratti	-	-	IV	Free living gonochorist	Absent	Present (high)	Present diffusely (nuclear localization distinct from H3K4me3)	Present high
S. papillosus	Closest Strongyloides species	Yes	IV	Free living gonochorist	Absent	Present (high)	Present (entire nucleus)	Present high
P. trichosuri	Closest outgroup species	Yes	IV	Free living gonochorist	Absent	Absent	Absent	Absent
Rhabditophanes KR3021	Closest sister family 'Alloionematoda'	Yes	IV	Free living parthenogen	Absent	Present (high)	Absent	Present
Panagrolaimus PS1159	Panagrolaimidae member	No (similar to *C. elegans*)	IV	Free living parthenogen	Present (mitotic cells)	Present	Present (meiotic cells)	Present
Panagrellus PS1163	Panagrolaimidae member	No (similar to *C. elegans*)	IV	Free living gonochorist	Present (mitotic cells)	Present	Present (meiotic cells)	Present
C. elegans	Farthest species in analysis	No	V	Free living hermaphrodite	Present (mitotic cells)	Present	Absent	Present

13

14

Nematode (male)	Phylogenetic distance to *S. ratti*	Gonadal organization similar to *Stongyloides*	Nematode clade	Life style/ Mode of reproduction	Distal arm		Gonadal loop	
					H3Pser10	H3K4me3	H3Pser10	H3K4me3
S. ratti	-	-	IV	Free living gonochorist	Absent	Present (high)	Present high (nuclear localization distinct from H3K4me3)	Present high
S. papillosus	Closest *Strongyloides* species	Yes	IV	Free living gonochorist	Absent	Present (high)	Present high (entire nucleus)	Present high
P. trichosuri	Closest outgroup species	Yes	IV	Free living gonochorist	Absent	Absent	Present (condensed meiotic chromosomes)	Absent

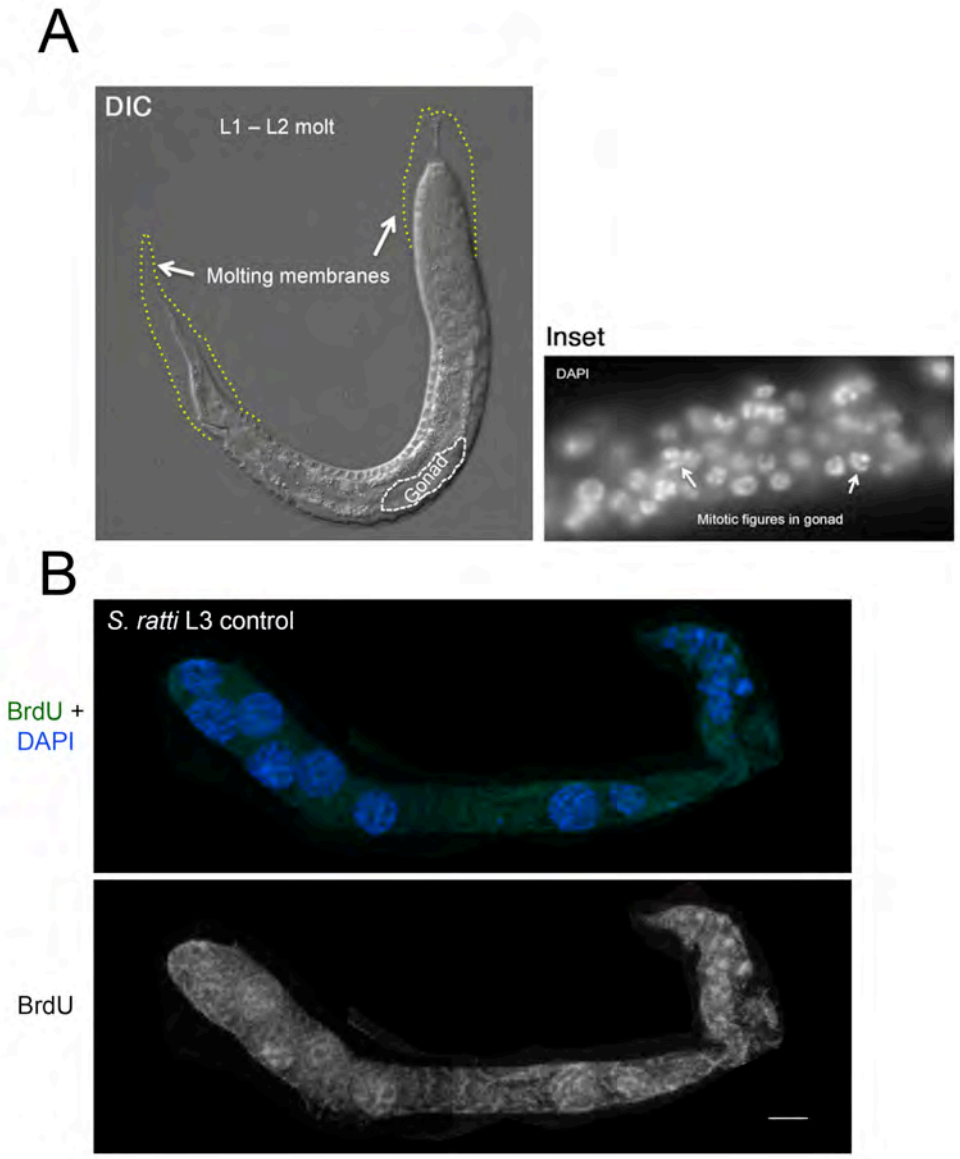
15

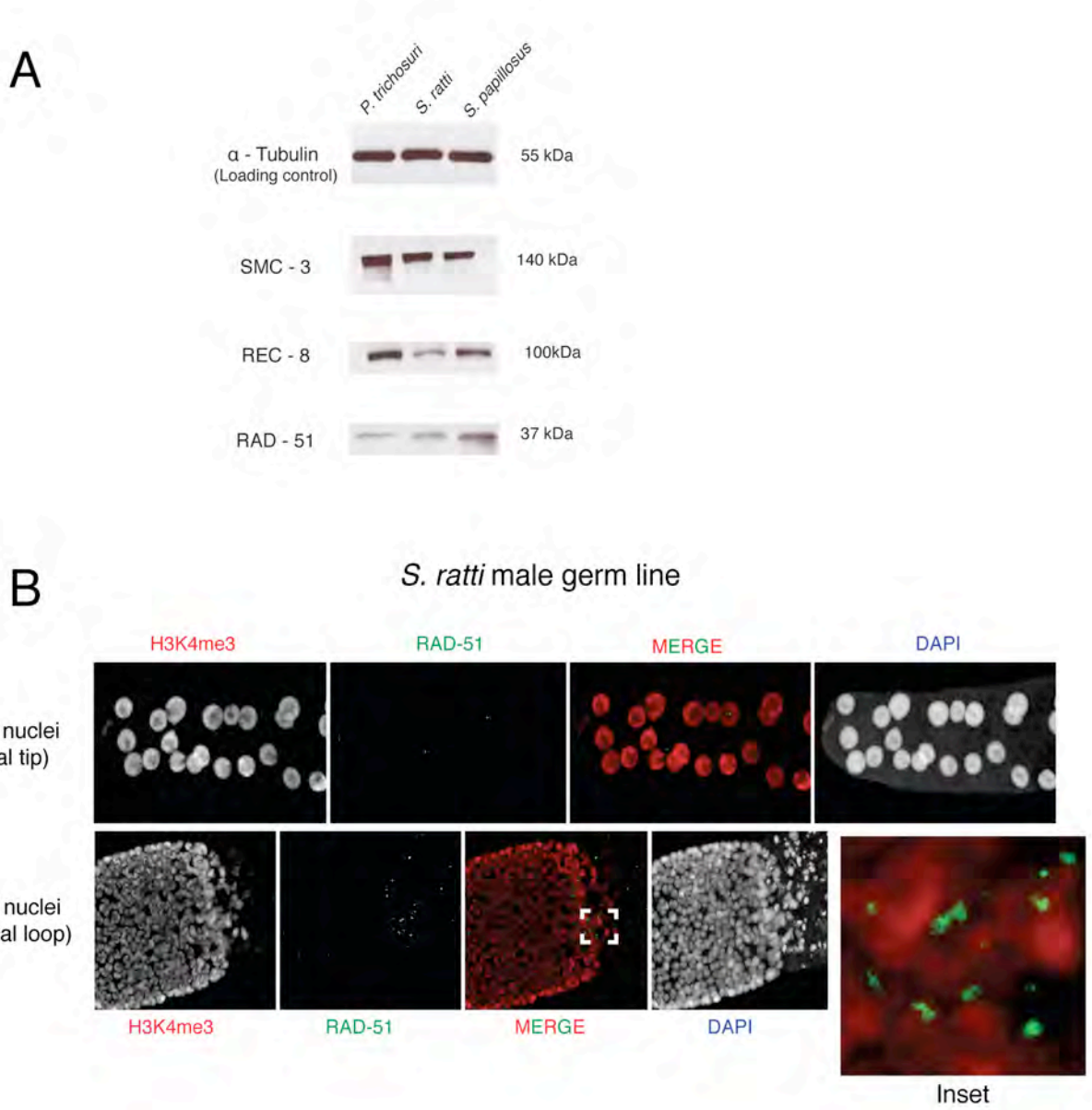
A In female germlines:

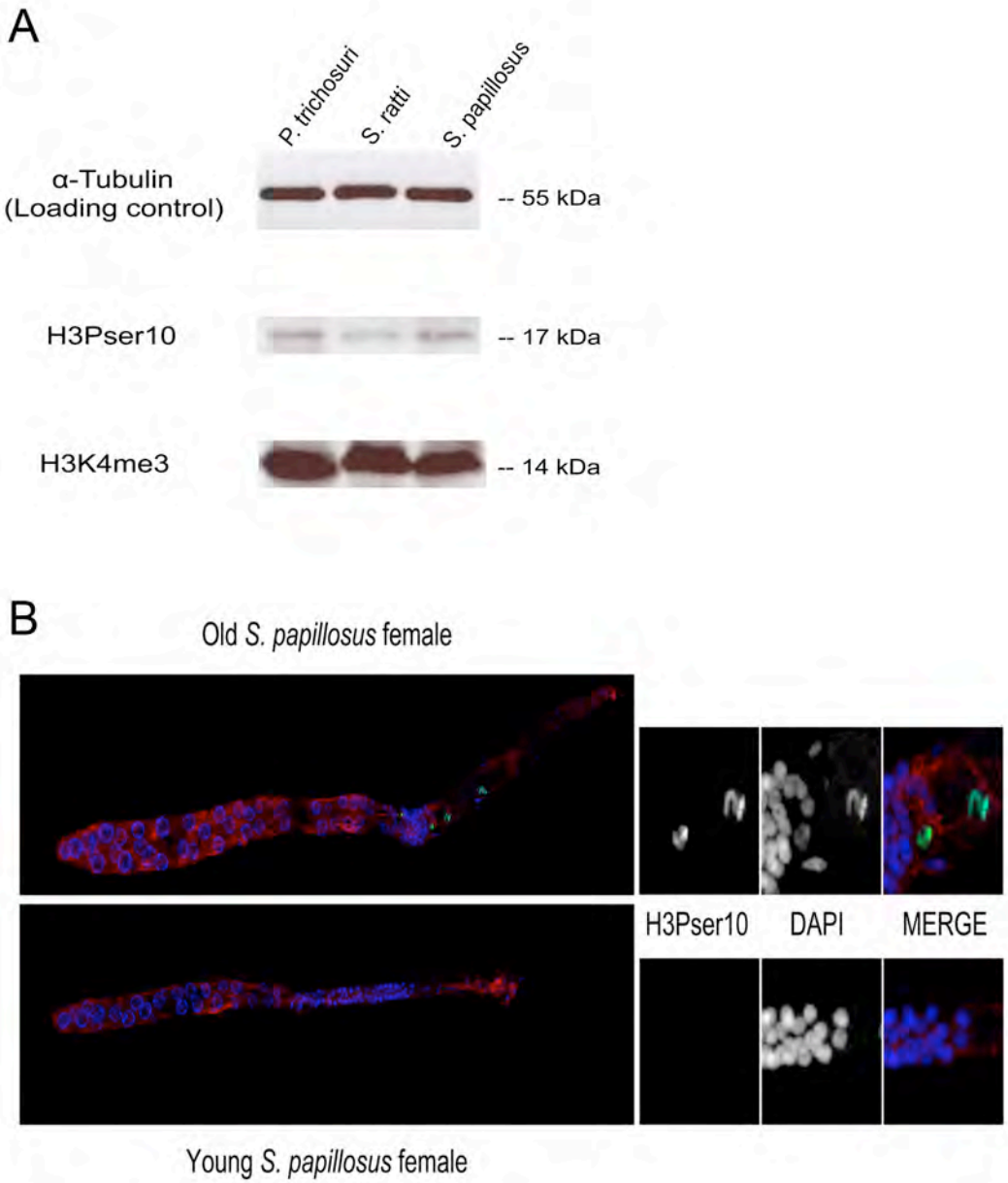
Histone modifications	*Distal arm (Giant nuclei)*	*Band of small compact nuclei*	*Diakinesis*	*Chromosome specificity*
H3^{P}ser10	Absent	Present (very low)	Present	Yes -- X chromosomal
H3K4me3	Present (high)	Present (high)	Present	Yes -- Autosomal
H3K9me	Present	Present (very low)	Absent	No -- entire nucleus
H3K27me3	Present (punctate)	Present	Present	Yes -- Autosomal
H3K9/K14ac	Present	Present (high)	Present	Yes -- Autosomal
H3K27ac	Present (high)	Present (high)	Absent	Yes -- Autosomal
H4K20me	Present	Present	Absent	No -- entire nucleus

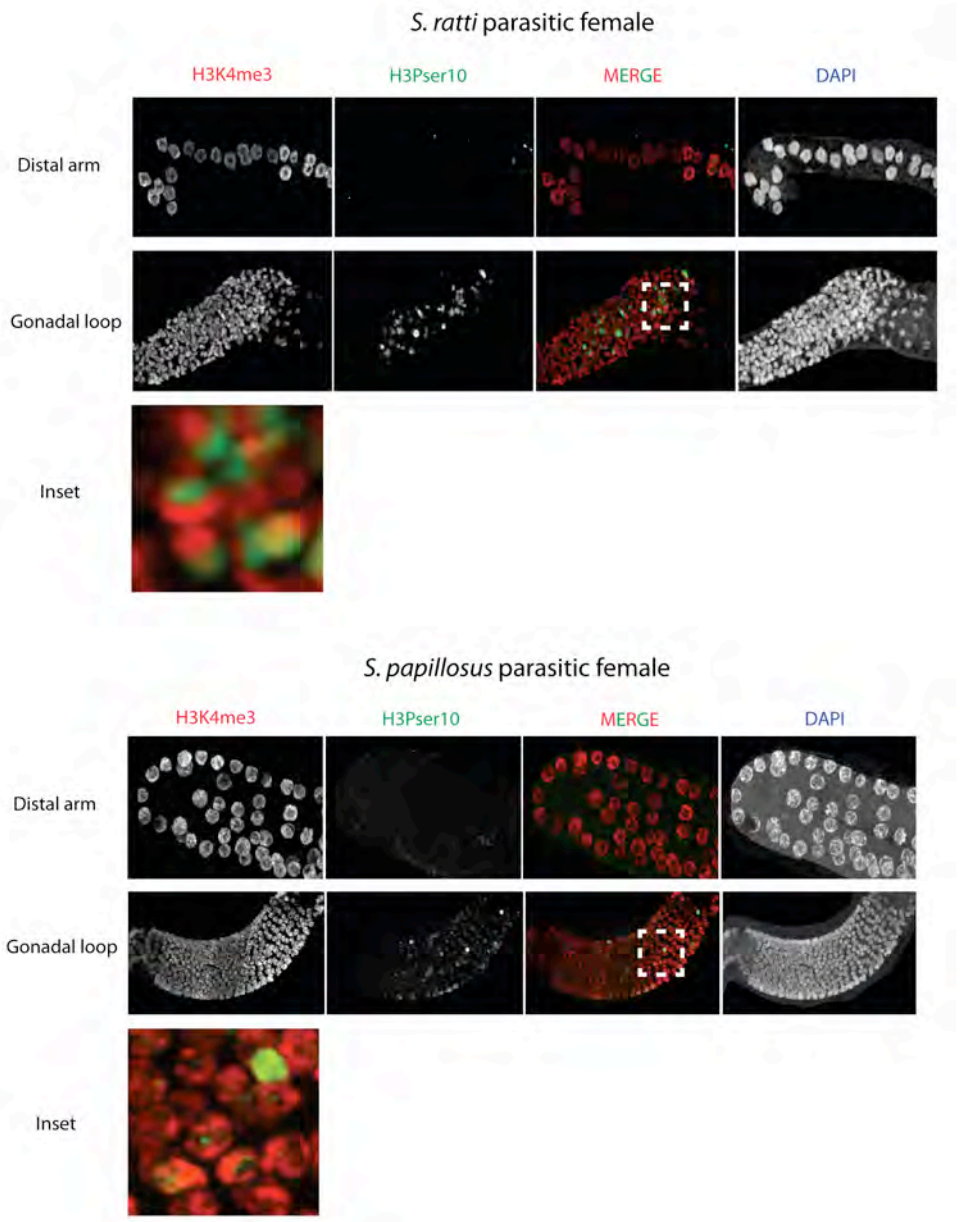
B In male germlines:

Histone modifications	*Distal arm (Giant nuclei)*	*Band of small compact nuclei*	*Spermatids*	*Chromosome specificity*
H3^{P}ser10	Absent	Present (high)	Absent	Yes -- X chromosomal
H3K4me3	Present (high)	Present (high)	Absent	Yes -- Autosomal
H3K9me	Present	Present (very low)	Absent	No – entire nucleus, maybe more on X
H3K27me3	Present (punctate)	Present	Absent	Yes -- Autosomal
H3K9/K14ac	Present	Present (high)	Absent	Yes -- Autosomal
H3K27ac	Present (high)	Present (high)	Absent	Yes -- Autosomal
H4K20me	Present (diffuse, high in some parts)	Present (high)	Absent	No – entire nucleus, stains X chromosome intensely


A
DIC
L1 – L2 molt
Molting membranes
Gonad
Inset
DAPI
Mitotic figures in gonad
B
S. ratti L3 control
BrdU +
DAPI
BrdU


A
P. trichosuri
S. ratti
S. papillosus
α - Tubulin
(Loading control)
55 kDa
SMC - 3
140 kDa
REC - 8
100kDa
RAD - 51
37 kDa
B
S. ratti male germ line
H3K4me3
RAD-51
MERGE
DAPI
Giant nuclei
(distal tip)
Small nuclei
(gonadal loop)
H3K4me3
RAD-51
MERGE
DAPI
Inset


A
P. trichosuri
S. ratti
S. papillosus
α-Tubulin
(Loading control)
-- 55 kDa
H3Pser10
-- 17 kDa
H3K4me3
-- 14 kDa
B
Old S. papillosus female
H3Pser10
DAPI
MERGE
Young S. papillosus female


S. ratti parasitic female
H3K4me3
H3Pser10
MERGE
DAPI
Distal arm
Gonadal loop
Inset
S. papillosus parasitic female
H3K4me3
H3Pser10
MERGE
DAPI
Distal arm
Gonadal loop
Inset