Molecular taxonomic characterization of the human parasitic population of the nematode *Strongyloides stercoralis* in Cambodia and development and evaluation of methods for the genetic study of *Strongyloides ratti*

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1 Summary

Nematodes of the genus *Strongyloides* are common small intestinal parasites of vertebrates. They have a complex life cycle, which in addition to parthenogenetic parasitic adults also contains a facultative free-living adult generation, with males and females. The presence of sexually reproducing adults outside of the host offers opportunities for genetic research, which are quite unique for an endo-parasitic organism. Accordingly, *Strongyloides* spp. is developing into a model system for parasitological, basic biological and evolutionary studies.

In the first part of my thesis, I examined the Small Ribosomal Subunit rDNA (SSU) sequences from *S. stercoralis* larvae isolated from human patients in Cambodian highly *S. stercoralis* prevalent areas. Three polymorphic positions and three different haplotypes were identified within a region of the SSU normally considered to be essentially invariable within a nematode species. Interestingly, no hybrid individuals were found. These results suggested a low frequency of interbreeding between the different haplotypes in this area, either because *S. stercoralis* in this region reproduces only asexually or because crossing happens only within rather than between haplotypes.

Many research tools and techniques routinely used in model organisms like *Caenorhabditis elegans* are not yet available for *Strongyloides* spp. One of these methods is mutagenesis using chemical mutagens. In the second part of my thesis I devised a protocol to mutagenize *S. ratti* with the chemical mutagen Ethyl Methanosulfonate (EMS). Using this protocol, I generated *S. ratti* mutants with a higher proportion of animals developing into the parasitic form. As a control, I also attempted to obtain the same effect by selection only, in absence of EMS. Next I evaluate the possibility of identifying the mutated genes by whole genome sequencing of multiple mutagenized and selected strains. While this approach appeared promising, I also found that the currently used laboratory strain is not sufficiently isogenic such that the number of resulting candidate mutations, which need to be tested is rather high. The strong population bottlenecks associatiated with the mutagenesis and selection procedures reduced the genetic complexity of the populations significantly. This demonstrated that generating a more isogenic *S. ratti* strain for genetic work is possible.

In the third part of my thesis I isolated and characterized *Strongyloides* mariner-like transposons (SMARTs). Contrary to *S. ratti,* in *S. papillosus* several copies of SMART appeared potentially active. These transposons have the potential to be used as genetic tools as it has been demonstrated for the related Tc1 transposons in *C. elegans*.

2 Zusammenfassung

Nematoden der Gattung *Strongyloides* sind weit verbreitete Dünndarmparasiten von Wirbeltieren. Der komplexe Lebenszyklus beinhaltet neben parthenogenetischen parasitischen Adulttieren auch eine fakultative frei lebende Generation mit Männchen und Weibchen. Die Existenz von Geschlechtstieren außerhalb des Wirts eröffnet Möglichkeiten für genetische Studien, die für Endoparasiten außergewöhnlich sind. Dementsprechend entwickelt sich *Strongyloides* spp. zu einem Modellsystem für parasitologische, grundlagenbiologische und evolutionsbiologische Studien.

Im ersten Teil meiner Doktorarbeit untersuchte ich die Sequenzen der rDNA für die kleine ribosomale Untereinheit (SSU) von *S. stercoralis* Larven aus Patienten in Regionen Kambodschas mit hoher *S. stercoralis* Prävalenz. Ich identifizierte drei polymorphe Positionen und drei verschiedene Haplotypen in einem Abschnitt der SSU, der normalerweise als praktisch invariable innerhalb einer bestimmten Art gilt. Interessanterweise gab es keine Hybride. Dieses Resultat lässt vermuten, dass sich *S. stercoralis* in dieser Gegend ausschließlich parthenogenetisch vermehrt, oder dass Kreuzung nur innerhalb, aber nicht zwischen den Haplotypen vorkommt.

Viele Methoden, die in Modellorganismen wie *C. elegans* routinemäßig angewandt werden, sind für *Strongyloides* spp. noch nicht verfügbar. Eine davon ist die Mutagenese mittels chemischer Mutagene. Im zweiten Teil meiner Doktorarbeit erarbeitete ich ein Protokoll zur Mutagenese von *S. ratti* mit Ethyl Methanosulfonat (EMS). Ich isolierte mutante *S. ratti* Linien, die sich zu einem größeren Anteil zu parasitischen Individuen entwickelten. Als Kontrolle versuchte ich den gleichen Effekt auch durch reine Selektion, ohne Mutagen zu erzeugen. Ich testete ich, ob es möglich ist die mutierten Gene durch Genomsequenzierung der mutanten und selektierten Linien zu identifizieren. Der Ansatz erwies sich als vielversprechend, Allerdings fand ich, dass der momentan verwendete Laborstamm nicht ausreichend isogen ist. Die Anzahl der isolierten Kandidatenmutationen, die getestet werden müssten, stellte sich deshalb als recht hoch heraus. Die mutagenisierten und selektierten Linien, die in Folge des Experiments durch starke "population bottlenecks" gegangen waren zeigten eine deutlich reduzierte genetische Variabilität. Dies zeigte, dass es möglich ist, einen mehr isogenen *S. ratti* Stamm für genetische Arbeiten zu erzeugen.

Im dritten Teil meiner Doktorarbeit isolierte und charakterisierte ich "*Strongyloides* mariner-like Transposons" (SMARTs). Anders als in *S. ratti* fand ich in *S. papillosus* mehrere möglicherweise aktive SMART Kopien. Diese Transposone haben das Potential als genetischen Werkzeugen benutzt zu werden, wie dies für die verwandten Tc1 Transposone in *C. elegans* der Fall ist.

3 Introduction

3.1 Strongyloidiasis and *Strongyloides* spp.

Strongyloidiasis is a parasitic disease caused by nematodes, or roundworms, of the genus *Strongyloides* and is most common in tropical or subtropical countries (Viney & Lok 2007). Members of the genus *Strongyloides* are small intestinal parasites of vertebrates and over 50 species were described from different hosts (Speare 1989). These nematodes are only rather distantly related to the nematode model organism *Caenorhabditis elegans* based on the phylogeny of the phylum Nematoda according to Blaxter et al. (1998). *Strongyloides stercoralis* is the major cause of human Strongyloidiasis, and recently attracts more and more interest in human parasitology.

In 1876 the French physician Louis Alexis Normand first recognized Strongyloidiasis and examined the worm from faecal samples with the microscope (Normand, 1876). Meanwhile, Bavay described the worm as *Anguillula stercorale* and *Rhabditis stercoralis* (Bavay 1876). In 1883 Rudolf Leuckart reported initial observations on the life cycle of this nematode (Leuckart 1983), and Paul Van Durme described the mode of infection through the skin (Van Durme 1902). The interest increased in the 1940s when a study revealed disseminated infections in immunosuppressed patients (Gill & Bell 1979).

3.1.1 Strongyloidiasis

Strongyloidiasis is worldwide spread and over 370 million people are estimated to be infected (Bisoffi et al. 2013). It is most common in tropical or subtropical climates and has been reported in Southeast Asia, Australia, Africa, Europe, Latin America and south

eastern United States (Johnston et al. 2005; Genta 1989; Liu & Weller 1993; Berk et al. 1987).

The parasitic nematodes *Strongyloides* spp. enter the host through exposed skin and establish infection in the small intestine (Streit 2008). Heavy infection with *S. stercoralis* leads to the symptoms of Strongyloidiasis. They are usually diarrheal and weight loss. Chronic infections with very low worm burdens may last for decades through an auto-infective cycle with the infective L3 larvae even in absence of new infection (Siddiqui & Berk 2001). The current record appears to be 65 years (Liu & Weller 1993).

Because of the low parasite load and the irregular larval output, Strongyloidiasis is sometimes difficult to diagnose (Siddiqui & Berk 2001). Strongyloidiasis is determined by clinical symptoms, such as eosinophilia and serologic finding (Grove 1996; Liu & Weller 1993; Genta 1989; Berk et al. 1987). Definitive diagnosis of Strongyloidiasis is usually made on the basis of larvae detection from the stool. The larvae of *S. stercoralis* are more easily detected in the case of strong autoinfection, because large numbers of worms are involved in disseminated infections (Heyworth 1996; Liu & Weller 1993). Detection from a single stool examination by use of conventional techniques may fail to detect larvae in up to 70% of cases. The detection rate can approach 100% if 7 serial stool samples are examine (Nielsen & Mojon 1987). Serological methods, like enzyme-linked immunosorbent assays (ELISAs) and indirect immunofluorescent test (IFAT), were successfully introduced in the detection of Strongyloidiasis (Boscolo et al. 2007; Conway et al.). Antibodies (IgG, IgG4 and IgE) are widely used in the detection against *S. stercoralis* (Norsyahida et al. 2013). Schär reported the first application of real time PCR in the diagnosis of *S. stercoralis* (Schär et al. 2013).

A molecular method is widely applied to identify nematodes of different species by

amplifying the small subunit ribosomal DNA. The ribosomal DNA consist of the small subunit 18S DNA (SSU) and the large subunit 5.8S and 28S DNAs (LSU), which are separated by internal transcribed spacer I and II (ITS1 and ITS2) (Ellis et al. 1986). About 55 copies of the rDNA were found in *C. elegans*, and a similar number is likely present in other nematodes (Dorris et al. 1999; Ellis et al. 1986). The SSU is highly conserved in sequence and is about 1700 base pairs in length and used in phylogenetic analysis across all organisms. The ITS regions are highly variable and can be used for the measurement of genetic distance within species (Chilton et al. 1995).

3.1.2 The nematode phylogenetic classification

Nematodes consist of large and widely distributed groups of animals in marine, freshwater, and terrestrial habitats. Estimates of the number of species in this phylum range from 40,000 to 100 million (Dorris et al. 1999). The traditional nematode phylogenetic classifications rely on morphological traits, such as buccal, pharyngeal structure, intestine, tail and etc. Because of the microscopic size of these structures, comparative morphological studies in nematodes are rather prone for observer bias and error, compared to studies on other taxa with various species (Dorris et al. 1999). The results consequently lead to some disagreements on nematode phylogeny. The application of the electron microscope gave more exact details for morphological research in nematodes (Gibbons 1986). However, the high expense and inconvenience prevent the use of electron microscopy for large scale phylogenetic analyses of nematodes. Recently, DNA sequence analysis was employed as an alternative method to determine phylogenetic relationships.

Blaxter directly compared free-living and parasitic taxa by amplifying rDNA sequences, which are found in all nematodes and keep a conserved function (Blaxter et al. 1998). This method allows comparing also distant taxa and gives more information about the phylogenetic structure of Nematoda. Parasitic species can be found in all five major clades within the phylum (Blaxter et al. 1998). Within the nematodes parasitism has evolved at least seven times independently from one another (Blaxter et al. 1998). Within the phylum Nematoda, over 16,000 of the about 28,000 described species are parasitic (Hugot et al. 2001).

While in the old phylogenies based on morphology *Stronghlyoides* spp. was considered to be fairly closely related to *Caenorhabditis elegans*, in the original molecular phylogeny based on the SSU *Strongyloides* was included in clade IV, and with this rather distant from *C. elegans*, which falls into clade V (Blaxter et al. 1998). The SSU sequences were later used as molecular markers in *Strongyloides* spp. in a number of taxonomic studies (Hasegawa et al. 2009; Dorris et al. 2002; Eberhardt et al. 2008; Hasegawa et al. 2010).

3.1.3 The parasitic model organism

In biological research, model organisms as the nematode *Caenorhabditis elegans* have been frequently used. Because it is easy to keep and breed, the small free-living soil nematode *Caenorhabditis elegans* is used as an experimental model organism in a variety of study areas, such as genetics, cell biology, developmental biology and neurobiology (Riddle et al. 1997).

Currently, there is no comparatively well-studied parasite model to investigate for example the co-evolution and the interaction between host and parasite. However, *S.*

stercoralis, S. papillosus and *S. ratti* and the closely related facultative parasite *Parastrongyloides trichosuri* are increasingly well studied and develop into an ideal group of organisms for the study of nematode parasitism (Viney & Lok 2007).

The maintenance of *Strongyloides* species relies on a vertebrate host. Because of the ability to tolerate infection with large numbers of infective larvae and the higher larvae output, the human parasite *S. stercoralis* is usually maintained in dogs in the laboratory environment (Schad et al. 1989). Gerbils are used as alternative lab host when requiring many replicate host infections (Lok 2007). The laboratory cultures of *S. papillosus* can be maintained in rabbits, which are non-natural but permissive hosts for the parasite of sheep (Eberhardt et al. 2007). *S. ratti* is maintained in its natural host but also infects gerbils under laboratory conditions.

3.1.4 Life cycle of *Strongyloides* spp.

The life cycle of *Strongyloides* species is quite different from other nematodes (Figure 1.1). The parasitic females infect the vertebrate host by skin penetration, establish infection in the small intestine, and reproduce parthenogenetically. The progeny leaves the host with the faeces and can undergo either of two fundamentally different life cycles. i) Direct or homogonic life-cycle: the larvae develop to infective third-stage larvae (iL3s) and invade a new host by skin penetration; all homogonically developing larvae are females; ii) Indirect or heterogonic life cycle: the larvae develop into non-infective L3s, which develop into free-living adults; this free-living generation consists of males and females and reproduces sexually; usually, all the progeny of the free-living generation are female and develop into iL3s (Streit 2008). The existence of the free-living generation provides a unique opportunity for the experimental manipulation (e.g. crossing) of a true parasite.

The human parasite *S. stercoralis*, shows a third developmental cycle, which is of great medical importance. A fraction of the larvae develop into infective larvae within the gut of the host and are able to re-infect the same host individual without leaving it. In immunocompetent patients an *S. stercoralis* infection can be maintained through this auto infective cycle for decades at very low level without clinical symptoms. If later the person becomes immunocompromised, the infection can self-enhance and result in a hyper infection syndrome and disseminated Strongyloidiasis, which, when untreated, is frequently fatal (Grove 1989).

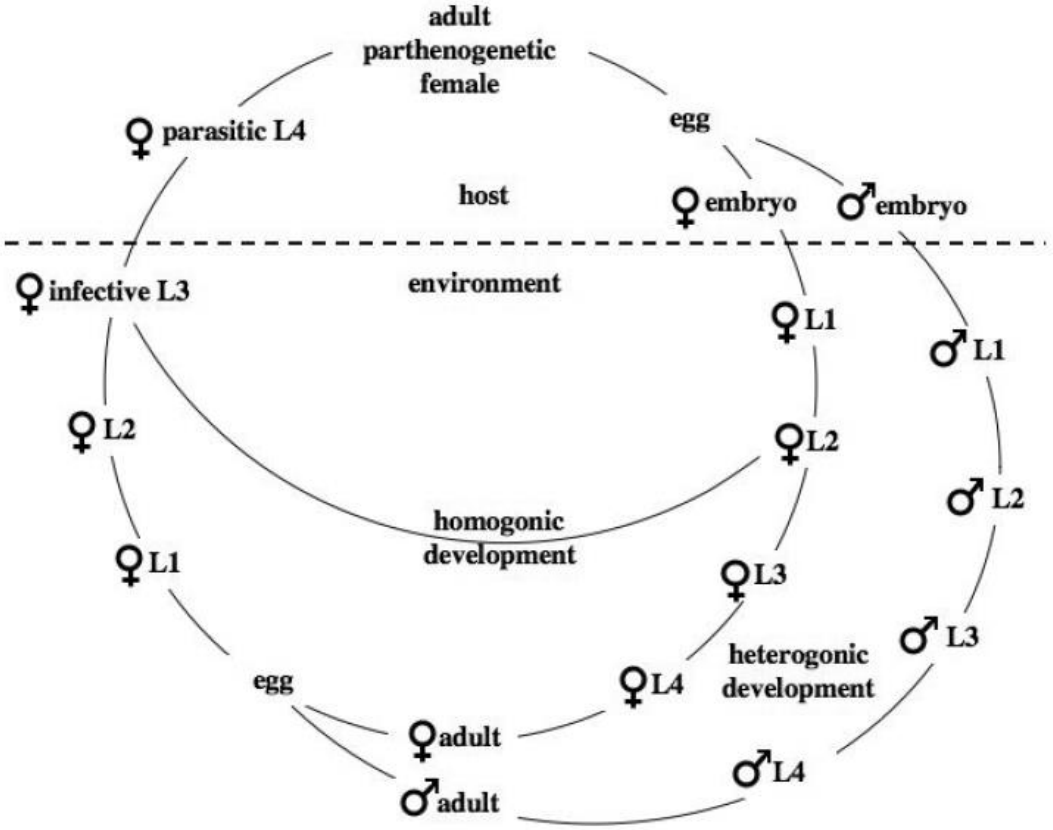

Figure 1.1: Generalized life-cycle of *Strongyloides* (Streit 2008).

Two genetic switches are important in the life history of *Strongyloides*. The first one is sex determination. The sex of an embryo is determined very early in development and is influenced by the immune status of the host (Gemmill et al. 1997). The second switch is

between homogonic and heterogonic development. In this female only choice, the larvae choose to develop directly into iL3s or develop into free-living adult females. The genetic background and the immune status of the host, affect both, the sex choice and the homogonic versus heterogonic switch, such that increased host immunity against *Strongyloides* leads to a higher proportion of males and predisposes females to undergo heterogonic development (Crook & Viney 2005; Harvey et al. 2000; Triantaphyllou & Moncol 1977). The homogonic versus heterogonic switch, but not the sex, can still be influenced by multiple environmental factors the larvae encounter only after leaving the host (Streit 2008). But the mechanism of this genetic switch is still not clear.

3.1.5 Sex determination in *Strongyloides* spp.

In the animal kingdom, the mechanisms of sex determination have been studied. The predominant one is considered as sex chromosome based mechanism. Of this mechanism, the sex determination employ either heterogametic chromosome based mechanism (XX/XY and ZW/ZZ system), or sex chromosome ratio based system (XX/XO), the ratio between the number of sex chromosome and autosomes lead the consequence of females and males. In the XX/XY system, the male is heterogametic (XY); in the ZW/ZZ system, female is heterogametic (ZW) (Namekawa & Lee 2009). However, some organisms like zebra fish have no sex chromosome. The sex is influenced by environmental factors (Slanchev et al. 2005).

The nematode *C. elegans*, uses a sex chromosome ratio based system (XX/XO). There is no Y chromosome. The male is heterogametic and denoted as XO; the hermaphrodite is homogametic as XX. In *C. elegans* male (XO) embryos, low X dosage activate the male-

specific switch gene *xol-1*. In contrast, the female (XX) embryos develop as a result of *xol-1* inactivation (Miller et al. 1988). XX/XO sex determination mechanism is very common across the phylum Nematoda (Walton 1940). Only a few of nematodes employ XX/XY system: *Brugia malayi, Onchocerca volvulus, Baylisascaris transfuga, Contracaecum incurvum*, and *Trichuris muris* (Zanetti & Puoti 2013).

The sex determination of *Strongyloides* species was studied cytologically and genetically (Bolla & Roberts 1968; Nigon & Roman 1952; Harvey & Viney 2001; Hammond & Robinson 1994). The cytological evidences in *S. stercoralis* and *S. ratti* indicate that the species employ an environmentally controlled XX/XO system (Figure 1.2) (Streit 2008). The females have 2 pairs of autosomes and 1 pair of X chromosomes; the males have the same autosomes and only 1 X chromosome (Nigon & Roman 1952; Bolla & Roberts 1968; Hammond & Robinson 1994). Nevertheless, although a sex chromosome is present, the proportion of males produced by the parasitic females is influenced by the immune response of the host (Gemmill et al. 1997). The cytological studies suggested that *S. papillosus* and *S. ransomi* do not have sex chromosomes (Triantaphyllou & Moncol 1977). Unlike in a conventional XX/XO system, *S. papillosus* was shown to achieve a chromosomal difference between the sexes through sex specific chromatin diminution (Figure 1.2)(Albertson et al. 1979; Nemetschke, Eberhardt, Hertzberg, et al. 2010). In males an internal portion of one of the homologues of one pair of chromosomes gets eliminated, creating a hemizygous region, which corresponds to the X chromosome in *S. ratti*. The result suggests that a chromosome fusion event may have occurred in the predecessor of *S. papillosus* but not *S. ratti* (Triantaphyllou & Moncol 1977; Nemetschke, Eberhardt, Hertzberg, et al. 2010; Kulkarni et al. 2013).

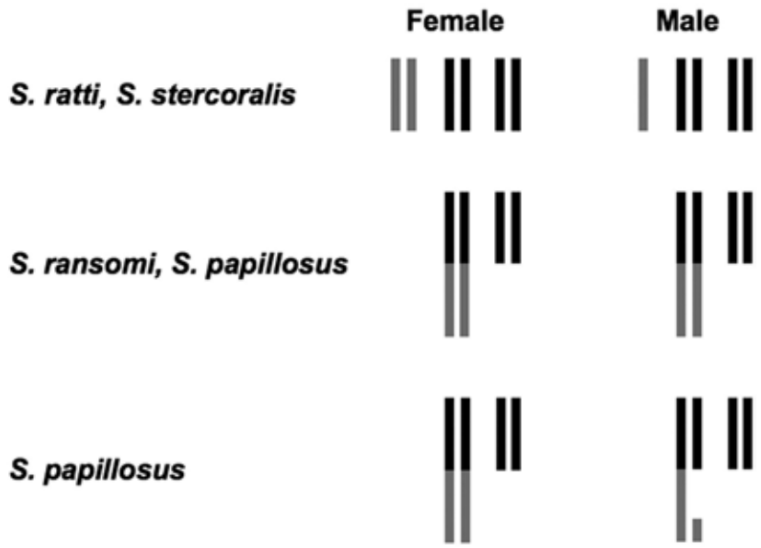

Figure 1.2: Schematic representation of the various karyotypes in females and males proposed for different species of *Strongyloides* (Streit 2008). (A) XX/XO sex determination. Autosomes (two pairs) are in black, X chromosomes are in grey. (B) No karyotypic difference between the sexes. (C) Sex specific chromatin diminution in males. In (B) and (C) the regions that were proposed to be derived from an X chromosome are in grey.

3.1.6 *Strongyloides* infective larvae development

In the response to harsh environmental conditions, nematode *C. elegans* undergo a dauer arrest after the second molt (Cassada & Russell 1975). The *Strongyloides* species have a complex life-cycle (Figure 1.1), which includes both an obligatory parasitic generation as well as a facultative free-living generation. The parasitic larvae are morphologically similar to dauer larvae of *C. elegans*.

The molecular mechanisms that govern the dauer developmental transition in *C. elegans* have been well characterised in past decades. The regulation of morphological development in *C. elegans* served as a model case for the study of organismal development. The regulation of *C. elegans* dauer development is undertaken through several interacting

signal transduction pathways (Hu 2007). Recent studies give more details and imply more complicated regulatory pathways. Identified pathways for controlling dauer formation include cGMP signalling, insulin/IGF-1 signalling (IIS), TGF-beta signalling and a hormone pathway (Kimura et al. 1997; P Ren et al. 1996; Birnby et al. 2000; Hu 2007). The cGMP signalling pathway regulates the dauer switch upstream of the parallel TGF-beta and IIS pathways (Fielenbach & Antebi 2008). The hormone pathway works downstream of the cGMP, TGF-beta and IIS pathways (Fielenbach & Antebi 2008). Neurons were found intensively involved in the dauer switch of *C. elegans*. The environmental signals are transformed into endocrine signals by amphid neurons (ASI, ADF, ASG, ASJ, ASK, AWA, and AWC) (Bargmann & Horvitz 1991; Schackwitz et al. 1996; P. Ren et al. 1996; Sze et al. 2000; Li et al. 2003). Serotonergic signalling in the neuron ADF relays environmental information and control dauer formation through DAF-16 (Liang et al. 2006).

The dauer stage in *C. elegans* is considered to be evolutionarily related to dauer-like developmental stages in parasitic nematodes i. e. infective larvae (Viney 2009; Hotez et al. 1993; Bürglin et al. 1998). Recent studies suggest that some regulatory pathways controlling the morphological development are conserved. Akira Ogawa in Ralf Sommer's lab, in collaboration with our lab, showed that the hormone Δ7-dafachronic acid (Δ7-DA) which block the dauer formation in *C. elegans*, can also inhibit the development of iL3s in *S. papillosus* (Ogawa et al. 2009). This result strongly suggests that these two distantly related nematodes share a conserved endocrine pathway in developmental regulation. In *C. elegans*, this endocrine module is at the very end of the genetic cascade that controls dauer formation. Numbers of homologs from the pathways were cloned in *S. ratti*, *S. stercoralis*, and their close relate species *Parastrongyloides trichosuri*, a parasite of Australian possums (Massey et al. 2001; Massey et al. 2006; Massey et al. 2005; Stoltzfus, Massey, et al. 2012; Siddiqui et al. 2000; Castelletto et al. 2009; Crook et al. 2005; Viney 2006). To understand the expression of these cloned gene in *Strongyloides* species, the

transcriptomes of *S. stercoralis* were analysed by comparing the expression profiles with that of *C. elegans* (Stoltzfus, Minot, et al. 2012). The result suggested significant differences in the patterns of gene expression. The dauer regulatory pathway genes are present in *S. stercoralis*. While the expression patterns of IIS and cGMP signalling pathway genes appear to be conserved with those in *C. elegans*, the TGF-beta signalling pathway genes appear to be expressed differently.

3.1.7 Transposable elements in *C. elegans*

After the first transposable element had been found in maize, the first transposon isolated from *C. elegans* was *Tc1* (Rosenzweig et al. 1983). It is a marine-like transposon and functions by "cut and paste" mechanism. Like other members of *mariner*-like super family, *Tc1* consists of two inverted terminal repeats (ITRs), flanking one open reading frame, which encodes the protein necessary to accomplish the transposition reaction (Rosenzweig et al. 1983). In addition to *Tc1*, Colloms et al identified another *marine*-like transposon gene, *Tc3*, in *C. elegans* (Colloms et al. 1994). In the genome of *C.elegans* N2, there are 31 *Tc1*s and 22 *Tc3*s, but these numbers are strain dependent. Greenwald took advantage of the presence of transposons to generate mutations which were tagged by the transposon using a mutator strain in which the transposons are activated due to a mutation (Greenwald 1985). However, as mentioned above, the endogenous transposons are present in multiple copies rendering the tag not unique and spontaneous re-excision can lead to tag loss. To circumvent these drawbacks, the exogenous transposon Mos1 was introduced and became a most useful tool in *C. elegans* studies (Jacobson et al. 1986), which is discussed in more detail in sections 1.2 and 2.3.

3.2 Research strategies and methodology for the study of *Strongyloides* spp.

3.2.1 Next generation sequencing in nematode and parasitic nematode

The genome of *C. elegans* was the first sequenced genome from a multicellular organism. The complete assembly of the genome without gap is available for comprehensive analysis (Hillier et al. 2005). The nematode genomes, for which information is available, vary in size from about 50 to about 250 Mb (Rödelsperger et al. 2013). The genome of *C. elegans* is ~100 Mb and has ~20,000 genes. The genome of the related species *C. briggsae* is about 5 Mb bigger, but has roughly the same number of genes as *C. elegans* (Stein et al. 2003). The difference in size between two genomes is in part the consequence of different amounts of repetitive sequence. Parkinson et al. used over 250,000 ESTs to analyse 30 species across the phylum (Parkinson et al. 2004). Of the in total 90,000 analysed genes, around 15,000 genes were found across all clades, only ~1300 genes were nematode-specific, meaning that they were present in most if not all nematodes but absent from all other phyla.

After the sequencing of the *C. elegans* genome, the genomes of a variety of nematodes were determined and used in recent research. The genome sequences of seven additional *Caenorhabditis* species are available on WormBase and can be used in comparative analyses (Yook et al. 2012). In addition, genomes of several parasitic nematodes were finished, such as *Meloidogyne hapla*, *M. incognita, Ascaris suum,* genomes of *S. ratti* and *S. stercoralis* are in process (Martin et al. 2012; Yook et al. 2012).

Among the *Strongyloides* species, *S. ratti*, and *S. stercoralis* are the most intensively studied ones. For *S. ratti*, Thompson et al. analysed 15,000 ESTs from free-living and

parasitic cDNA libraries, which grouped into over 4000 clusters (Thompson et al. 2005). One quarter showed non-significant BLAST alignments; around three quarter had significant alignments. This result probably reflects the large evolutionary distance between taxa (Parkinson et al. 2004). A similar result was shown by Mitreva et al, who obtained about 11,000 ESTs which grouped into over 3000 clusters (Mitreva et al. 2004). Only about 15% of the clusters had no significant alignment. The analysis of secretome data showed that 89.8% excretory/secretory protein had homologues in the free-living nematode *C. elegans* and 86.3% in other parasitic nematodes (Garg & Ranganathan 2011). In another *S. stercoralis* transcriptomic analysis, 56% putative protein sequences had orthologs/homologs in public databases (Marcilla et al. 2012). The rest sequences remained unannotated.

Thompson et al. used microarray for gene expression analysis of the free-living and parasitic stage in *S. ratti* (Thompson et al. 2006). Ramanathan et al. analysed the differences between non-infective first stage and infective third-stage larvae of *S. Stercoralis* (Ramanathan et al. 2011). Twenty five genes were found highly expressed in infective L3. A RNAseq analysis of *S. stercoralis* revealed divergent regulation of genes involved in the control of dauer formation in *C. elegans* (Stoltzfus, Minot, et al. 2012).

Most genes in parasitic nematode are probably inherited from their free-living ancestors (Blaxter 2003). Some genes essential for parasitism in worms were novel. One explanation is that this kind of genes may derive from gene duplication, which make one duplicate available for the acquisition of a new, i.e. parasitism related, function while the other copy retains the original function (Gomez-Escobar et al. 2002). The other explanation is horizontal gene transfer (Dieterich & Sommer 2009). Plant parasite *Meloidogyne* species were reported to carry genes acquired from bacteria (Bird et al. 2003; Dieterich & Sommer 2009).

Although, the free-living stages of *Strongyloides* can be grown on bacterial lawns like *C. elegans*, large-scale culturing of *Strongyloides* is still limited. This makes it more difficult to conduct, for example, proteomic studies of *Strongyloides* as it has been done in free living nematodes *C. elegans* and *P. pacificus* (Audhya & Desai 2008; Borchert et al. 2010).

3.2.2 Transgenesis

First attempts to establish transgenes in *S. stercoralis*, *S. ratti* and in the related nematode *P. trichosuri* were based on protocols developed for the model nematode *C. elegans* (Li et al. 2006; Grant et al. 2006; Rieckher et al. 2009; Li et al. 2011). This was successful in *P. trichosuri* (Grant et al., 2006) but in *Strongyloides* spp. the results were not satisfactory. While reporter constructs containing promoters, and 5' and 3' untranslated regions (UTR) were expressed in a tissue-specific manner in the first generation, they were silenced in subsequent generations (Junio et al. 2008; Li et al. 2006; Lok & Massey 2002). This problem was later solved by employing a strategy that is based on the piggyBac transposon such that the desired construct is flanked by transposon derived terminal repeats and injected into the gonad of *Strongyloides* spp. females along with a helper plasmid containing the piggyBac transposase gene (Shao et al. 2012). When lacking the helper vector, the transgenes were only transiently expressed in F1 generation after transformation and became silenced in later generations, although their physical presence could still be detected.

3.2.3 Mutagenesis

A protocol of chemical mutagenesis for *S. ratti* was applied to isolate invermectin resistant worms (Viney et al. 2002). However, so far this is the only report of a forward genetic screen in any species of *Strongyloides* and the mutations causing the phenotype were not identified. In *C. elegans* a completely sequenced genome and a dense genetic map allow the identification of the mutations, which cause a particular phenotype (Kutscher & Shaham 2014). Although the genome of *S. ratti* is being sequenced at the Sanger Institute and a first genetic map was established in our lab, these tools are still very limited in *Strongyloides* (Nemetschke, Eberhardt, Viney, et al. 2010). As mentioned above transposons were used to generate tagged mutations in several systems, among them *C. elegans* where the transposable element Tc1 was used successfully to generate mutants (Greenwald 1985; Moerman et al. 1986). No attempts to use transposons as mutagen have been made in *Strongyloides* spp. yet.

3.2.4 Cell ablation

The cell deactivation through laser ablation became an important method, which allows for example neuronal studies in model nematode *C. elegans* (Bargmann & Avery 1995). The morphological similarities between *C. elegans* and *Strongyloides* spp. gives the possibility to identify homologous cells in *Strongyloides* species. Ashton introduced this protocol to *S. stercoralis* as the first parasitic nematode and analysed neurons in detail (Ashton et al. 1995; Ashton et al. 1998). From his results, 13 amphidial neurons were identified and named by using *C. elegans* nomenclature (Ashton et al. 1995). The ALD neurons in *S. stercoralis* are likely the homologs of the AWC neurons in *C. elegans* (Lopez et al. 2000). Nolan et al. reported that the ALD neurons in *S. stercoralis* controls the

temperature-sensitive choice of alternative developmental pathways (Nolan et al. 2004). The homologs of *C. elegans* ASJ in *S. stercoralis* were found to trigger the resumption of development of infective larvae, an equivalent to dauer stage (Ashton et al. 2007). These findings suggest conserved neurons in *S. stercoralis*. In the process of interaction with external environment, neurons transmit the signals and cause downstream regulations.

3.2.5 RNA interference in parasitic nematodes

Gene silencing through double stranded RNA (dsRNA) interference was successfully established in *C. elegans* (Fire et al. 1998). However, the introduction of RNAi to parasitic nematodes is not straightforward (Britton et al. 2012). The efficiencies were found to vary among species. The technique was successfully applied in the plant parasitic nematodes *Heterodera glycines*, *Globodera pallida* and *Meloidogyne incognita* (Urwin et al. 2002; Dalzell et al. 2010). Unsuccessful attempts to use of RNA interference were reported in the animal parasite *Ostertagia ostertagi, Haemonchus contortus* and *Heligmosomoides polygyrus* (Lendner et al. 2008; Geldhof et al. 2006; Visser et al. 2006). The hypotheses to explain the difficulties with RNAi in parasitic nematodes that were proposed are a) the delivery of dsRNA to parasitic nematodes is inappropriate (Viney & Thompson 2008). Parasitic nematodes seem to be generally refractory to systemic RNAi from dsRNA present in the intestinal lumen; b) genes required for RNAi are functionally incapable to initiate gene silencing in animal parasitic nematodes (Viney & Thompson 2008). In *C. elegans* the uptake of environmental dsRNA requires the transmembrane proteins SID-1 and SID-2 (Winston et al. 2002; Winston et al. 2007). The intercellular spread of double stranded RNA in *C. elegans* requires RSD-3, *rsd-3* null mutants are unable to distribute dsRNA into germline (Dalzell et al. 2011). However, recent reports suggest that SID-2 is not widely conserved even among the *Caenorhabditis* species (Winston et al. 2007;

Hunter et al. 2006). No homologs of SID-1/2 were found in the animal parasitic nematodes *Haemonchus contortus* and *Heligmosomoides polygyrus*, and the plant parasites *G. pallida* and *M. incognita* (Geldhof et al. 2006; Lendner et al. 2008; Britton et al. 2012). Nevertheless, without identifiable orthologs of SID-1/2 and RSD-3, the plant parasitic nematodes *Meloidogyne* and *Globodera* species are susceptible to RNA interference by soaking in dsRNA/siRNA (Rosso et al. 2009; Dalzell et al. 2011; Dalzell et al. 2010; Kimber et al. 2007). The results suggested that alternative mechanisms for dsRNA distribution are involved (Dalzell et al. 2011).

3.3 Aim of this thesis

Given that *Strongyloides* spp. is not only an emerging model system for both basic biological and parasitological research but also a medically relevant parasite of humans and livestock, in this thesis I attempted to contribute to three different areas of *Strongyloides* research.

a) Development of methods for the analysis of *Strongyloides* spp. To this end I isolated an endogenous transposon from *S. papillosus*, which has the potential to be developed into a tool for mutagenesis and transgenesis and I devised a protocol for chemical mutagenesis of *S. ratti*.

b) Epidemiology of human *Strongyloidiasis*. In collaboration with a research group at the Swiss Tropical and Public Health Institute in Basel I molecular genetically characterized *Strongyloides* spp. isolated from humans and animals in rural Cambodia. I identified multiple non-interbreeding genotypes of *S. stercoralis* in humans.

c) Basic biology of an *S. ratti* developmental switch. Combining mutational analysis and selection experiments with genomic approach I characterized potential mutations in the developmental switch between the homogonic (direct) and the heterogonic (indirect) development.

4 Results and discussion

4.1 *Strongyloides stercoralis* genotypes in humans in Cambodia

Li Guo[1], Fabian Schär[1], Adrian Streit, Virak Khieu, Muth Sinuon, Hanspeter Marti, Peter Odermatt

[1]Shared first authorship. The paper was published as Schär, Guo et al.

4.1.1 Synopsis

The nematode *Strongyloides stercoralis* is one of the worldwide distributed human parasitic nematodes. However, it is one of the most neglected helminth infections in tropical areas. The larvae infect human host by skin penetration and reproduce parthenogenetically in the small intestine. The adult females can give rise to either parasitic female progeny or to a facultative free-living generation reproducing sexually. As a suitable host, humans can be also infected by another species, *S. fuelleborni* in Africa and Papua New Guinea.

The small subunit (SSU) rDNA is for the most part highly conserved among organisms, allowing the design for PCR primers that work for many different species over a fairly broad phylogenetic range. However, it also contains so called hyper variable regions (HVR-I to -IV) in which species tend to differ. Therefore this region is popular for phylogenetic studies of various organisms including of the genus *Strongyloides*. The SSU sequences of *S. stercoralis* from different locations and hosts had been described. However, a study of the genetic structure of *S. stercoralis* populations within a certain location had not been reported.

To examine the *SSU* genotypes of *S. stercoralis* larvae from Cambodian highly *S. stercoralis* endemic regions, larvae were collected from infected persons. The portions of the *SSU* including HVR I and HRV IV were sequenced from individual *S. stercoralis* larvae. Three polymorphic positions and three different haplotypes were identified. Interestingly, no hybrid individuals were found. These results suggested that interbreeding between the different haplotypes in the study area is very rare, either because in this region *S. stercoralis* reproduces asexually only or because mating occurs only within but not between haplotypes.

4.1.2 Contribution

Fabian Schär and Virak Khieu collected samples in Cambodia. I genotyped all larvae from all samples. Analysis of sequencing data was done by myself and Fabian Schär. The results were interpreted together with Adrian Streit, Hanspeter Marti and Peter Odermatt. Fabian Schär and Peter Odermatt wrote the manuscript, Adrian Streit, Simuon Muth, Hanspeter Marti and I assisted with manuscript revisions.

4.2 A Protocol for chemical mutagenesis in *Strongyloides ratti*

Li Guo, Zisong Chang, Christoph Dieterich and Adrian Streit

4.2.1 Synopsis

Forward genetics was first established in fruit fly *Drosophila melanogaster* and became an important approach in the analysis of many model organisms. Mutations are generated and followed by screening for the desired phenotypes. Since the chemical mutagen ethyl methanesulfonate (EMS) induced mutations efficiently in *D. melanogaster*, it has been used for the nematode *Caenorhabditis elegans* and later also applied for the studies of other free-living nematodes like *C. briggsae* and *Pristonchus pacificus*.

In endoparasitic nematodes, however, the mutagenesis techniques used in *C. elegans* cannot be easily adapted, primarily because of the location of the reproductive adults within their hosts. The exception are nematode worms of the genera *Strongyloides* and *Parastrongyloides* which can form facultative free-living generations in between parasitic generations. Transgenesis was introduced from *C. elegans* into the human parasitic nematode *S. stercoralis*. The first and so far only genetic map of an animal parasitic nematode was established in *S. ratti*, which improves the experimental usefulness in genetic research. A single attempt to mutagenize *S. ratti*, was reported but never followed up. One of the reasons for this was that even if a desired phenotype could be obtained, at the time it was virtually impossible to identify the gene that carried the mutation causing the phenotype. Recent development in sequencing technology, however is now offering new opportunities to identify mutations by whole genome sequencing of mutants.

Here I present a detailed protocol the mutagenesis of *S. ratti* with the chemical mutagen EMS. Starting from protocols for *C. elegans* and *S. ratti* I devised a protocol and

systematically varied different parameters in order to improve the initially unusably low survival and reproduction of the EMS treated worms. Then I mutagenized a strain of *S. ratti* with a very high tendency to form free-living worms and screened for an increase in the development to infective larvae. As a control I also attempted to obtain the same phenotype by selection, in absence of the mutagen. Finally, in order to directly assess the effect of the mutagenesis and the screening/selection procedures and to explore the possibility of identifying mutations by whole genome sequencing, I sequenced the genomes of several mutagenized and selected strains. The main results were: i) the currently used standard laboratory strain of *S. ratti,* still contains a rather high number of polymorphic positions, ii) the strong populations bottlenecks associated with the mutagenesis and selection procedures reduced the genetic complexity of the populations considerably, but also made some originally rare alleles detectable which were not seen when the starting population was sequenced. As a consequence that total number of candidate mutations to be further tested in order to identify the one causing the phenotype of interest was in the several hundred to a few thousands. This is too high for practical feasibility but our results clearly indicate that the number could be strongly reduced by additional inbreeding of the starting population. Our results also demonstrate that such further inbreeding is tolerated by *S. ratti*.

4.2.2 Contribution

I carried out all mutagenesis, screening and selection experiments and I maintained the mutant and selection lines in the lab, and collected worms for genome sequencing. Zisong Chang extracted DNA and prepared the libraries for sequencing. I analyzed the data together with Christoph Dieterich, Adrian Streit and Zisong Chang. The manuscript was written by Adrian Streit and myself with input from Christoph Dieterich.

4.3 Identification of a *Strongyloides* MARiner-like Transposon (SMART) in *Strongyloides papillosus*.

4.3.1 Introduction

Transposons are genetic element capable of moving around in the genome. Varieties of transposable elements have been identified in prokaryotes and eukaryotes since it was first identified in maize (McClintock 1950). They are divided into two main classes, namely class I retrotransposons and class II DNA transposon. Retrotransposons are reverse-transcribed into DNA and inserted into genome via an RNA intermediate, and result in a new, additional copy of themselves (Feschotte & Pritham 2007). Whereas DNA transposon function without RNA mediated replication. In transposition, it is excised from donor site and paste into a target site without increase in number, called "cut-and-paste" mechanism (Haren et al. 1999).

Tc1, the best studied member of *Tc1/mariner* transposable element superfamily, was identified from *C. elegans* (B Rosenzweig et al. 1983). *Tc1* elements, which are about 1,3 – 2,4 kb in length and contain a single open reading frame, were also found in ecdysozoans and vertebrates (Plasterk et al. 1999). *Tc1/mariner* elements were applied in *C. elegans* as mutagens which generates mutations with special tags simplifying screening (Greenwald 1985). A new strategy combining transgenesis was introduced in *C. elegans* genome engineering. When transgenes contains transposable element and DNA template, Tc1 excision cause breakages and the point mutations can be copied into the genome during the repair process (Plasterk & Groenen 1992). However, usage of Tc1 has some drawbacks as an endogenous transposon. There are several copies in genome, which

leads the identification of mutagenic insertion more complicated. Furthermore, in some strains, such as mutator strain, transposition is not controlled and spontaneous re-excision can lead to the loss of the tag. *Mos1*, a member of *Tc1/mariner* elements, was isolated from *D. mauritiana* (Jacobson et al. 1986). It circumvents the limitations by providing an efficient exogenous transposase and unique recognition site (Williams et al. 2005). Recently, *Mos1* was used for site directed mutagenesis, targeted transgene insertion and gene deletion to engineer the genome of *C. elegans* (Frøkjaer-Jensen et al. 2010; Frøkjaer-Jensen et al. 2008; Robert et al. 2009).

In the study of the model organism *C. elegans*, molecular strategies are well developed for forward and reverse genetics (Kutscher & Shaham 2014). The successful introduction of extrachromosomal transgenes into *S. stercoralis* was described (Lok & Massey 2002). These transgenes, however, are silenced in the second generation, such that transgene expression is limited to the first transgenic generation, severely limiting their usefulness. Nevertheless, expression in one generation should be sufficient to mobilize a transposon included in the transgene. Therefore, I would like to establish endogenous and exogenous transposons as tools for mutagenesis and transformation in *Strongyloides* sp., as it has been done successfully in *C. elegans*. As a first step towards this goal, I describe here the isolation and characterization of a *mariner* type transposon from *S. papillosus*.

4.3.2 Isolation of SMART ITRs and transposases from *S. papillosus*.

To isolate SMART sequences from *S. papillosus* ($SMART^{pa}$), degenerate primers were designed based on the ITR sequences of *S. stercoralis* SMART ($SMART^{st}$) and *S. r*atti

SMART (SMART^{ra}), which were provided to me by James Lok, University of Pennsylvania (unpublished). These primers were used to amplify first transposase sequences from *S. papillosus*. Then, new primers against these sequences were made and used to amplify additional ITRs by inverse PCR. Finally, degenerate primers based on the *S. papillosus* ITRs were designed and used to amplify additional copies of the SMART transposase (for details see Materials and Methods). In total I isolated 9 ITRs and 17 transposase genes or pseudogenes.

The SMART^{pa} ITR sequences are 28 bp in length, similar to most mariner-like elements, which are about 30 bp. TA dinucleotides were found adjacent to the 5' ends of ITRs. Such a duplication of the target sequences is common to all transposable elements and is thought the result from a staggered cleavage on the target site during transposition (Craig 1997). The alignment of the ITR from *S. papillosus*, *S. stercoralis*, *S. ratti* and *Hsmar1*, an ancient transposon in man, reveals a highly conserved region within the ITRs (Figure 2.1). This conserved region is probably important for site recognition during the protein-DNA binding process. Although it is unknown if SMART^{pa} has preferred integration sites, the target sequence studied in Hsmar1 suggested a common preference of the target sites among eukaryotic DNA transposons (Claeys Bouuaert & Chalmers 2010). Of the seventeen different SMART^{pa} transposon sequences, fourteen had at least one premature stop codon. Three sequences had one uninterrupted open reading frame, which is 1020 bp in length. The presence of long open reading frames suggested that these SMART^{pa} putative proteins might function in the element's transposition. Three contigs (Contig12904, FKHPLC401CY67Z, Contig6445) from the *S. papillosus* EST libraries shared high similarities with the region of the consensus sequence encoding the catalytic domain (Figure 2.2). The sequences of these contigs were not fully identical to each other, or to any of the three putative SMART^{pa} transposase sequences described above. Of the three

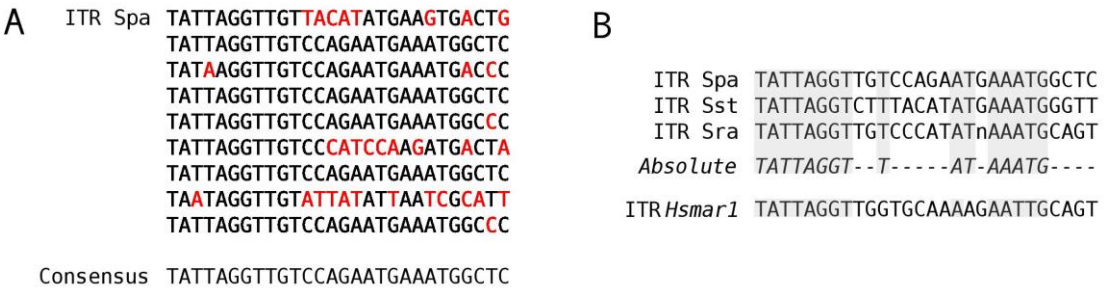

Figure 2.1: SMART^{pa} ITR sequences. - A. Nine *S. papillosus* ITR sequences (Spa ITR) were aligned. Letters in red represent the not conserved base pairs. The consensus Spa ITR sequence is listed below. B. The consensus ITR sequences of SMART^{pa},SMARTst, SMART^{ra}, and Hsmar1 were aligned with each other. The conserved bases among the ITRs are shaded.

contigs, Contig6445 was found in the library derived from infective stages, the other two were in the database of free-living. These analysis suggested that there are more putative transposases in *S. papillosus* and that they are probably expressed in both, the free-living and the infective stages. The structural organization of SMART^{pa} shares some common features with eukaryotic *Tc1/mariner* transportable elements. The consensus sequence of the full element is composed of 1,290 base pairs in length. Three of sixteen cloned candidates were putatively active, and had only one open reading frame, which encodes a deduced 340 amino acid protein. The common eukaryotic polyadenylation signal AATAAA was found at the 3' end of the coding region, overlapping with the stop codon TAA (Figure 2.3). The SMART^{pa} consensus sequence was used to BLASTN search the *S. ratti* database. Twenty one hits returned with high similarities (e-value less than 7.4e-11), but none of them was putatively active. All contained at least one premature stop codon in transposase sequence.

A BLASTN search of the *C. elegans* (WS217) database and the *Pristionchus pacicus* genomes produced no significant hits. The SMART^{pa} consensus sequence was translated

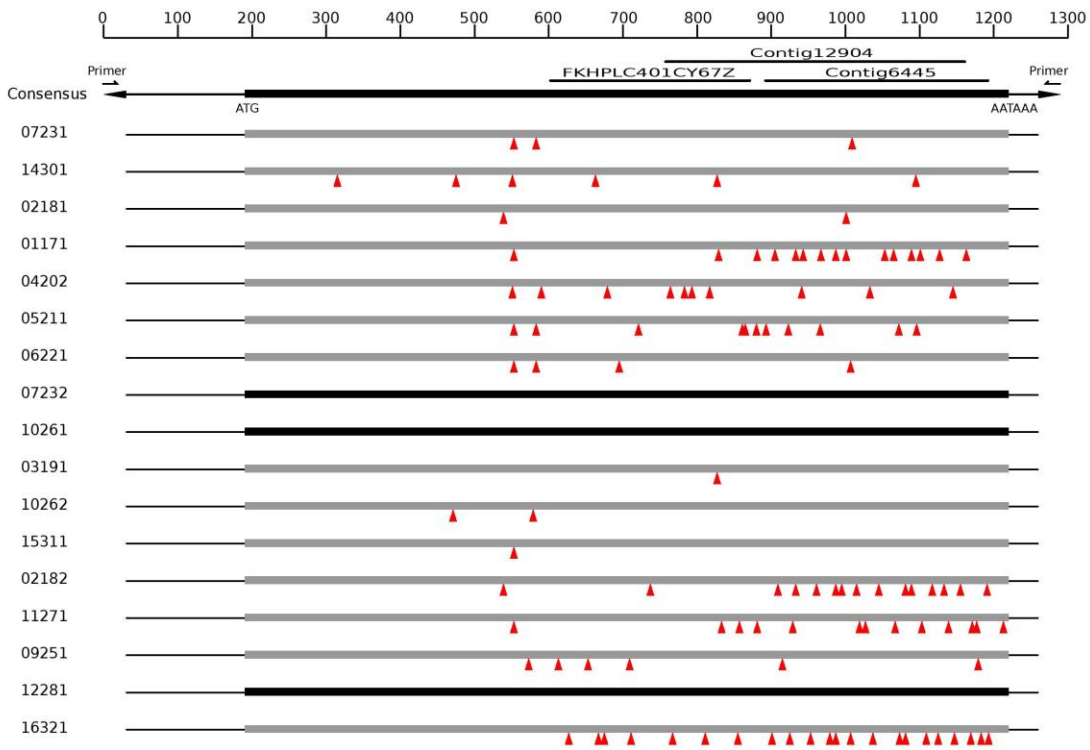

Figure 2.2: SMART^{pa} sequences. - Seventeen SMART^{pa} sequences were amplified with primers against the consensus ITR sequence. The consensus sequence is schematically shown above the 17 sequences, with the ITRs on both sides, indicated as arrow. The three EST contigs found in the data base were aligned above the consensus sequence. The start (ATG) and stop (TAA) codons are shown below the sequence. The polyadenylation signal overlaps with the stop codon. The sequences with premature stop codons (red arrow heads) are shown as grey boxes, sequences with coding potential for putatively active transposase as black boxes.

and BLASTP searched against the swissprot protein database. The predicted peptides shared a high similarity with the *Hsmar1* synthetic sequence, the *SETMAR* of *Homo sapiens* and *Mos1* of *Drosophila mauritiana*, with e-value of 3e-132, 3e-129 and 1e-57 respectively.

To compare conservation among *Tc1/mariner*-like transposases, SMART^{pa} was aligned with *Hsmar1* (AAC52010), *Mos1* (AAC16609), *Tc1* (P03939), *Tc3* (P34257), Sleeping Beauty (AAP49009) in ClustalW. The *S. papillosus* transposase has the major features of a *mariner* transposase (Robertson 1995), in particular the $DD_{34}D$ putative catalytic domain

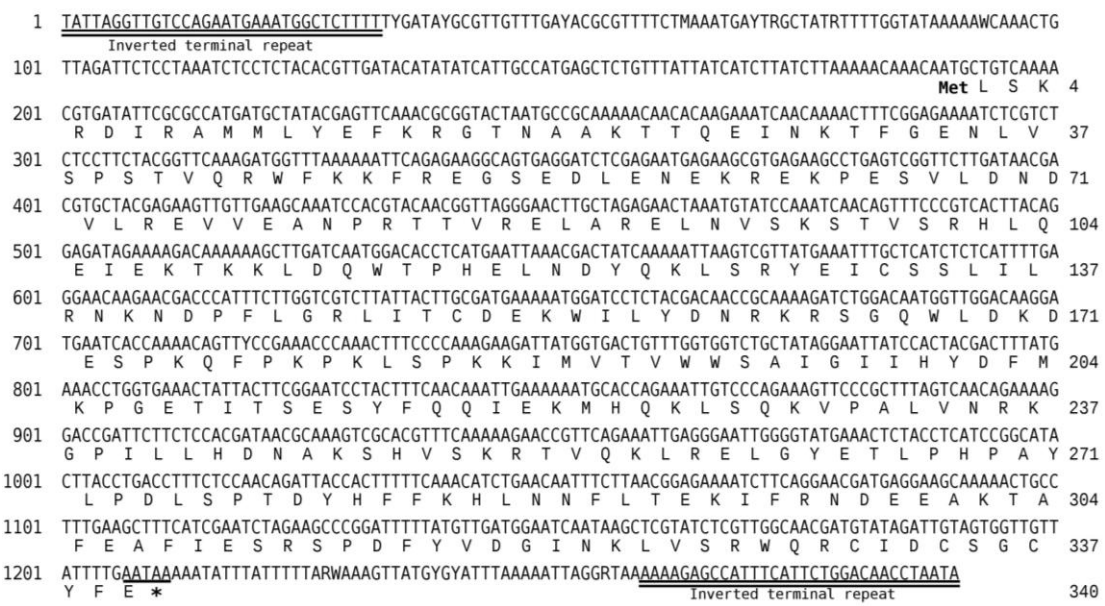

Figure 2.3: The consensus DNA sequence of SMART^{pa} and conceptual translation of the encoded transposase. - The inverted terminal repeats are double underlined. The polyadenylation site that overlaps with the stop codon is underlined. Nucleotide and amino acid numbers are shown on the left and right side respectively.

(Doak et al. 1994). Unlike *Tc*-like elements, of which the third amino-acid in the motif is E, a common mariner-like DDD motif was found in the catalytic domain of SMART^{pa}. In addition to the catalytic triad, SMART^{pa} has most of the additional canonical features of *mariner*-like elements (MLEs), the WPHEL motif (WTPHEL in SMART^{pa} transposase) and the YSPDLAP motif (YLPDLSP in the transposase) (Robertson 1993). The conserved FLHDNARPH motif that contains the second D of the catalytic triad in most MLE transposases was replaced with a LLHDNARSH motif (Robertson & Walden 2003; Witherspoon & Robertson 2003), a feature shared with Hsmar1 (Figure 2.4).

In the ancient transportable element *Hsmar1*, all identified copies appear to be dead remnants which are inactivated by mutations (Robertson & Zumpano 1997). However, the coding region of this transposase is still active as part of the SETMAR gene, with a

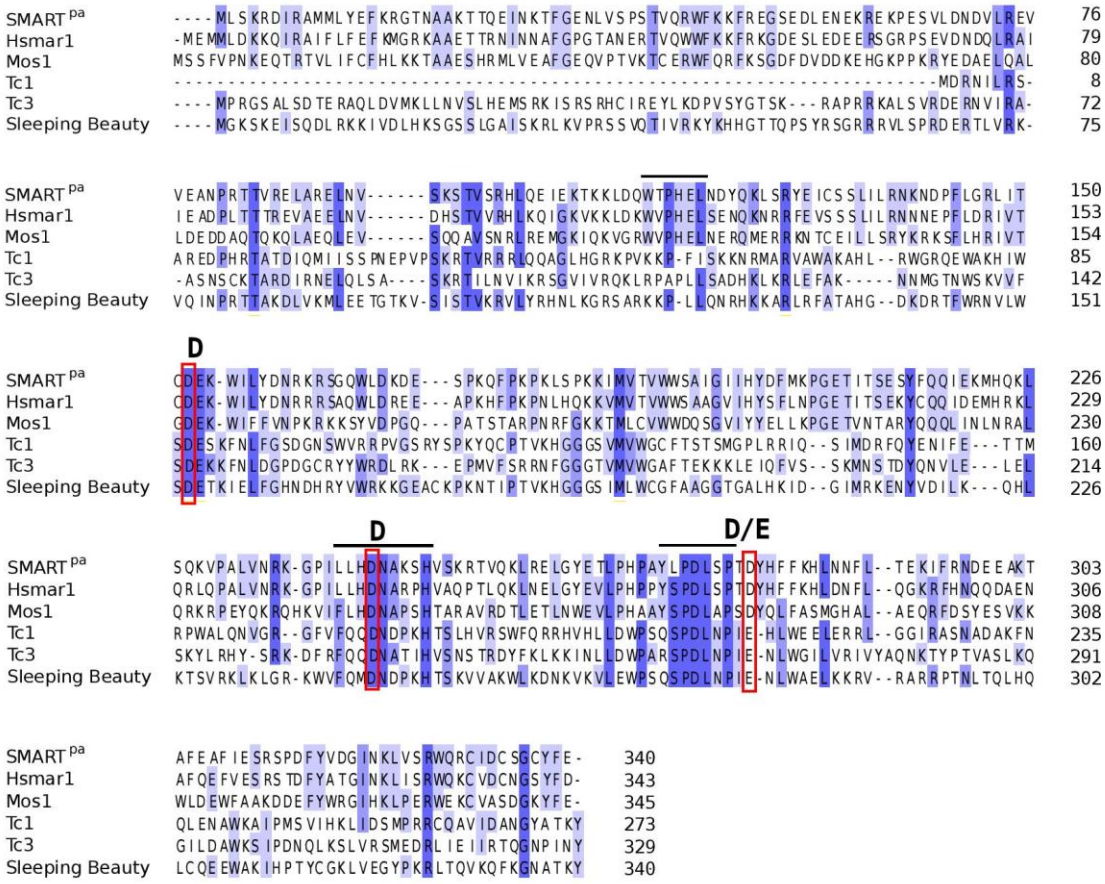

Figure 2.4: Multiple sequence alignment of the transposases of SMART with those from related transposons. - The position of the catalytic triad domain $DD_{34}(D/E)$ is indicated. The conserved motifs of mariner-like elements are overlined. Conserved residues are shaded. The GenBank accession numbers of these aligned transposons are Hsmar1 (AAC52010), Mos1 (AAC16609), Tc1 (P03939), Tc3 (P34257), Sleeping Beauty (AAP49009).

histone methylatransferase SET domain fused to the N-terminus of *Hsmar1* (Miskey et al. 2007). These studies of *Hsmar1* revealed two possible fates of this ancient transposon: 1) inactivation by mutations or 2) becoming part of a functional protein of the host. To date, the only natural *mariner* elements proven to be active are *Mos1* and *Famar1* (Barrett et al. 2004; Jacobson et al. 1986).

4.3.3 Phylogenetic position of SMART

The *mariner* superfamily transposases were classified into six sub families (Prasad et al. 2002). A phylogenetic neighbor-joining tree with 1000 boot strap replicates was constructed based on the alignment of the entire SMART^{pa} and SMARTst consensus amino acid sequences, and 26 other transposase protein sequences available in public databases. This showed that SMART is most closely related to Hsmar1 from *Homo sapiens* (Figure 2.5).

SMART formed a clad within elements from the *cecropia* subfamily with solid bootstrap support. This diphyletic clade includs a branch containing SMART and three primate-originated MLEs, and a branch with Funmar1 from *Fungia* sp., Aamar1 from *Attacus atlas* and Gtmar1 from *Girardia tigrina*. The transportable elements Tc1 and Tc3 from *C. elegans*, and Mos1 from *Drosophila* are only distantly related with SMART. The remarkable identity between SMART^{pa}, SMARTst and the primate MLEs, Hsmar1 and SETMAR, strongly suggest horizontal transition of this element from host to parasite (or vice versa).

The exchange of genetic material between hosts and parasites influences their genomic evolution. Feschotte and Pritham reviewed DNA-mediate transposons among eukaryotic species and also suggested that their movement and accumulation contribute a major force shaping the genes and genomes of almost all organisms and therefore led to the emergence of genetic innovations in different eukaryotic lineages (Feschotte & Pritham 2007).

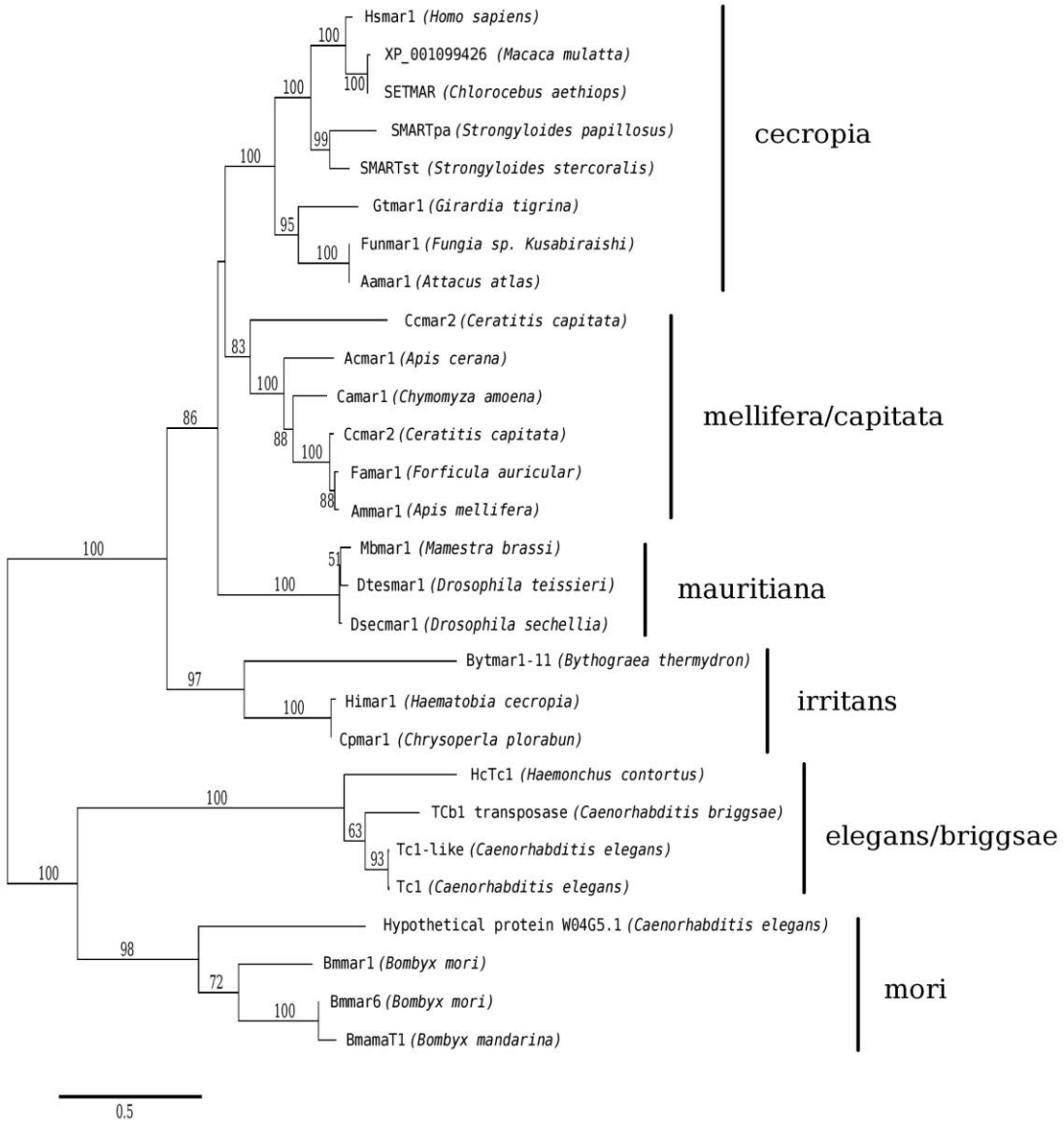

Figure 2.5: Phylogram of transposases of representative mariner superfamily transposons. - Representatives of six clades of *mariner*-like elements were included in the analysis. The elements used in the tree includes Tc1-like and Tc1 from *C. elegans*, HcTc1 from *Haemonchus contortus*, TCb1 from *C. briggsae*, Bmmar1 and Bmmar6 from *Bombyx mori*, Himar1 mutagenesis vector pFNLTP16H3, Cpmar1 from *Chrysoperla plorabunda*, Bytmar1-11 from *Bythogreae thermydron*, Dtesmar1 from *D. teissieri*, Dsecmar1 from *D. sechellia*, Mbmar1 from Mamesta brassicae, XP_001099426 from *Macaca mulatta*, SETMAR from *Cercopithecus aethiops*, Hsmar1 from *Homo sapiens*, Aamar1 from *Attacus atlas*, Funmar1 from *Fungia sp.*, Famar1 from *Forcula auricularia*, Ammar1 from *Apis mellifera*, Ccmar2 from *Ceratitis capitata*, Camar1 from *Chymomyza amoena*, Acmar1 from *Apis cerana*, Ccmar1 from *Ceratitis capitata*.

In conclusion, SMART^{pa} is a *mariner*-like transposable element. The similarity among *mariner*-elements in structure and sequence suggest a common origin of these

transposons. In the genetic study of *Strongyloides*, *SMART* has the potential to be served as a tool for genetic manipulation, comparable to *Tc1* in *C. elegans*.

4.3.4 Materials & methods

Culturing Strongyloides

S. papillosus used this study (isolate LIN) was originally isolated from naturally infected lambs at the agricultural experimental field station Oberer Lindenhof of the University Hohenheim, Stuttgart that is located in Baden-Württemberg, Germany (Eberhardt et al. 2007).

The infected rabbits were kept in cages with perforated bottoms and feces were collected in a tray with wet paper towels. The fecal pellets were crushed and mixed with sterile sawdust such that the sawdust contributed one third to one half of the total volume. The mixture was moisturized with tap water and placed in a 9 cm Petri dishes and incubated at 25 °C in a moisture saturated atmosphere.

To isolate iL3s, from which later DNA was extracted, the cultures in the 9 cm Petri dishes were placed without a lid in a 15 cm Petri dish that was filled with tap water up to 1-3 mm below the edge of the inner dish. The 15 cm dish was covered with a lid and incubated at 25 °C in a water saturated atmosphere. Since infective L3 have a tendency to disperse out of the culture dish, the iL3s were collected at the bottom of the 15 cm Petri dish after 6-9 days.

Genomic DNA preparation

Infective L3s of *S. papillosus* were collected as described above. Subsequently, Genomic DNA was isolated from the parasites using the Sigma GenElute Mammalian Genomic DNA Miniprep Kit (G1N350) according to the manufacturer's instructions. Briefly, worms (50-100 mg) were lysed in lysis buffer supplemented with RNase at 50 °C for 4 hours. Proteinase K was added to the extract and incubated at 50 °C for 2 hours. The lysate was applied to a column. The eluted genomic DNA was ethanol precipitated and dissolved in TE buffer, and its concentration and purity determined using a Nanodrop spectro photometer.

Isolation of *S. papillosus* SMART sequence

The initial partial *S. papillosus* SMART transposase consensus sequence "consensus_minus9_10" (see Appendix) was provided by Adrian Streit. It had been obtained in the following way. Based on the ITR sequences of *S. stercoralis* (communicated by H. Massey and J. Lok, University of Pennsylvania) primer AS2819 (5' TATTAGGTTGTCCCATATKAAATG 3') was designed and used for PCR amplification with genomic *S. papillosus* DNA as template. The PCR products were cloned into TOPO4 and several clones were sequenced resulting in consensus_minus9_10. This consensus sequence (consensus_minus9_10) was used to design inverse primers (see Table 2.1 below) to obtain the sequence of inverted terminal repeats. According to their directions on SMART^{pa} sequence, these primers are used as pair as AS2890 & AS2891, LG2924 & 2925, LG2947 & LG2948, LG2948 & LG2949, LG3225 & LG3226, LG3229 & LG3230, LG3247 & LG3247, LG3256 & LG3257. The primers (LG3120, 5' AATATTAGGTTGTCCAG-

AATGAAATG 3'; LG3121, 5' AATATTAGGTTGTCCCATCCAAGATG 3') against consensus ITR sequence were used to amplify SMART^{pa} sequences.

Primer ID	Primer sequence (5' → 3')	Direction
AS2890	GAGAAAACACGCGTCAAACAACG	Reverse
AS2891	AAGCTTGTATCTCGTTGGCAAC	Forward
LG2924	CATTAGTACCGCGTTTGAACTC	Reverse
LG2925	AGAAATGACCCATTTCTARGTCG	Forward
LG2947	GCTTCATCCTTGTCCAACCATT	Reverse
LG2948	GAATMAATAAGCTYGTATCTCGTTGG	Forward
LG2949	CTAATTCSCTCARTTTCTGRASG	Reverse
LG3225	TCGTTATTGTTTCCTGCCCGTA	Forward
LG3226	CGTCTTCCCAAAATCATGGAAT	Reverse
LG3229	GTTATTGTTTCCTGCCCGTAG	Forward
LG3230	TTACGTCTTCCCAAAATCATGG	Reverse
LG3246	TACTTATAATAATGAGAAAACAGTCAG	Forward
LG3247	GAAAGTTTGGTGGTCAATAAAG	Reverse
LG3256	TAATAATGAGAAAACAGTCAGA	Forward
LG3257	AATAAAGTATGCTATCGCC	Reverse

Table 2.1: The transposase primers in reverse PCR.

Copies of *S. papillosus SMART* ITRs were isolated by inverse PCR as follows: per reaction, one microgram each of genomic DNA of *S. papillosus* was digested with the restriction endonucleases EcoRV, HincII, HaeIII, RsaI respectively in a total volume of 20 µl. One hundred nano gram digested DNA fragments were ligated in the volume of 1 ml in order to circularize the molecules. The ligation product was then extracted with phenol/chloroform; precipitated with 2 volumes of ethanol; washed with 70% ethanol;

re-suspended in 20 µl DNase free water. The ligation mix was used as template for inverse PCR using inverse primers against transposase sequences. The PCR products were gel purified and isolated with the QIAquick Gel Extraction kit from Qiagen (Cat. No. 28706) and TA cloned into TOPO TA cloning vector (Cat. No. K4575-02) from Invitrogen and subsequently sequenced with T3 and T7.

To amplify whole length transposase genes, primer LG3120 (5' AATATTAGGTTGT-CCAGAATGAAATG 3') and LG3121 (5' AATATTAGGTTGTCCCATCCAAGATG 3') were designed against the consensus sequence of the ITRs. Fifty microgram genomic DNA was used as template in PCR reactions (94 °C for 30 sec, 53 °C for 30 sec and 72 °C for 3 min, 35 cycles). The PCR products were purified, cloned and sequenced as described above. The sequences were aligned using MegAlign of Lasergene.

Sequencing reaction

The cloned ITR and SMART sequences in TOPO vector were sequenced in the reaction mix of 2 µl 5X Sequencing Buffer, 0.5 µl Cycle Sequencing Mix of BigDye Terminator Sequencing Kit from Applied Biosystems, 0.5 µl 10 pmol sequencing primer in a volume of 10 µl, following the condition 94 °C for 20 sec, 50 °C for 10 sec and 72 °C for 4 min, 35 cycles.

Phylogenetic analysis

The entire transposase ORFs of *SMART*^{pa} and related representative elements were selected for construction of the phylogenetic tree. The following sequences were included

in the analysis: (Tc1-like (AAD12818) and Tc1 (P03934) from *C. elegans*, HcTc1 (AAD34306) from *Haemonchus contortus*, TCb1 (CAA30681) from *C. briggsae*, Bmmar1 (U47917) and Bmmar6 (AAN-06610) from *Bombyx mori*, Himar1 (ABB59013) mutagenesis vector pFNLTP16H3, Cpmar1 (AAC46945) from *Chrysoperla plorabunda*, Bytmar1-11 (CAD45369) from *Bythogreae thermydron*, Dtesmar1 (AAC28261) from *D. teissieri*, Dsecmar1 (AAC16609) from *D. sechellia*, Mbmar1 (AAL69970) from *Mamesta brassicae*, XP 001099426 from *Macaca mulatta*, SETMAR (ABC72092) from *Cercopithecus aethiops*, Hsmar1 (AAC-52010) from *Homo sapiens*, Aamar1 (BAA21826) from *Attacus atlas*, Funmar1 (BAB-32436) from Fungia sp., Famar1 (AAO12863) from *Forfiula auricularia*, Ammar1 (AAO12861) from *Apis mellifera*, Ccmar2 (AAO12864) from *Ceratitis capitata*, Camar1 (AAO12862) from *Chymomyza amoena*, Acmar1 (BAB86288) from *Apis cerana*, Ccmar1 (AAB17945) from Ceratitis capitata). Alignments of amino acid sequences of functional domains were accomplished with MUSLE (Edgar 2004). Alignment curation was finished with Gblocks (Castresana 2000). A phylogenetic analysis was performed on this blocked sequence alignment using PROTDIST of PHYLIP and a tree was constructed using the neighbor joining method with BioNJ (Gascuel 1997). The resulting phylogenetic trees were displayed using Njplot. Statistical significance of branching points was evaluated with 1,000 repetitions of a bootstrap analysis. The protein sequences used in this analysis were obtained directly from the GenBank entries, or the protein sequences were predicted by translating the nucleotide sequences provided in GenBank.

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6 Appendix

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Short communication

Strongyloides stercoralis genotypes in humans in Cambodia

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ABSTRACT

Little is known about the genetic variability of the soil-transmitted nematode, *Strongyloides stercoralis*, in humans. We sequenced portions of the small subunit rDNA (SSU), including the hyper variable regions (HVR) I and IV from *S. stercoralis* larvae derived from individuals living in a rural setting in Cambodia. We identified three polymorphic positions, including a previously reported one within the HVR I. HVR IV was invariable. Six different SSU alleles existed in our sample. Although different genotypes of *S. stercoralis* were found in the same individuals, no heterozygous larvae were found. This indicates that there is no or very little interbreeding between the different genotypes. Further studies are needed to examine if this is because sexual reproduction, which is facultative, is rare in our study area's *S. stercoralis* population or because what is considered to be *S. stercoralis* today is actually a complex of closely related species or subspecies.

1. Introduction

Strongyloides stercoralis is one of the most neglected helminth infections among the so-called neglected tropical diseases (NTDs) [1]. This soil-transmitted nematode is found world-wide [2,3], with the highest concentrations being in the tropical regions of Africa, South-East Asia and Latin America [3]. The parasitic adults are all parthenogenetically reproducing females that give rise to infective (parasitic) female progeny (homogonic cycle) and progeny that form a facultative free-living generation of male and female adults (heterogonic cycle) [4–6]. A suitable host is infected when filariform larvae penetrate the skin. Humans may also carry *Strongyloides fuelleborni* infections in Africa and Papua New Guinea [4,5,7].

The small ribosomal small subunit (SSU) rDNA sequence is highly conserved. In nematodes, it is frequently used for phylogenetic studies among and within species [8–11]. In the genus *Strongyloides*, SSU sequences are useful markers for molecular taxonomy [8,12–14]. A few studies on within-species variations in the SSU and in mitochondrial DNA sequences have been conducted on *S. stercoralis* originating from different locations and hosts [8,14–17]. Nevertheless, information on genetic variation in *S. stercoralis* is still scarce. In particular, we are not aware on any studies of the genetic structure of *S. stercoralis* populations within a particular location.

We examined the SSU genotypes of *S. stercoralis* larvae from humans in highly *S. stercoralis* endemic villages in Cambodia and report on the presence of different co-existing but not regularly interbreeding *S. stercoralis* genetic strains.

2. Methods

2.1. Study area and design

The study was part of a cross-sectional survey of intestinal helminth infections conducted in randomly selected villages in Preah Vihear and Takeo provinces in Cambodia, in March 2010 and 2011, respectively. Two stool samples were collected from members of selected households on two consecutive days and examined for intestinal helminth infections. If *S. stercoralis* was diagnosed, larvae were collected and fixed in 70% ethanol for genotyping.

2.2. Field and laboratory procedures

Stool samples were collected each morning and analysed in the laboratory within 3 h. A Baermann test [18] was performed and *S. stercoralis* larvae were collected for genotyping. In brief, stool was placed in a funnel fitted with a wire mesh and gauze. The funnel was filled with tap-water while a clamp keeps the water in the funnel. Artificial light was placed in

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front of the funnels to animate larvae to move out of the stool. After 2 h, the clamp was released and the water containing the larvae was collected in a tube. After centrifugation at 2000 rounds per minute (rpm), the effluent was examined for the presence of *S. stercoralis* larvae. If larvae were present on the first slide, the sediment was conserved with 70% ethanol.

2.3. Genotyping single S. stercoralis larvae

We sequenced about 450 bp covering the hyper variable region (HVR) I (c.f.[8]) from 269 individual *S. stercoralis* larvae. This region had been used for molecular taxonomy in multiple studies of *Strongyloides* and other nematodes [9,10,12,13]. It is frequently invariable within nematode species [11]. For *S. stercoralis*, a within-species polymorphism has been reported [8]. For 151 of our larvae, we also sequenced a fragment containing HVR IV, which is known to vary between, but not normally within, *Strongyloides* species [8]. The exact procedures were as follows: Single larvae were prepared for polymerase chain reaction (PCR) amplification, as described by Eberhardt and colleagues [19]. In brief, single larvae were picked out and incubated in 10 µl 2× lysis buffer (20 mM Tris–HCl pH 8.3, 100 mM KCl, 5 mM MgCl2, 0.9% NP-40, 0.9% Tween 20, 0.02% Gelatine, 240 µg/ml Proteinase K) at 65 °C for 2 h, followed by 95 °C for 15 min. The HVR I region was amplified using primers, RH5401 (5′-AAAGATTAAGCCATGCATG-3′) and RH5402 (5′-CATTCTTG GCAAATGCTTTCG-3′) and sequenced with RH5403 (5′-AGCTGGAATTAC CGCGGCTG-3′), as described by Eberhardt and colleagues [19]. The HVR IV region was amplified with primers, 18SP4F (5′-GCGAAAGCATTTGC CAA-3′) and 18SPCR (5′-ACGGGCGGTGTGTRC-3′) as described by Hasegawa and colleagues [8] and sequenced using the same primers.

2.4. Ethics statement

The study was approved by the National Ethics Committee for Health Research (NECHR), Ministry of Health, Cambodia and the ethics committee of the Cantons of Basel-Stadt and Basel-Land (EKBB), Switzerland. All participants were informed of the study aims and procedures and provided written informed consent prior to enrolment. All data handled remained strictly confidential. All individuals infected with *S. stercoralis* were treated with ivermectin (200 µg/kg BW) over two days.

3. Results and discussion

We sequenced the region around HVR I from 269 *Strongyloides* larvae isolated from 29 different people (Fig. 1) and compared the sequences with each other; with the sequence with accession number AF279916, reported by Dorris and colleagues [12]; and with the three sequences with accession numbers AB453314, AB453315, and AB453316, reported by Hasegawa and colleagues [8] (Fig. 2). All our sequences differed at two positions from AF279916. Position 27 was T, not G and starting at position 38 there were three As not four. The sequences reported by Hasegawa and colleagues [8] do not cover these positions.

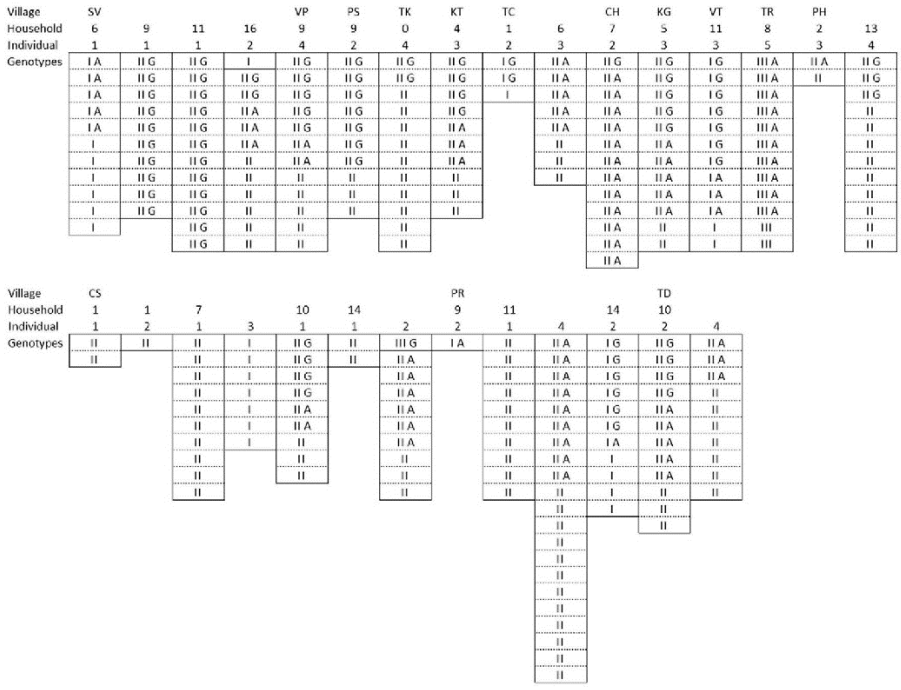

Fig. 1. SSU genotypes of *S. stercoralis* worms isolated from individual persons. Each cell corresponds to one worm, worms isolated from one patient are in the same column. The three different variants of the region around HVR I are indicated by roman numerals as follows:

I: positions 176–179 = 4 T; position 458 = T (KF926658)
II: positions 176–179 = 5 T; position 458 = T (KF926659)
III: positions 176–179 = 5 T; position 458 = A (KF926660).

The GenBank accession numbers are given in ().
If position 1454 was determined the nucleotide (A or G) is given after the roman numeral (accession numbers KF926662 and KF926661 respectively).

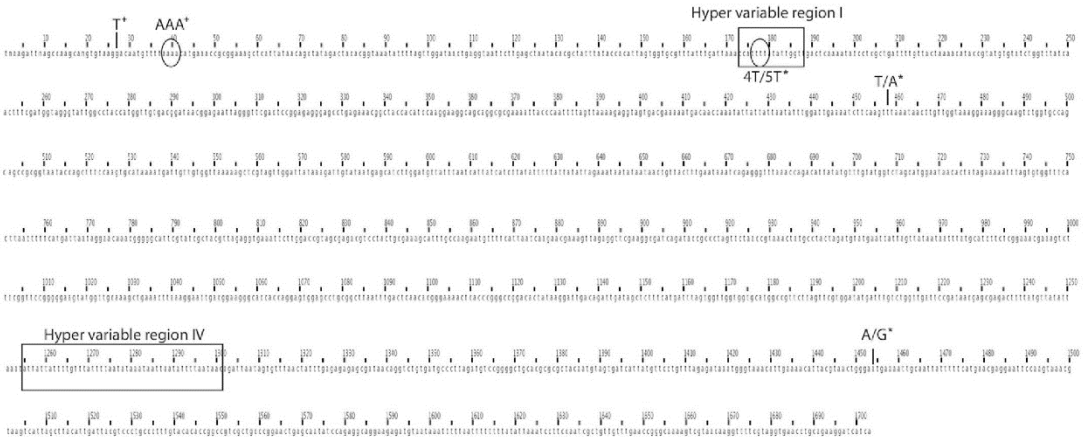

Fig. 2. *S. stercoralis* 18S SSU (AF279916) used as reference sequence. Positions found to be polymorphic in our human derived samples are indicated with a star *. Positions that were not polymorphic in our samples but differed from the reference sequence are marked with a plus +. Hyper variable regions I and IV are boxed.

Furthermore, among our samples, we found two polymorphic positions. First, the stretch of Ts starting at position 176 consisted of either 4Ts or 5Ts (Fig. 2). This is the same polymorphism Hasegawa and colleagues reported earlier [8]. Second, at position 458 we found either a T or an A (Fig. 2). At this position all three sequences from Hasegawa and colleagues had an A and differed from AF279916, which has T.

Of the four possible combinations, three (accession numbers KF926658–KF926660) existed in our samples, namely 4T + T (read four Ts at position 176 and T at position 458) (like AF279916; in Fig. 1 represented by I in a yellow cell), 5T + T (not previously described; in Fig. 1 represented by II in a red cell) and 5T + A (like AB453315; in Fig. 1 represented by III in a blue cell). No worms heterozygous for any two of these versions were found. Since studies on other nematodes found this region to be virtually invariable within a particular species [10,13,20], it was surprising that we found two polymorphisms. Therefore, for a fraction of our samples (Fig. 1), we also determined the sequence of the HVR IV. Hasegawa and colleagues [8] found the HVR IV to be invariable among the different isolates of *S. stercoralis* analysed and, at the same time, to be quite different between most species. All 151 HVR IV sequences were identical to the previously reported sequence for *S. stercoralis*. However, we did identify a polymorphic position somewhat outside of HVR IV. Position 1454 was either A or G (accession numbers KF926662 and KF926661, respectively). AF279916 has an A in this position, while AB453314, AB453315, AB453316 do not cover this nucleotide. When all three polymorphic sites described here were combined, we found six different SSU alleles in worms isolated from humans that one would consider as *S. stercoralis*. At least 93 worms were isolated from hosts that contained worms of multiple genotypes (for another 22 individuals, worms with different genotypes were isolated from the same household) demonstrating that the different genotypes co-exist sympatrically, thus there should be ample opportunity for crossing. Nevertheless, we did not identify a single heterozygous worm. This suggests that the carriers of the different SSU alleles in our study area interbreed only very rarely, if at all. An obvious explanation for this would be that the different SSU alleles represent separate populations of interbreeding animals and would have to be considered separate species. However, at the moment, we have no proof for within group sexual reproduction. It is also possible that sexual reproduction is rare or absent in at least certain lines of *S. stercoralis* in our study area. This could be either because successful reproduction occurs exclusively through the parthenogentic direct cycle or because reproduction in the free-living generation is non-sexual, in spite of the existence of males. Indeed, based on cytological studies, it was proposed that reproduction in the free-living generations of multiple species of *Strongyloides* is by sperm dependent parthenogenesis (pseudogamy) (for *Strongyloides ratti* [21]; for *Strongyloides papillosus* [22]; for *S. stercoralis* [23]). Using molecular genetic approaches, this hypothesis was disproved and sexual reproduction has been demonstrated for *S. ratti* [24] and for *S. papillosus* [19]. To our knowledge, no genetic investigation has been conducted with *S. stercoralis* and pseudogamic reproduction remains a possibility in this species. Nevertheless, although, based only on this study, it is too early to draw any firm conclusions, one should consider the possibility that what is traditionally considered to be *S. stercoralis* is actually a complex of closely related species or subspecies.

Authors' contributions

FS, LG, HM, AS and PO designed the study; FS and VK collected samples in 2010; FS collected samples in 2011 and conducted field laboratory analysis; SM had the overall responsibility of data collection; LG and AS conducted the genotyping of the larvae, LG and FS analysed data and interpreted results together with AS, HM and PO; FS and PO wrote the manuscript; AS, LG, SM and HM assisted with manuscript revisions; all authors read and approved the final submitted manuscript; AS and PO are guarantors.

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A protocol for chemical mutagenesis in *Strongyloides ratti*

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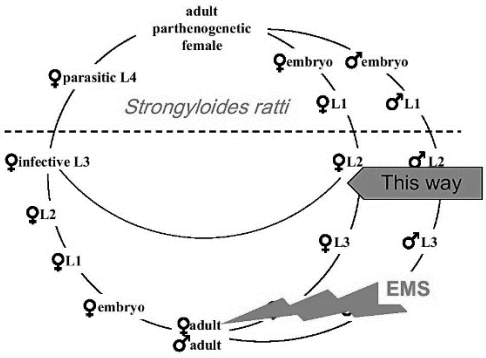
HIGHLIGHTS

- We present an improved protocol for chemical mutagenesis of *Strongyloides ratti*.
- Relatively high genetic variability complicates the identification of mutations.
- Further inbreeding of *S. ratti* is desirable and tolerated by the organism.

GRAPHICAL ABSTRACT



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ABSTRACT

Genetic analysis using experimentally induced mutations has been a most valuable tool in the analysis of various organisms. However, genetic analysis of endoparasitic organisms tends to be difficult because of the limited accessibility of the sexually reproducing adults, which are normally located within the host. Nematodes of the genera *Strogyloides* and *Parastrongyloides* represent an exception to this because they can form facultative free-living sexually reproducing generations in between parasitic generations. Here we present a protocol for the chemical mutagenesis of *Strongyloides ratti*. Further we evaluate the feasibility of identifying the induced mutations by whole genome re-sequencing.

1. Introduction

Ever since Morgan's pioneering work in the fruit fly *Drosophila melanogaster*, genetic analysis has been a crucial approach in the investigation of various model organisms. The random introduction of mutations followed by screening for the desired phenotype remains an important strategy in biological research. Although less specific than modern reverse genetic techniques this classical genetic approach has the advantage that it not only leads to simple loss of function mutations or alterations with already known consequences (e.g. dominant negatives) but also to sometimes highly informative hypomorphic, hypermorphic or neomorphic alleles. As a prominent example serves the *D. melanogaster* mutation *nasobemia* in the gene

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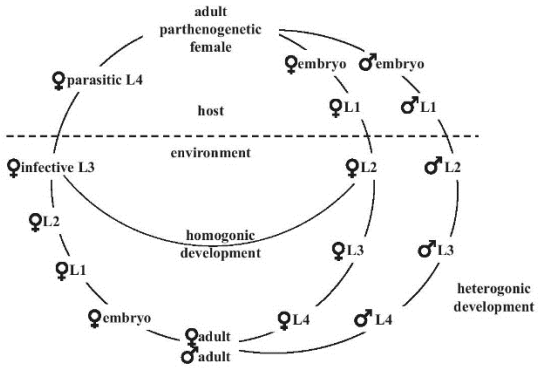

Fig. 1. Life cycle of *Strongyloides ratti*.

antennapedia, which eventually led to the discovery of the conserved homeobox (Gehring et al., 1994; McGinnis et al., 1984). The phenotype of this mutation is caused by the mis-expression of the gene and would not have been found in the context of a systematic gene knock out analysis (Schneuwly et al., 1987).

Genetic analysis of endoparasitic organisms is normally rather difficult because the sexually reproducing adults, which are required for crossing, are located within the host. Exceptions to this are nematode worms of the genera *Strogyloides* and *Parastrongyloides* (Grant et al., 2006b; Viney and Lok, 2007). These worms can form facultative free-living sexually reproducing generations in between parasitic generations, which, in the case of *Strongyloides* sp., reproduce parthenogenetically (Streit, 2008) (Fig. 1). A variety of techniques for the molecular genetic analysis of multiple species of *Strongyloides* and *Parastrongyloides trichosuri* have been developed over the last few years (Grant et al., 2006a; Lok, 2007; Lok and Unnasch, 2013; Nemetschke et al., 2010). Nevertheless, in comparison with the free-living, self-fertilizing model nematodes *C. elegans*, *C. briggsae* and *P. pacificus* there remain substantial technical and logistic obstacles for forward genetic screens in *Strongyloides* sp., which are related to the need for host passages (Sommer and Streit, 2011). Indeed, except for one report (Viney et al., 2002), which suggested that it is in principle possible, mutagenesis has not been employed yet in *Strongyloides* sp. The incentive to overcome the technical challenges for forward genetic screen was very limited because, even if a mutant phenotype would have been successfully isolated and secured, the identification of the mutation causing this phenotype was technically virtually impossible. With the recent progress in sequencing technology, requiring less material and becoming more affordable in the model nematodes *Caenorhabditis elegans* (Sarin et al., 2008) and *Pristionchus pacificus* (Ragsdale et al., 2013) it became possible identifying mutations by sequencing the genomes of mutant animals and comparing them with the wild type.

Here we describe a detailed protocol for mutagenizing *S. ratti* with the chemical mutagen EMS. We used this protocol to screen for dominant mutations that alter the ratio of larvae that develop into free-living adults and the ones who become infective. We attempted to obtain the same phenotype by simple selection. Finally, we evaluate the chances of identifying mutagen induced mutations by whole genome sequencing using the currently available and feasible technology and starting materials.

2. Materials and methods

2.1. S. ratti *isolate and husbandry*

The *S. ratti* strain ED321 was used. ED321 is a derivative of ED5 (Viney et al., 1992) selected for predominantly heterogonic development (Viney, 1996). *S. ratti* was maintained in female Wistar rats and cultured at 19 °C as described (Viney et al., 1992). All experiments with animals were done following the German animal wellfare legislation and all necessary permits were obtained. The rats were kept in an in-house animal facility, which is subject to regular inspections by the local authorities.

2.2. *Creation of mutant lines*

Two independent mutagenesis experiments were performed as descibed in Box 1. Two thousand L3i, progeny of EMS treated parents, were hand picked and used to infect two rats with 1000 L3i each. The feces of these rats were harvested from the sixth day on and cultured for 2 days. The worms were isolated using a Baerman funnel and differentiated L3i (from the direct cycle) were hand picked. This was repeated each day until 1000 L3i were reached. The L3i were stored in PBS at 19 °C. Two new rats were infected with 500 L3i each. In total three rounds of selection were done.

After the third round of selection rats were infected with individual L3i as described (Viney et al., 1992) resulting in a total of four (one from the first and three from the second mutagenesis experiment) isofemale lines with a clearly increased proportion of homogonic development. One line from the second experiment grew very poorly and we were unable to maintain it.

Box 1 Protocol for the mutagenesis of *S. ratti* with ethyl methanosulfate (EMS)m.

1. Harvest about three thousand L4 larvae from rat feces using Baermann funnels.
2. Wash larvae in a watch glass with M9 buffer. Suspend them in 1 ml of M9.
3. Pellet the *E. coli* OP50 bacteria from 5 ml of an over night culture and resuspend the bacteria in 1 ml of M9.
4. In a fume hood, add 80 µl of ethyl methanosulfate (EMS, Sigma M0880) to 4 ml of M9 buffer and mix.
5. Add the larvae and the *E. coli* to the EMS solution and incubate in dark at room temperature for 2 h. Shake it every 30 minutes to resuspend the worms and the bacteria.
6. Centrifuge (1 min, 700 g) and remove as much of the supernatant as possible.
7. Wash with 15 ml of M9 buffer three times. After each wash centrifuge and remove the supernatant as described above.
8. Transfer the worms onto a NGM plate seeded with OP50 and supplement with a pellet of autoclaved rat feces.
9. Incubate the plate at 19 °C in water saturated atmosphere to let the worms recover and reproduce.
10. After 3 days transfer the fecal pellets into a Baermann funnel (with PBS) and wash the plate with PBS and store the worms in a watch glass. After a few hours add the worms from the Baerman funnel to the watch glass and wash them with PBS several times. The vast majority of the worms should be infective L3 larvae.
11. Use the infective larvae to infect rats.

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2.3. Selection experiment

L3i from two infected rats were collected using a Baermann funnel after incubation at 19 °C for 5 days. The vast majority of these L3i were from the heterogonic cycle. Ten rats were infected with 500 L3i per rat to start ten selection lines. From the sixth to the eight day after the infection feces were collected and L3i from the homogonic cycle were collected 2 days later. For each selection line 500 L3i were hand picked and used to infect a new rat. This was repeated for seven consecutive generations/rounds of selection.

2.4. Whole genome sequencing

Genomic DNA (gDNA) was extracted by using the Qiagen® DNA preparation kit. Five micrograms of gDNA was fragmentated in 130 µl nuclease-free water at 4–7 °C using a Covaris® device with cooling unit. We used the following parameter settings: duty cycle 20%, intensity 5, cycler/burst 200, total time 45 s to shear DNA into fragments of about 200 bp. The gDNA fragments were cleaned up with 180 µl of AMPure® XP beads. All DNA concentrations were determined by Qubit® Fluorometer and the size distribution of gDNA fragments were checked with an Agilent Bioanalyzer using a DNA1000 chip.

Standard DNA sequencing library preparation was performed following the Illumina® Truseq DNA kit protocol. Each of the 8 sample libraries was tagged with a different sequencing barcode. We conducted two independent sequencing runs on a HiSeq 2000 sequencer. To this end, all 8 samples were pooled into a single HiSeq flow cell lane for each experiment. Each library was sequenced to a depth of ~50 million reads (1 × 50 bp). We mapped the reads to the *S. ratti* draft genome available from the Welcome Trust Sanger Institute web server (reference strain 321, assembly version 5) with Bowtie2 (release 2.1.0) (Supplementary Fig. S1). Mapping rates were above 90% for all samples. We performed SNP calling from BAM files with samtools (version 0.1.19) as outlined on the samtools homepage (Supplementary Table S1).

3. Results and discussion

Starting from the procedures for mutagenesis proposed by Viney et al. (2002) and established protocols for *C. elegans* (Kutscher and Shaham, 2014), we systematically varied different parameters in order to optimize the survival and the reproductive output of *S. ratti* worms mutagenized with ethyl methanesulfonate (EMS). We noticed that upon isolation from fecal cultures (2 hours in a Baermann funnel) and incubation in EMS for 4 hours the *S. ratti* adult females displayed symptoms reminiscent of what has been described as adult reproductive diapause in *C. elegans* (Seidel and Kimble, 2011). In particular the germ line appeared to be loosing volume and cells. This was not an effect of the EMS since MOK treatment had the same effect. Although the females did resume feeding when placed on *E. coli* bacteria and survived for up to a few days, their reproductive output remained very low. We therefore introduced three main changes. First we shortened the incubation time in EMS to 2 hours and at the same time increased the EMS concentration. Second, we added *E. coli* bacteria as food to the EMS solution. Third, we transferred the mutagenized worms onto NGM plates containing a small pellet of rat feces derived from a non-infected animal. A detailed protocol is given in Box 1.

The *S. ratti* isolate we mutagenized normally develops almost exclusively through the heterogonic cycle (Figs. 1 and 2). In two independent experiments we infected rats with the progeny of mutagenized females. From the progeny of these worms we selected manually the few homogonic L3i and infected another rat. This was repeated for two more times. The ratios of homogonic L3i after the thee selection rounds were respectively 18%, 48% and 44% in the first experiment and 13%, 16% and 41% in the second experiment. Then we infected rats with single L3i and established a total of three iso-female lines that showed a markedly increased proportion of homogonic development (EMSI-7 from the first, EMSII-5 and EMSII-9 from the second experiment) (Fig. 2). Because they share most sequence variants when compared with the reference sequence (see discussion later and Supplementary Table S1) EMSII-5 and EMSII-9 are likely not independent but were probably derived from siblings. Although, all three lines did produce free-living females and males, these animals appeared to be hardly fertile and we were not successful systematically backcrossing the mutant lines.

Although these results appear to indicate that the mutagenesis worked and produced dominant mutations causing a higher tendency to undergo homogonic development, there is a caveat. Our protocol, like the one in the only previous report of experimental mutagenesis of *S. ratti* (Viney et al., 2002), involved a selection step for the desired phenotype (homogonic development in our case and anhelminticc resistance in the case of Viney et al. (2002)). In order to assess if mutagenesis was indeed necessary in order to obtain the change in the proportion of homogonic development or if the phenotype could have been obtained simply by selecting we established ten independent cultures, which were propagated exclusively through the homogonic cycle for seven consecutive generations. This represents more than twice the number of rounds of selection we had done in the context of the mutagenesis experiments. In each generation we determined the proportion of homogonic development (Fig. 3). The proportions of homogonic L3i varied greatly not only between cultures but also between generations of the same culture. We did not observe a steady increase over the generations, in particular not over the first three generations as we had seen it with the mutagenized lines but by generation 7 most cultures had markedly increased their homogonic development. However, it should be noticed that several of these cultures did not maintain their high proportion of homogonic development over time (compare for example 10G_5 in Figs. 2 and 3). This indicated that selection probably contributed to the phenotype of the mutagenized lines but is not sufficient to explain

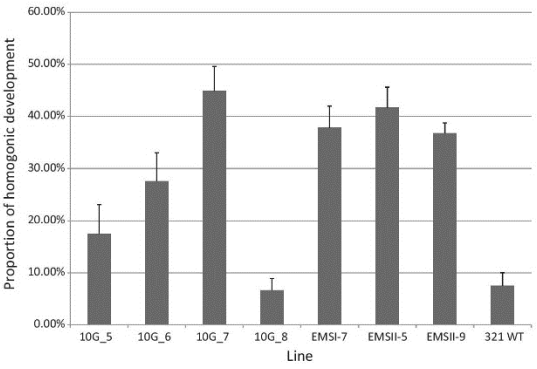

Fig. 2. Proportions of homogonic L3i of the populations submitted for whole genome sequencing. Worms were collected over three consecutive days. From each culture a sample >150 worms was used to determine the proportion of homogonic L3i. The rest of the culture was used for DNA extractin for sequencing. Error bars are standard errors. 10G_5–10G_8 are four strains from the selection experiment (cf. Fig. 3). EMSI-7 is an iso-female line derived from the first mutagenesis experiment, EMSII-5 and EMSII-9 are iso-female lines derived from the second experiment and are not independent. Notice that these numbers are for the cultures that were actually used for sequencing. 10G_5–10G_8 correspond to the F7 populations in Fig. 3 but had been expanded trough several passages in order to obtain enough material for sequencing.

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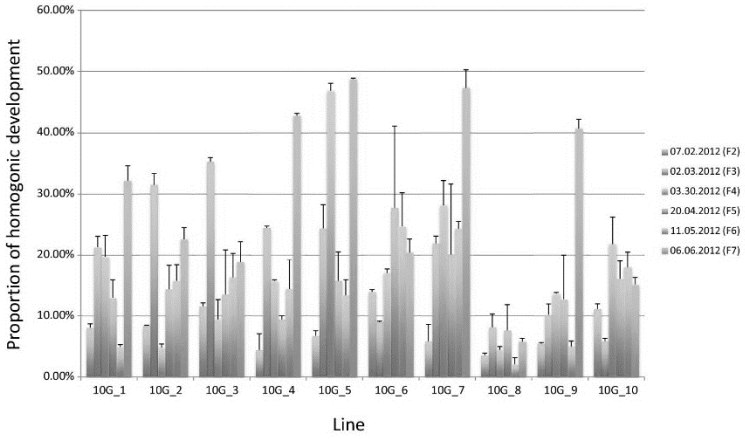

Fig. 3. Proportions of homogonic L3i in 10 lines maintained exclusively through the homogonic cycle. FX denotes the number of generations of selection.

it fully. It is interesting to note that neither by selection nor by mutagenesis and selection were we able to obtain a strain that had reverted to a proportion of homogonic development close to ED5 (Viney et al., 1992), the highly homogonic strain from which ED321 had originally been derived by selection for heterogonic development (Viney, 1996).

A great challenge is to identify the mutation causing a particular phenotype. In genetic model organisms including the model nematode *C. elegans* this has traditionally been achieved by positional cloning, a strategy that involves very precise genetic mapping followed by molecular analyses (Sommer and Streit, 2011). For a parasite like *S. ratti* this approach is virtually non-feasible. Recently whole genome re-sequencing of mutant strains in combination with rough genetic mapping has been employed sucessuflly in *C. elegans* (Sarin et al., 2008) and in *Pristionchus pacificus* (Ragsdale et al., 2013). This strategy should, at least, in theory also be applicable for *S. ratti*. However, even in *C. elegans* where an excellent reference genome, which is based on an almost perfectly inbred strain, is available, only a rather small fraction of the sequence differences identified represent genuine mutagen induced mutations (Sarin et al., 2008). The rest are sequencing errors and differences that arose during the cultivation of the reference strain in different laboratories for an ever increasing number of generations. In order to estimate how difficult it would be to identify mutations by whole genome sequencing we re-sequenced the genomes of our mutant lines, three of the selected lines did show an increased homogonic development (10G_5, 10G_6 and 10G_7) and one line where selection had no effect on the proportion of homogonic development (10G_8). Further, we also re-sequenced the parental line ED321, which we had received in 2010 from Mark Viney, University of Bristol, and maintained in our lab since then. The proportion of homogonic L3i of the cultures used for sequencing were determined and are shown in Fig. 2. First we asked how much our ED321 differed from the reference sequence, which is also derived from ED321. It must be noticed that the reference sequence, with the exception of 186 N base calls, contains one defined nucleotide at each position. However, ED321 is not perfectly inbred and homozygous at every position. First we compared the re-sequencing of our ED321 with the reference sequence. We identified 19,222 positions at which our strain appeared to be homozygous for a variant (single nucleotide difference or small indel) different from the reference sequence and 26,245 positions where our isolate was polymorphic. Next we created a combined data set (321 pool) with all sequence information from the non-mutagenized samples (ED321 and the selections lines 10G_5, 10G_6, 10G_7 and 10G_8). This added a total of 9898 variants. These variants must have been present in the parental strain at a frequency below detection level but have reached detectable frequencies in the derived lines either due to the selection for homogoic development or stochastically because of the population bottle necks imposed during the selection experiments. In comparison with the reference sequence in 321 pool we detected 16,342 different apparently monomorphic positions and 33,263 polymorphic positions. Given the assembley size of 43,150,242 bp this means that within our 321 strain we detected in average about one polymorphic position per 1.3 kb of genomic sequence. In order to further characterize the effect of our selection and mutation/selection treatments we compared the corresponding lines with our ED321 and with 321 pool (in the case of samples included in 321 pool with 321 pool minus the sample in question). The results are summarized in Table 1. Row number 1 lists positions at which the reference is polymorphic and the derivates have become monomorphic (homozygous). These numbers reflect the loss of genetic complexity caused by the selection or stochastically by the severe population bottle necks the populations went through during the selection procedure. It is striking that the reduction of genetic complexity in the selection lines was only marginally less, if at all, than in the mutant lines, which at one point had been reduced to a population size of 1. This indicates that our selection lines consisted of individuals derived from very few or even only one worm in the original infection. Row number 2 represents positions where the derived strain had aquired a new mutation and this mutation has gone to fixation. Given our experimental design no such positions are expected to exist. Row 3 represents positions where the parental animal was heterozygous and one of the alleles was mutated. Given the relatively low degree of heterozygozity in ED321 (see discussion earlier) such mutations are expected to be very rare. Row 4 represents cases where in a heterozygous animal both copies were mutated to different nucleotides. This case is expected to be even less frequent than row 2. Rows 2 and 4 are rather theoretical possibilities and are included for completeness. It is, however, reassuring that such cases were virtually not detected. Row 5 represents positions monomorphic in the original and one new mutation in the derived strain. In theory, this row represents the newly induced mutations. However, a number of false positives, which are not caused by mutation but by the detection limits in the reference sample, are to be expected. Consistent

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Table 1
Genomic comparison of the selection and mutation lines with the parental strain.

Number	Description	10G5	10G6	10G7	10G8	EMSI-7	EMSII-5	EMSII-9
1	Reference polymorphic – sample monomorphic for a nucleotide present in the reference	15,260/19,745	8017/11,943	8309/11,506	6483/10,480	14,405/18,497	8561/14,102	8634/14,177
2	Reference polymorphic – sample monomorphic for a nucleotide not present in the reference.	2/1	1/2	2/0	2/0	1/1	0/0	0/0
3	Reference polymorphic – sample polymorphic but one nucleotide not present in the reference.	5/0	7/4	3/3	2/3	5/2	5/2	3/2
4	Reference polymorphic – sample polymorphic but both nucleotides different from the reference	0/0	0/0	0/0	0/0	0/0	0/0	0/0
5	Reference monomorphic – sample polymorphic with one nucleotide not present in the reference.	2460/663	3044/818	3801/586	3026/352	3498/557	2026/534	3034/544
6	Reference monomorphic – sample monomorphic for nucleotide different from the reference	928/88	286/60	166/36	189/11	549/24	1286/63	1204/58

Numbers in comparison with ED321/321pool as reference.

with this, including more information (321 pool as reference instead of ED321) strongly reduces this number in all samples. The numbers in row 5 for the mutagenized strains are in the same range as for the non-mutagenized strains. However, this is not surprising given that, based on the experiments in *C. elegans* (Sarin et al., 2008), the number of mutagen induced mutations is expected to be only in the range a few dozens. Further, the mutagenized strains would be expected to have slightly fewer false positives in this row because they have fewer polymorphic positions to start with due to the somewhat more severe population bottle neck they went through. Row 6, finally, lists cases where a new mutation arose and went to fixation. Again, due to the experimental design such cases were not expected to occur. Most likely also these numbers reflect positions for which the derived strain has an allele that also is present in the parental strain but at a frequency below detection level. However, rows 2 and 6 can be viewed as an estimate for the false detection rate of homozygous differences from the reference, which is what one would be looking for in the case of a screen for recessive mutations.

As conclusions, whole genome re-sequencing as done here alone is clearly not sufficient to identify the molecular lesions causing our dominant non-crossable mutations The number of candidates that would have to be tested is to big (see Table 1 rows 3 and 5). Nevertheless, with respect to future screens for recessive mutations, our results are encouraging. The number of false homozygous differences from the reference is fairly low (see Table 1 rows 2 and 6). Whole genome re-sequencing approaches could have a great potential for the identification of induced mutations in *S. ratti*, in particular if strategies established in model nematodes to reduce the number of false candidates are employed. (Ragsdale et al., 2013; Sarin et al., 2008) e.g. parallel sequencing of non-mutant siblings and rough genetic mapping, which is tideous but possible in *S. ratti* (Nemetschke et al., 2010). Although this might work even with the current laboratory strain ED321 as starting material, we think that an important conclusion from this work is that a less gentically variable parental strain of *S. ratti* should and can be established. Both the selection and the sequencing experiments demonstrated that there is a considerable amount of genetic variation within the strain including many rare alleles. This variation leaves room for effects of selection and stochasticity in addition to mutagenesis and may complicate both the definition of a clear phenotype and the identification of the mutation by whole genome re-sequencing. Table 1 row 1 clearly indicates that a considerable reduction of heterozygocity and genetic complexity is straightforward to achieve and readily tolerated by *S. ratti* (our selection lines did grow well). Therefore several rounds of inbreeding and single worm infections should be conducted prior to engaging in large scale mutagenesis screens.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.exppara.2015.03.001.

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