Comparative Transcriptomics and Genetic Analyses in Animal Parasitic Nematodes

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Summary

Animal parasitic nematodes cause a wide range of diseases in humans and animals. Most *Strongyloides* and *Onchocerca* species are highly host specific, being restricted to one or a few species, but a few of them have a broader host range (eg. *S. stercoralis*). If a parasitic nematode species can infect multiple hosts, the infection efficiency and dynamics, reproductive success and the gene expression patterns may vary depending on the host. Also within the species, different genotypes that are specialized for a particular host may exist.

In this thesis I: 1) compared the infection dynamics and the transcriptomes of *S. ratti* in its natural host rat and in the sub-optimal host gerbil; 2) analyzed the transcriptomes of different developmental stages of *S. papillosus*; 3) investigated the population structure of *S. stercoralis* from humans and dogs in rural Cambodia and 4) compared the genetic variation in the whole mitochondrial genomes of the *O. ochengi* population in northern Cameroon.

To 1) I show that the infection success of *S. ratti* in gerbils is lower than in rats, but those worms that are successful, survive and reproduce much longer compared with *S. ratti* in rats. They produce a very high percentage of male progeny. The gene expression patterns are very similar in both hosts. Hence, gerbils are good laboratory hosts for the long-term maintenance of *S. ratti*.

To 2) I report a comparative analysis of the transcriptomes of six developmental stages of *S. papillosus.* Different members of Astacin and CAP gene families, which are strongly expanded in *Strongyloides* spp., are specifically up-regulated particularly in parasitic adults and infective larvae suggesting biologic importance of these gene families for the parasitic life cycle of this organism.

To 3) Using the nuclear 18S rDNA locus and mitochondrial markers, I demonstrate the existence of multiple genotypes among *S. stercoralis* isolated from humans and dogs in rural Cambodia. Whilst some make up the vast majority of *Strongyloides* spp. in dogs and are specific for this host, others are predominant in humans but also occur in dogs. This illustrates the possibility of zoonotic transmission of this parasite. I never found hybrids between the different 18S genotypes. This indicates either that intermixing only occurs within the same 18S genotypes, and as a result *S. stercoralis* represents a species complex of closely related species, or that these worms reproduce only asexually, as it had been proposed for *Strongyloides* spp. by some authors (but disproven for *S. ratti* and *S. papillosus).* I provide preliminary evidence that at least the laboratory isolate of *S. stercoralis* is capable of sexual reproduction*.*

To 4) I present the complete mitochondrial genome sequences of eleven different individuals of *Onchocerca ochengi*, a filarial nematode of cattle. I confirm the existence of two *O. ochengi* mitochondrial clades at the whole genome level and resolve the mitochondrial phylogeny of the two *O. ochengi* clades and the very closely related human pathogen *O. volvulus*.

Zusammenfassung

Tier-parasitäre Nematoden verursachen eine große Bandbreite an Erkrankungen in Menschen und Tieren. Viele *Strongyloides* und *Onchocerca* Arten sind hoch Wirt-spezifisch und damit auf eine oder wenige Arten von Wirten beschränkt. Bei anderen Arten ist das Wirtsspektrum breiter (z.B. *S. stercoralis*). Wenn eine Art verschiedene Wirte infizieren kann, können Effizienz und Dynamik der Infektion, der Reproduktionserfolg und das Genexpressionsmuster vom Wirt abhängig sehr unterschiedlich sein. Innerhalb der Arten können verschiedene Genotypen existieren, welche auf betimmte Wirte spezialisiert sind.

In dieser Doktorarbeit habe ich 1) die Effizienz und die Dynamik der Infektion sowie die Transkriptome von *S. ratti* in dessen natürlichem Wirt Ratte mit denen im suboptimalen Wirt Wüstenrennmaus verglichen; 2) die Transkriptome von verschiedenen Entwicklungsstadien von *S. papillosus* analysiert; 3) die Populationsstruktur von *S. stercoralis* aus Menschen und Hunden im ländlichen Kambodscha untersucht und 4) die genetischen Variationen im mitochondrialen Genom von *O. ochengi* im nördlichen Kamerun untersucht.

Zu 1) zeige ich, dass der Infektionserfolg von *S. ratti* in Wüstenrennmäusen niedriger ist als in Ratten aber dass die Würmer, die in Wüstenrennmäusen Erfolg haben, viel länger überleben und reproduzieren als *S. ratti* in Ratten. Sie produzieren einen hohen Anteil an männlichen Nachkommen. Die Genexpressionsmuster sind in beiden Wirten sehr ähnlich. Somit sind Wüstenrennmäuse gute Wirte für eine langzeitige Erhaltung von *S. ratti* für Labore.

Zu 2) lege ich eine vergleichende Analyse der Transkriptome von sechs Entwicklungsstadien von *S. papillosus* vor. Aus den bei *Strongyloides* spp. sehr verbreiteten Gen-Familie Astacin und CAP sind manche Gene spezifisch in parasitären adulten Würmern oder infektiösen Larven hochreguliert. Dies legt nahe, dass diese Genfamilien eine biologisch wichtige Rolle für den parasitären Lebenszyklus dieser Organismen spielen.

Zu 3) zeige ich mit dem nukleären 18S rDNA Lokus und mitochondrialen Makern, dass in Menschen und in Hunden im ländlichen Kambodscha mehrere *S. stercoralis* Genotypen vorkommen. Während manche die große Mehrheit von *Strongyloids* spp. in Hunden ausmachen und für diesen Wirt spezifisch sind, kommen andere überwiegend in Menschen, aber auch in Hunden, vor. Dies legt die Möglichkeit von zoonotischer Übertragung dieses Parasiten nahe. In dieser Studie wurden keine Hybride zwischen den verschiedenen 18S Genotypen gefunden. Das bedeutet, dass Kreuzung entweder nur innerhalb eines 18S Genotypen stattfindet und *S. stercoralis* damit ein aus nah verwandter Arten bestehender Artenkomplex ist, oder dass sich diese Würmer nur asexuell vermehren. Letzteres wurde von älteren Autoren für *Strongyloides spp.* postuliert aber später für *S. ratti* und *S. papillosus* widerlegt. Ich liefere erste Evidenzen, dass zumindest ein Laborisolat *S. stercoralis* fähig zur sexuellen Reproduktion ist.

Zu 4) vergleiche ich die kompletten mitochondrialen Genom Sequenzen von elf verschiedenen Individuen von *Onchocerca ochengi*, einer Filarie in Rindern. Ich bestätige, gestützt auf das vollständige Genom, die Existenz von zwei mitochondriellen Kladen innerhalb der Art und ich charakterisiere die phylogenetische Verwandtschaft zwischen den beiden *O. ochengi* Varianten und dem sehr nah verwandten human pathogenen *O. volvulus*.

1. Introduction

1.1 Parasitism in the phylum Nematoda

Nematodes commonly known as roundworms are the largest phylum in animal kingdom [1]. Nematodes live in a variety of environments such as marine or fresh water, every type of soil, both the polar and the tropical regions, as well as in the highest and lowest elevations either as free-living individuals or in plants, animals and humans as a parasites [2][3]. Many nematode species are free-living in nature and play an important role in the decomposition process, aid in recycling of nutrients in marine environments, and are sensitive to changes in the environment caused by pollution [2][3][4]. They also effectively regulate bacterial populations and community composition, for instance they may eat up to 5,000 bacteria per minute [3]. For example, *Caenorhabditis elegans* (*C. elegans*) is a free-living nematode model organism for studying development, aging and some aspects of proteins involved in human diseases [5] as 40% of genes known to be associated with human diseases have clear orthologs in the *C. elegans* genome [6]. The phylum Nematoda consists of more than 1 million species, of which only about 25,000 species have been described so far, more than half of which are parasitic nematodes [2]. Phylogenetically, the phylum Nematoda has been classified into five clades, each clade containing at least one parasitic species [7], suggesting that parasitism has arisen multiple times throughout evolution [7][8][9]. The exact mechanism by which nematodes developed the parasitic life histories is not clear [8][9][10][11]. A phylogenetic relationship between nematodes and their evolution of parasitism is presented in Figure 1 below.

Many parasites are highly host specific, being restricted to one or a few species of hosts [12]. Host specificity can evolve through either through long-term associations of hosts and parasites, persisting through speciation events that can restrict sister parasite lineages to sister host lineages, or alternatively a parasite lineage that is initially capable of utilizing several species of hosts

may become restricted to a subset of them [12]. This could happen when a parasite population inhabits a geographically isolated area where some potential hosts are absent or due to a genetically based loss of ability to parasitize certain species that formerly served as hosts even when they are present [12]. Lateral transfers of parasites across host lineages could involve an expansion of host range, allowing colonization of a new host, and then loss of ability to use the ancestral host [13].

As far as the parasitic nematodes of interest for this thesis are concerned, *Strongyloides* spp. are considered to be rather host specific (naturally infect one or very few specific hosts), but some species like *Strongyloides stercoralis (S. stercoralis)* and *Strongyloides fuelleborni (S. fuelleborni)* are known to infect a wider range of vertebrate hosts [14][15][16][17]. Also artificial infections of suboptimal non-natural hosts with some *Strongyloides* spp. have been reported (for example: *Strongyloides ratti* (*S. ratti*) in mice and gerbil [18][19], *Strongyloides papillosus* (*S. papillosus*) in rabbit [20]; *Strongyloides venezuelensis* (*S. venezuelensis*) [21] and *S. stercoralis* in gerbil [22]). Also in the genus *Onchocerca* most species appear host specific [23]. This species provides an example for a recent speciattions even assotiated with a host switch. It has been hypothesized that *O. volvulus* and *O. ochengi* evolved from a common ancestor after humans acquired this infection from their cows upon cattle domestication in Africa about 10,000 years ago [24]. This host switching might have been followed by fusion of two chromosomes in *O. volvulus. O. ochengi* has 2n=5 chromosomes, where as *O. volvulus* has 2n=4 [25]. *O. ochengi* infection in cattle has no pathology, where as *O. volvulus* cause serious eye (river blindness) and skin diseases [26]. This might be due to long term of co-evolution and adaptation to each otherr of cattle and *O. ochengi* [27], where as *O. volvulus* infection in humans is much more recent and has therefore a more pronounced pathological effect on the host [24].
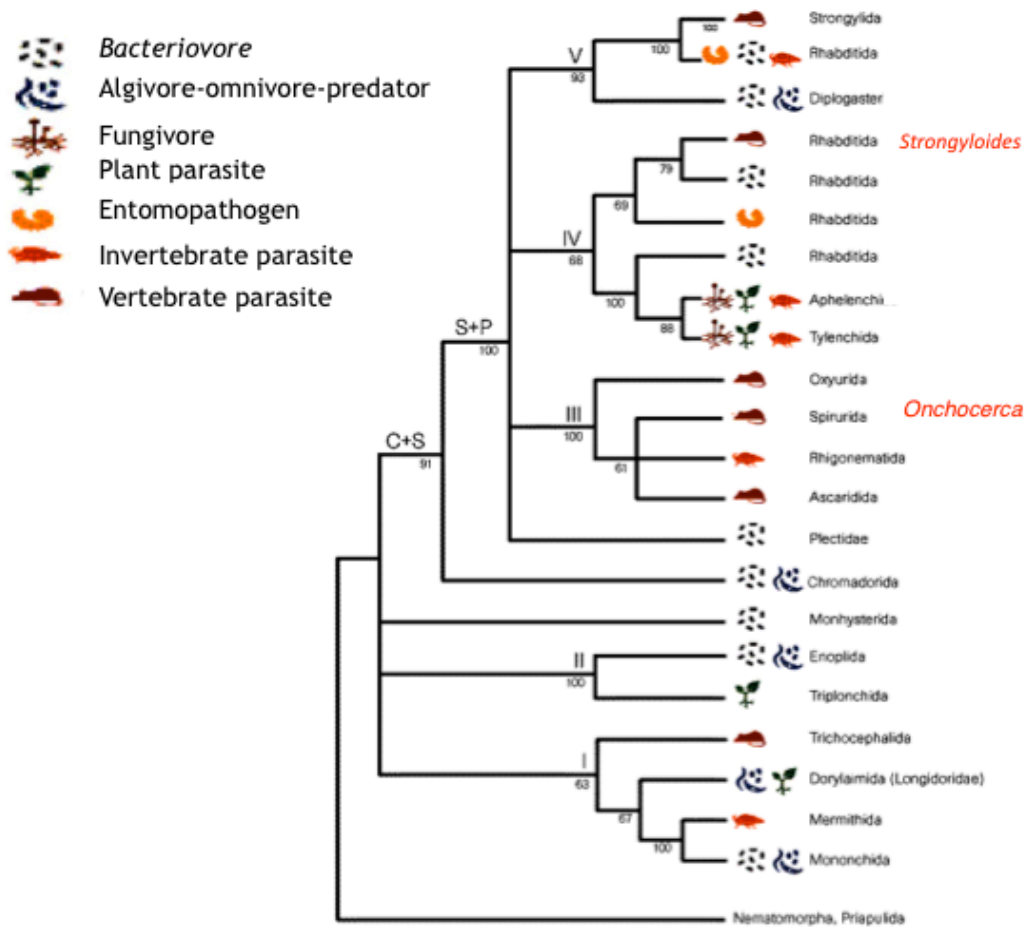
Figure 1. Phylogenetic relationships between nematodes based on SSU sequences (adapted from [7]).

Parasitic nematodes cause a wide range of diseases in humans, animals and plants [28]. In domestic and wild animals, parasitic nematode infections reduce the productivity and affect general welfare [29]. The economic importance of parasitic nematodes is enormous [30]. Plant parasitic nematodes infect most cultivated plant species and cause crop production losses, thereby exacerbating global food shortages [10]. Global total annual yield losses caused by plant parasitic nematodes are estimated to be greater than US$ 125 billion [31].

Nematode infections in humans lead to serious health problems causing substantial suffering, especially in children [32][33]. Globally, more than 2.9 billion people are infected by parasitic nematodes that can impair physical and

educational development in children [34] often resulting in significant morbidity and mortality [35]. The infectious forms of many human parasitic nematodes are developmentally arrested third-stage larvae (iL3s), which reside in soil, from which the designation soil transmitted helminthes (STH) originated [35]. The most common STH in humans are hookworm disease (*Necator americanus* and *Ancylostoma duodenale*)*,* ascariasis (*Ascaris lumbricoides*)*,* trichuriasis (*Trichuris trichiura*)*,* lymphatic filariasis (*Wuchereria bancrofti* and *Brugia malayi*)*,* onchocerciasis (*Onchocerca volvulus*), and strongyloidiasis (*S. stercoralis*)*.* These are reported to infect between 576-740, 807-1221, 604-795, 120, 37 and 30-100 million people worldwide respectively [35][36]. Strongyloidiasis caused by *S. stercoralis* and *S. fuelleborni* causes serious health problems in humans [37], dogs and non-human primates [16][17][38][39]. In domestic animals, *S. papillosus, S. ransomi* and *S. westeri* [40] are the most important parasite of sheep, pigs and horses that cause serious disease in nursing lambs, piglets and foals respectively. During my PhD studies, I have conducted comparative transcriptomics and genetic analyses in *S. ratti, S. papillosus, S. stercoralis and Onchocerca ochengi.* Below, I provide basic information on the taxonomy, biology, disease, genomics and genetic tools in these species.

1.2 The genus *Strongyloides*

1.2.1 Taxonomy

The genus *Strongyloides* is grouped in the order Rhabditida of clade IV in the phylum Nematoda [7]. This genus contains more than 50 described species that are obligate gastrointestinal parasites of vertebrate hosts ranging from mammals and birds to reptiles and amphibians [40]. In the wild or in natural infections, hosts become infected when iL3s penetrate the skin [37]. Artificial infections can be achieved by bringing the iL3s close to the host skin or by subcutaneous injection in the laboratory [41]. The *Strongloides* iL3s are considered homologous to *C. elegans* dauers that are developmentally arrested larvae that can live in the external environment for more than 2 weeks [42].

4

Taxonomic classifications of *Strongyloides* spp. are mainly based on molecular phylogeny [40] due to the fact that different species are often very similar morphologically and cannot be identified solely on this criterion. Recently Hino et al. [43] reported a very informative phylogenetic tree for the most important *Strongyloides* spp. using the full length sequence of the 18S rDNA. Humans can be infected with *S. stercoralis* and *S. fuelloborni*, which are phylogenetically distant from each other [40][43]. Rats can be infected with *S. ratti* and *S. venezuelensis*, which are also far apart from each other on this tree [43]. Phylogenetically, *S. ratti* and *S. stercoralis* are very close to each other [40][43]. *S. papillosus, a* parasite of sheep is phylogenetically very close to *S. fuelleborni* and to *S. venezuelensis* [43]. These examples illustrate that in *Strongyloides* spp., the phylogenetic relationships of the parasites and of their respective host species do not correlate, suggesting that host switching was fairly common during the evolution of the genus *Strongyloides* [40][43]. The phylogenetic relationship between different *Strongyloides* spp. and their respective hosts they infect are given in Figure 2 below.
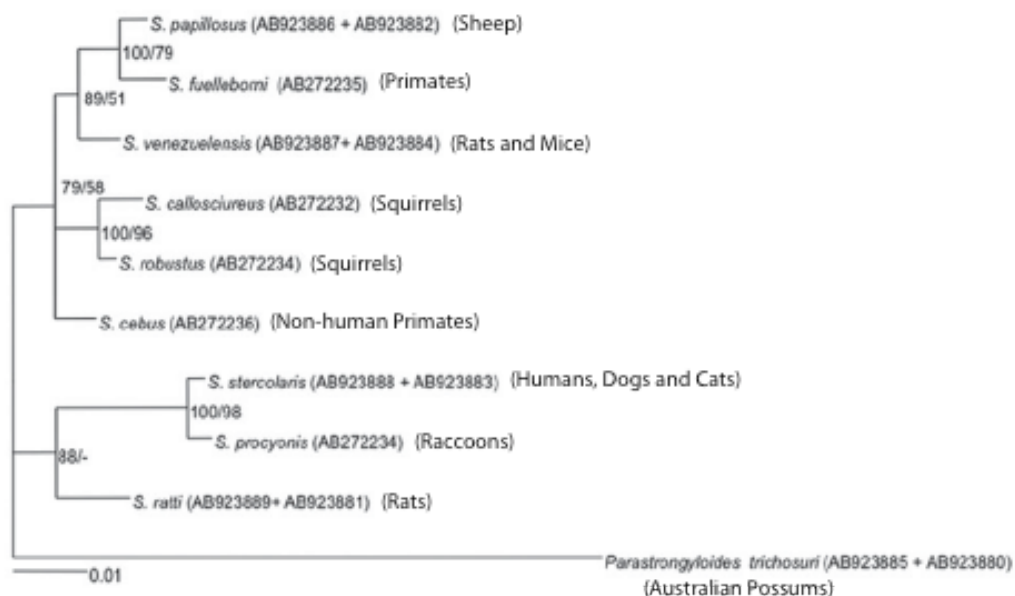
Figure 2. The molecular phylogenetic relationship between *Strongyloides* spp. and their hosts (according to [43]).

1.2.2 Morphology

There are clear morphological differences between different developmental stages and sexes of *Strongyloides* spp. [44]. The parasitic females are approximately 2-10 mm in length, depending on the species, with either blunt-ended or pointed tails (this is used for species morphological identification) and an elongated filariform esophagus [37][43][44][45]. The ovary is didelphic and opens at the vulva, that is positioned ventrally approximately two thirds along the body length [37][43][44]. The parasitic female of the genus *Strongyloides* contains no male gonads and, with the exception of two reports in the early 1930s which were later contested, the existence of parasitic males is not yet reported [43][44][45][46]. The free-living female is 0.64-1.7 mm in length, with didelphic ovaries and a vulva at the mid-point of the body [37][44][45][46]. The free-living male is 0.7-1 mm in length, with a short and conical tail consisting of 1 or 2 pairs of per-anal and 1 or 2 pairs of post-anal papillae, the spicules and the gubernaculums [37][45][46]. Both sexes in free-living stages have a rhabditiform esophagus. The length of the L1 is about 0.18-0.3 mm, whilst the L2 is about 0.25-0.54 mm [37][45][46]. The iL3 is approximately 0.4-0.63 mm in length and radially constricted, with a filariform esophagus approximately half the length of the larva and tripartite pointed tail that looks like a simple fork [37][44]. Morphological features useful for identification of *Strongyloides* spp. include the angle of vulval rotation, the presence or absence of post-vulva narrowing in adult free-living females, the natures of the tip and ventral membrane of the spicules, and the locations of the perianal papillae in adult free-living males. In parasitic females, the shape of the stoma in apical view, presence or absence of spiralling ovaries, the shape of the tail and the number of lobes on the circumoral elevation can be used for identification [37][47].

1.2.3 Life cycle and reproduction

Strongyloides spp. have unique and complicated life cycles compared to some other gastrointestinal nematodes and alternate between free-living and parasitic generations [44][48]. The hosts are infected when iL3 larvae

penetrate the skin (in experiemental infecions, the iL3 are frequently subcutaneously injected) [37]. These larvae migrate through the host body and 24 hours after infection they reach at the lungs or the naso-frontal region (depending on the species) of the host, where they will be swallowed to reach the small intestine [41]. During this migration they moult to L4 stages and then to adult parasitic females [41]. The parasitic adults are all females and live in the mucosa of the small intestine of the host [37][45].

The parasitic adult females reproduce by mitotic parthenogenesis (asexual reproduction) [45][49]. From six days after infection, eggs and/or first stage larvae (L1) will appear in the host feces depending on the species [41]. The female L1s can have alternative developmental fates [45][50]. Either they develop via L2-L4 stages into rhabditiform free-living adult worms, as do all the males. This type of development is known as indirect, sexual or heterogonic development [45][51]. The free-living males and females undergo sexual reproduction by mating [45][51] and all their progenies develop to female iL3s that can live in the environment (soil) until they encounter a suitable host, where the iL3s penetrate the host skin to initiate the parasitic cycle [37]. Pseudogamy (sperm dependent parthenogenesis) has been proposed as mode of reproduction of free-living generations of *Strongyloides* spp*.* For *S. ratti* and *S. papillosus,* it has been confirmed by crossing experiments and molecular genotyping that the free-living generations of these species actually reproduce sexually [45][51]. It is crucial to investigate the mode of reproduction in the free-living generations of the medically important *S. stercoralis,* both in laboratory and wild populations*.*

Alternatively, the female progenies of parasitic adults that pass with host feces can develop directly to iL3s. This kind of development is called direct or homogonic development or clonal or asexual reproduction [45][51]. As a medically important particuliarity, *S. stercoralis* but not the other species of *Strongyloides* investigated, has an auto infective cycle in which larvae develop into iL3s within the host. This allows the parasite to re-infect the same host individual. Therefore, *S. stercoralis* infections may persist for much longer than the life duration of an individual parasitic worm, even in the

absence of new infection [37][52][53][54]. These unique alternating life cycles provide opportunity to apply available genetic and molecular tools for better understanding of the basic biology and evolution of parasitism in *Strongyloides* [48]. The generalized life cycle of *Strongyloides* spp. is given in Figure 3 below.

Figure 3. Generalized life cycle of *Strongyloides* spp. (modified from [55]). The autoinfective cycle (in red) is specific for *S. stercoralis*. The developmental stage that leaves the host varies between species.

1.2.4 Sex determination and Karyotype

Many species of *Strongyloides* and *Parastrongyloides trichosuri* have an XX/XO sex determination mechanisms suggesting that XX/XO sex determination system is ancestral [56]. In *S. ratti* and *S. stercoralis* both sexes have two pairs of autosomes, and the females have two but the males have only one X chromosome [50][58]. So in these species females have 2n=6 and males have 2n=5 [50]. In *S. papillosus* and *S. vituli*, the I and X chromosome are fused [56][59][60] and sex-specific chromatin diminution in males result in the formation a hemizygous region corresponding to the X chromosome in *S. ratti* [50][56][58][59]. The males have 2n=5 and the females have 2n=4

[56][59]. However, the chromosomal differences between the sexes are not the primary sex determining factors, but sex determination in *Strongylodes* spp. also depend on environmental factors such as the host immune status [61]. Increased immune response of the host against the worms leads to a higher proportion of males [61]. A schematic representation of the chromosomes of the *Strongyloides* spp. discussed here is presented in Figure 4 below.
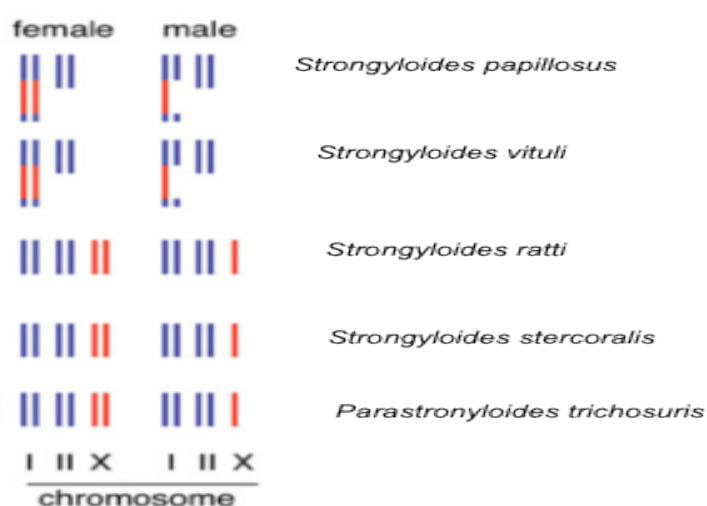
Figure 4. Schematic representation of *Strongyloides* and *Parastrongyloides* male and female karyotypes. Chromosomes/genomic regions present two copies in both sexes are depicted blue; chromosomes/genomic regions present in two copies in females and only in one copy in males are depicted in red (adapted and modified from [56]).

1.2.5 Genomes and transcriptomes

Whole genome and transcriptome sequencing of nematodes is becoming common and easy with the fast growing and improving next generation sequencing technologies [62][63][64][65][66]. *S. ratti* has the second most assembled and annotated nematode genome after the *C. elegans* reference genome, with a high-quality 43 Mb reference genome with the two autosomes [59] assembled into single scaffolds and the X chromosome [59] assembled into ten scaffolds [11][59]. High-quality draft assemblies ranging from 42 to 60 Mb were also released for *S. stercoralis*, *S. venezuelensis* and *S. papillosus* [11]. The *Strongloides* genomes (~43 Mb) are small compared to the

genomes of *C. elegans* (100 Mb) [67] and *P. pacificus* (169 Mb) [68]. The *S. ratti* and *S. stercoralis* genomes have GC contents of 21% and 22%, respectively, that make these species the most AT rich nematode genome reported so far [11]. The total protein-coding content of *S. ratti and S. stercoralis* ranges between 18–22 Mb [11]. The predicted numbers of genes for *S. ratti*, *S stercoralis*, *S. papillosus* and *S. venezuelensis* are 12,451, 13,098, 18,457 and 16,904 respectively [11].

Very little is known of the spatial and temporal regulation of parasitic nematode genes. Knowledge of expression pattern of these genes will help to understand their biological functions. Microarray and transcriptomic studies of *Strongyloides* began with analyses of Expressed Sequence Tags (EST) from different developmental stages of *S. stercoralis* and *S. ratti* [69][70]. These analyses resulted in the generation of about 11,000 and 15,000 ESTs that were grouped into 3,311 and 4,152 clusters in *S. stercoralis* and *S. ratti* respectively. Transcriptome analyses of different developmental stages of *S. ratti* and *S. stercoralis* identified genes possibly important in parasitism [11] [70]. Recently Nagayasu et al. [72] reported robust transcriptome analyses in four developmental stages of *S. venezuelensis*. O'Meara et al. [64] reported relatively stable gene expression in *S. ratti* parasitic adult transcriptome raised in rats with different immunological status. In *S. stercoralis*, transcriptome analyses were mainly performed in iL3s, to identify potential candidate genes for drug targets [63][73][74]. With rapidly improving genomic and transcriptomic sequencing technologies becoming available, more effort can now be put on assembly and complete annotation of the genomes of *Strongyloides* spp., to identify and characterize genes involved in development and parasitism.

1.2.6 Genetic tools

Unlike for *C. elegans*, there is a lack of robust functional genomic and genetic tools for most of the parasitic nematodes, which severely limits functional studies in these organisms [44]. However, there are some promising progress

being made in the area of transgenesis [75], CRISPR-Cas9 [76] and RNA interference [77].

1.2.6.1 Transgenesis

Transgenesis is the process of introducing an exogenous gene called a transgene into the germ line of an organism so that a new property will be transmitted to its offspring [78]. It is an essential tool for studying gene function and is used to search and validate essential molecular targets, for new drugs and vaccines in disease causing agents especially parasitic nematodes [78][79][80]. The access to free-living females and its morphological similarities to *C. elegans,* have helped to adapt gonadal microinjections as a method for gene transfer in *Strongyloides* spp. [81]. The wealth of genome sequence data available could be used to identify promoter regions and regulatory motifs, and to construct promoter reporter constructs to examine expression patterns of major genes, that may play significant roles in the parasitic life cycle of *Strongyloides* spp. [80].

In *Strongyloides* spp., transgenesis was first developed for *S. stercoralis* [75] [81]. The same protocols and promoter constructs from *S. stercoralis* were also used in *S. ratti* transgenesis [82], however with considerably lower transformation efficiency. Initial attempts to establish stable lines of transgenic *S. stercoralis* and *S. ratti* by following standard *C. elegans* protocols for the injection of plasmid-constructs failed. Although transgenes were established, they were not expressed from the second generation onward [79][82]. This may be due to transcriptional silencing in the multi-copy episomal arrays [82]. Shao et al. [79] solved this issue by creating constructs consisting of regulatory elements of the piggyBac transposon system, allowing the creation of the first stable transgenic lines with close to 100% transgene expressions after the second generations. Low germline transformation efficiency and survival rate of injected worms in *S. stercoralis* and *S. ratti* is a bottleneck in transgenesis in these organisms [75][79]. Several successful transgenesis in *S. stercoralis* have been reported [74][83] and these encouraging results will

help scientists to investigate the expression patterns and functions of more genes that play role in development and parasitic life of these nematodes.

1.2.6.2 CRISPR-Cas9

CRISPR-Cas9 is a newly developed genome-editing tool that has a wide range of potential applications [76][84][85]. The CRISPR-Cas9 system is most commonly used to introduce mutations in specific, molecularly defined loci [76][84][85]. The system is derived from a natural type of bacterial adaptive immune system[84], the type 2 short palindromic repeats (CRISPR) system. It involves a bipartite RNA (single guide or sgRNA, which in most experimental approaches replaces the two RNA molecules present in the natural system) that provides sequence specificity by base pairing, which then recruits the endonuclease Cas9 to the recognition site, leading to endonucleolytic cleavage of the DNA. Mutations arise by imperfect repair of the chromosome break. By providing a DNA molecule with the desired sequence as template, it is possible to introduce specific alteration into the genome. In *C. elegans* and other model nematodes, CRISPR-Cas9 has been successfully used to generate heritable mutations (insertions and/or deletions), by microinjecting DNA or RNA constructs coding for the Cas9 and the guide RNA or a mixture of the two components made in vitro into the gonad of hermaphrodites (which replace females in this species) [76][85][86][87][88][89]. But in parasitic nematodes, successful CRISPR-Cas9 transformation has not yet been reported [48]. However, CRISPR-Cas9 is a promising genetic tool to induce gene knock out to study gene functions in parasitic nematodes [48][90].

1.2.6.3 RNA Interference

RNA interference (RNAi) is a mechanism for RNA guided silencing of gene expression in eukaryotic cells [91]. It can induce both transcriptional and post-transcriptional gene silencing [92]. The natural functions of RNAi and its related processes seem to be protection of the genome against the invasion

of mobile genetic elements (i.e. viruses or transposons), as well as orchestrated functioning of the developmental programs of eukaryotic organisms [92][93][94]. In RNAi, double stranded RNA (dsRNA), complementary to a portion of a gene, is processed into small interfering RNAs (siRNAs) [95][96][97]. This effect is amplified beyond the initiation site within the gene by secondary siRNAs [98][99][100]. The siRNAs bind to the endogenous mRNA, forming a mRNA-siRNA duplex, which results in the recruitment of the RNA interfering specificity complex (RISC); this complex degrades/suppresses the endogenous mRNA [101]. The whole process results in the cessation/reduction of the effective function of the gene, which may result in visible phenotypic defects [102]. A potent gene silencing effect by RNAi was achieved in *C. elegans* by injecting double stranded RNA [95]. In nematodes, dsRNA/siRNA can be delivered by injection [95], soaking [103], feeding [104]. In parasitic nematodes electroporation was also atempted [105][106][107]. A schematic representation of the exo-RNAi pathway in *C. elegans* is given Figure 5 below.
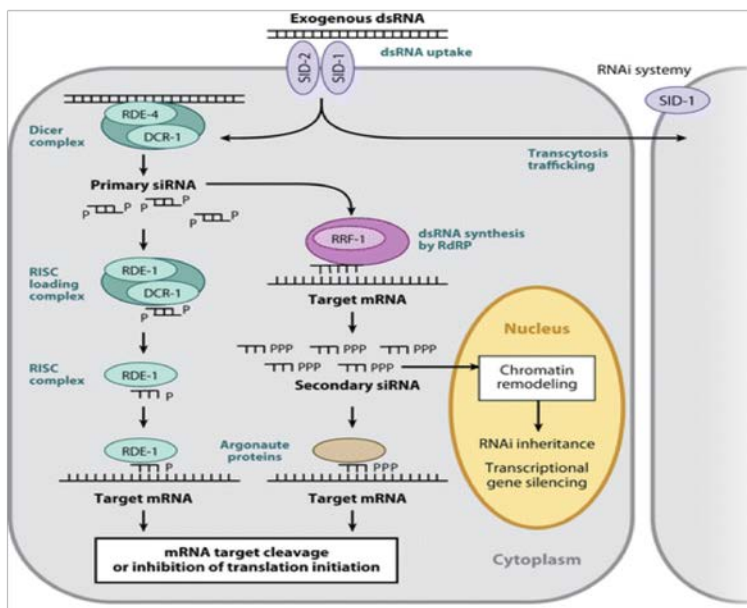
Figure 5. Schematic representation of the exo-RNAi pathway in *C. elegans* (adapted from [108]).

RNAi in several nematodes is becoming a very important tool to understand and characterize parasitism genes [77]. The first successful RNAi report in

gastrointestinal nematode was reported in *Nippostrongylus brasiliensis* [109]. Following this report, RNAi by soaking in dsRNA was reported for several parasitic nematodes: *Brugia malayi*, *Onchocerca volvulus*, *Haemonchus contortus*, *Ostertagia ostertagi*, *Ascaris suum* and *Trichostrongylus colubriformis* and has resulted in some RNAi mediated knock down [105][106] [110][111][112][113][114]. However, while transcript levels have been reduced for some target genes in parasitic nematodes, using current methods, RNAi seems to be inconsistent with variable results between species and between genes[77][108][115][116]. RNAi in plant parasitic nematodes seems to be more effective compared to animal parasitic nematodes [77][108]. In plant parasitic nematodes, usually the larval stages were soaked in dsRNA together with octopamine and/or serotonin to induce pharyngeal pumping [117], facilitating the ingestion of dsRNA. In animal parasitic nematodes, several difficulties are encountered when attempting RNAi such as reproducibility, specificity and susceptibility issues [118]. When using different siRNA/dsRNA delivery methods, RNAi was not successful for *Strongyloides* spp. and many other parasitic nematodes [119]. These mixed results suggest the importance of optimizing RNAi methods in animal parasitic nematodes to analyze gene functions which could be helpful in identifying candidate genes for drug and vaccine targets [120].

1.2.7 Strongyloidiasis in humans

In humans, strongyloidiasis is caused predominantly by *S. stercoralis,* but to a lesser extent also by *S. fuelleborni and S. fuelleborni kellyi* [37]. *S. stercoralis* infections have also been described in dogs, cats, and non-human primates [14][15][16][17][121]. *S. stercoralis* has a cosmopolitan distribution in tropical and subtropical regions and is the predominant species of *Strongyloides* in humans [36][122]. *S. fuelleborni* infection mainly occurs in African primates but is zoonotic and so infection can be transmitted to humans [15][37]. Human infections with *S. fuelleborni kellyi* has been reported only from New Guinea [123]. Humans become infected when iL3s come in contact with host skin and penetrate it, allowing the establishment of an infection [37].

Strongyloidiasis is one of the most neglected soil transmitted helminthiasis [36][124][125]. It is estimated to infect 100-200 million individuals worldwide [33][124], however this number could be an underestimate as it is often difficult to detect infections in single fecal examination [47]. The prevalence of *S. stercoralis* infections ranges from 5% to 40% in many tropical and subtropical countries [36] with higher prevalence among young individuals and children [44]. Factors such as high temperature, high moisture, poor sanitation and sharing premises with domestic animals may contribute to this high prevalence of *S. stercoralis* in developing countries within the tropical and subtropical regions [36][126].

In healthy individuals, strongyloidiasis is asymptomatic causing very little pathology [37]. However in immunocompromised individuals, it can result in serious complicated disease called hyperinfection syndrome (eventually leading to disseminated strongyloidiasis) due to massive invasion of autoinfective iL3s throughout the body [37][52][127][128]. This special ability to replicate in vivo and re-infect the same host is unique for *S. stercoralis* and infections have been shown to persist for many decades [37][54]. In chronic uncomplicated strongyloidiasis, major symptoms involve the gastrointestinal, respiratory and cutaneous systems. The common gastrointestinal signs are intermittent or persistent diarrhea, abdominal pain, pruritus ani, anorexia, vomiting (nausea), constipation and indigestion [37][129]. Chest pain, coughing and dyspnea are some of the symptoms involved in the respiratory system [37][129]. Skin rash, larva currens and urticarial are the major dermatologic signs from migrating infective larvae in the skin tissue [129].

In severe complicated strongyloidiasis, complication of gastrointestinal, respiratory, central nervous systems result in severe diseases [37]. The major gastrointestinal complications are abdominal distension, intestinal obstruction [130], dilation and thickening of the loops of the jejunum, necrotizing jejunitis, arteriomesenteric occlusion, small bowel infarction, biliary obstruction, aphthoid ulceration of the colon gastrointestinal hemorrhage and anorectitis [37][131][132][133][134][135][136][137]. It has been reported that some pulmonary complications like irritative or productive cough and shortness of breath, are often associated with wheezing that might lead to fatal adult

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respiratory distress syndrome [138][139]. Invasion of the central nervous system by migrating larvae, is often accompanied by secondary bacterial infection that might cause meningitis or brain abscess resulting in fever, headache, nausea, vomiting, neck stiffness, or convulsions or coma [37]. Septicemia due to enteric microorganisms like *Pseudomonas aeruginosa* may result in shock [132]. Pelvic inflammatory disease has also been ascribed to *S. stercoralis* [37]. Migration of larvae through skin may cause petechial or purpuric lesions [135][140][141]. In disseminated complicated strongyloidiasis, the mortality rate could reach up to 85 % [47] if anti-*Strongyloides* therapy is not given immediately. The most frequently used drugs for the treatment of strongyloidiasis are ivermectin (200 µg/kg body weight in a single dose) and albendazole (400 mg daily for 3 days) [142][143]. In a comparative study conducted on both regimens in Zanzibar, a cure rate of 82.9% and 45.0% was reported for ivermectin and albendazole respectively [142]. Zaha et al. [144] reported 96.0% efficacy for ivermectin used at 200 µg/kg that increased to 98.0% after a second follow-up treatment 2 weeks later. Patients with hyperinfection and disseminated strongyloidiasis are treated with repeated doses of Ivermectin several weeks apart to completely clear the infection [144]. Antibiotic therapy directed towards enteric pathogens and/or meningitis is also recommended in case of severe and complicated strongyloidiasis [37]. Ivermectin is contraindicated in children younger than 4 years old and pregnant women due to its toxicity [124]. Alternative drugs such as thiabendazole, cambendazole, and mebendazole can be used, but are less effective than ivermectin [122][143][145][146].

Commonly used standard parasitological methods for diagnosing strongyloidiasis are Bearmann technique, formalin-ether concentration, Kato-Katz technique, test-tube culture and agar plate culture [15][47][121]. These methods are laborious, time consuming, and need well-trained technicians [47][125]. Immunological and serological tests have been reported in the diagnosis of strongyloidiasis but with variable sensitivity and specificity [147][148][149][150]. The major clinical manifestations described above, coupled with the detection of rhabditiform larvae and/or embryonated eggs in patient stool by parasitological methods, will assist in the diagnosis of

strongyloidiasis in humans. However, these tests have to be repeated several times to detect the larvae/eggs in the stool, because of the low sensitivity and specificity of these methods [47]. *S. stercoralis* larvae are passed in the stool whereas in *S. fuelleborni,* embryos are passed as eggs. Autoinfection probably also does not occur in *S. fuelleborni* [37].

Specific detection of *Strongyloides* with improved sensitivity and specificity using DNA obtained from human stool samples, and by identifying using the 18S rDNA with real-time PCR, has been reported [151] as a promising diagnostic method. In *Strongyloides* spp.*,* the 18S rRNA sequence is highly conserved [152]. However, Hasegawa et al. [121] reported several nucleotide polymorphisms among different species of *Strongyloides,* in four of the hyper variable regions of the 18S rRNA (HVR-I to IV)*.* HVR-IV has been suggested for *Strongyloides* spp. diagnosis, because the nucleotide arrangements at this region is species specific and no within species variablility has been found [121]. As far as HVR-I and the rest of the SSU are concerned, some within-species variablity has recently been detected in *S. stercoralis* isolated from humans in rural Cambodia [153]. Hasegawa et al. [15] used the mitochondrial cytochrome oxidase subunit 1 (*cox*1) gene for identifying the cryptic variations in *S. stercoralis*, isolated from different host species. *S. stercoralis* is generally considered a zoonotic disease [37][125][126]; that transmits from humans to animals and vice versa but the infectivity of *S. stercoralis* of animal origin to human is a matter of debate [15][125]. To address this question I isolated *S. stercoralis* larvae from humans and dogs in the same household in rural communities in Northen Cambodia, where the living condition is ideal for strongyloidiasis (people walk barefoot, few or no toilet access). *Strongyloides* larvae were genotyped using 18S rDNA and *cox*1 molecular genetic markers. I also investigated the mode of reproduction of free-living generations of the laboratory isolate of *S. stercoralis*.

1.2.8 *Strongyloides* infections in animals

1.2.8.1 *Strongyloides ratti*

Strongyloides ratti is a common gastrointestinal parasite of rats (*Rattus norvegicus*) [154]. Although the worms normally reside in the small intestine, there has also been a report of isolation of *S. ratti* from the large intestine of wild rats [154]. *S. ratti* infection in rats is usually cleared 4 weeks after infection due to a strong anti-*S.ratti* immune response [155][156]. The prevalence of *S. ratti* in wild rats in the UK was reported to be 62% [157], demonstrating its high abundance in nature. *S. ratti* infections in rats can be detected by examination of host feces using Baermann technique and agar culture for the presence of rhabditiform larvae [47][158]. Immunological, serological and molecular diagnostic tools described above for *S. stercoralis* are also used in the diagnosis of *S. ratti* infections [148][150][159][160].

Phylogenetically, *S. ratti* is closely related to *S. stercoralis* [40][43], and is used as a laboratory model animal to study this human parasitic nematode [161]. The basic biology of *Strongyloides* including genetics [162], sex determination [50], reproduction [45] are well studied and documented in *S. ratti*. In addition, major molecular and genetic tools such as genetic linkage maps [163], transgenesis [79][82], transcriptomics [64] and a well-annotated genome [11] are available for *S. ratti*.

Mongolian gerbils (*Meriones unguiculatus*) have been found to be permissive hosts for many parasitic nematodes including *S. ratti* [19][21][22][164] [165][166]. Persistent *S. venezuelensis* [21][163] and *S. stercoralis* [22] infections have also been reported in gerbils. But there has been no report of natural infection of gerbils with any species of *Strongyloides*. The responses of both natural and permissive hosts to nematode infections can be quantified by infection dynamics and durations (parasitic worm burden, worm/egg output, sex ratios), and change in gene expressions [167][168][169][170]. In this thesis, I systematically compared the *S. ratti* infection dynamics in the

natural host rat and the permissive non-natural host gerbil. I also compared the transcriptomes of parasitic females isolated from rat and gerbil intestines.

1.2.8.2 *Strongyloides papillosus*

Strongyloides papillosus is a common gastrointestinal parasite of sheep and several domestic and wild ruminants [51][171][172][173]. *S. papillosus* infection is very common in sheep and cattle in tropical to temperate climate zones. There are reports of very closely related species of *Strongyloides* infecting ruminants [29][171][172][173][174][175]. Usually *S. papillosus* infections proceed without clinical manifestations, but heavy infections can result in fatal strongyloidiasis often called sudden death syndrome in young ruminants, due to sudden cardiac arrest without any premonitory signs [176]. The infection routes are peroral, percutaneous and galactogenic [177], but percutaneous is the most common route of infection, whereby infective larvae penetrate intact skin and establish infection [173][178].

The major clinical manifestations in S*. papillosus* infection in lambs and calves are diarrhoea, dehydration, anorexia, cachexia, anaemia, ataxia, nystagmus, slow growth and sudden death [172][179][180]. Commonly observed pathologies in *S. papillous* infected lambs and goat are ventricular fibrillations, enteritis, status spongiosus in the brain, hepatosis, hepatic rupture, nephrosis, pulmonary oedema and interstitial pneumonia [39][176][179][181]. Mortality rates of up to 25% with very high egg output (up to 5,000 eggs per gram of feces) were observed in calves on a beef farm located in South Bohemia, Czech Republic without previous clinical signs [38].

Strongyloides papillosus infections can be diagnosed by detection of the characteristic embryonated eggs in the feces [172][173][182]. The standard flotation technique is used for the eggs, but as the eggs hatch in few hours after fecal collection, the Baermann method is also suitable for detecting the larvae [60][179]. Parasitological and molecular diagnostic methods described for *S. stercoralis* above can also be applied in the diagnosis of *S. papillosus* infection. Ivermectin in a dose of 200 µg/kg administered subcutaneously,

albendazole and benzimidazoles are used for the treatment of adult *S. papillosus* in sheep, goat and cattle [183]. The biology, genetic and genomics of *S. papillosus* has been studied as in *S. ratti* [11]. The rabbit is a permissive sub-optimal host for *S. papillosus* and is commonly used to raise this parasite in the laboratory [60][173][184].However, successful infection using single iL3 to establish isofemale lines have so far not been achieved in rabbits, but are possible although technically difficult in lambs [59].

Transcriptomic information about the gene expression profiles of different developmental stages, have so far been reported for only three of the *Strongyloides* spp. (*S. ratti*, *S. stercoralis and S. venezuelensis)*, whose genomes were recently reported by Hunt et al. [11], but not for all of the life cycle stages. Hunt et al. [11] reported that CAP and Astacin gene families have undergone much duplication in *Strongyloides* spp. This extreme expansion coincided with the emergence of parasitism but continued during subsequent speciation events. They are possibly associated with the infection of novel hosts and makes members of these families primary candidates for genes associated with parasitism [11]. Blaxter et al. [7] suggested that parasitism evolved multiple times independently in the phylum Nematoda. Thus it seems unlikely, that there is only a single genomic basis for parasitism in nematodes [185]. To get more insight into the evolution of parasitism at the genomic level, multiple independent studies focusing on different parasitic species as well as non-parasitic outgroups from different clades would be needed. The identification of candidate genes associated with parasitism is very important for the development of potential treatments, especially in *Strongyloidides* spp. To contribute more to the ever-improving transcriptome areas of parasitic nematodes, I conducted a comparative transcriptomics analysis between different developmental stages of *S. papillosus* and compared the results with the published transcriptome data of *S. ratti*.

1.3 The genus *Onchocerca*

The genus *Onchocerca* consists of about 30 described species that can parasitize ungulates [24], humans [26] and dogs [23][186]. It is associated with endosymbiontic Wolbachia bacteria [221]. *Onchocerca* spp. undergo two host switches during their life cycle, between a vertebrate host and an insect intermediate host (in the case of the species discussed here black flies). Adult stages are only found in the vertebrate hosts. During the blood meal, the black fly takes up the first larval stage worms (microfilariae). The microfilariae then migrate to the muscle of the thorax of the fly, and molt to the second larval stage. After a few days they molt to the third larval stage, which is the infective stage. This infective stage migrates to the head of the fly and gets deposited into host tissue during the next blood meal [27]. It is very difficult to distinguish the infective larvae of different *Onchocerca* spp. morphologically. In the vertebrate host, the infective larvae undergo two more molts and develop to adult worms. Adult females can grow up to 30 cm while males are only about 5 cm long. The adult females of some species (among them the human parasite *O. volvulus)* induce the formation of a nodule made of host tissue consisting mainly of collagen, where they stay for the rest of their life. One adult female produce about 75, 000 to 100, 000 healthy embryos per reproductive cycle [27][187]. The embryos hatch to microfilarea in the female, and migrate to the subcutaneous tissues of the vertebrate host, to be taken up by black fly vector during a blood meal to continue their developmental cycle [27].

1.3.1 *Onchocerca ochengi* and *O. volvulus*

Onchocerca ochengi is a nodule-forming parasite of cattle. It is most closely related to *O. volvulus* (the causative agent of human onchocerciasis also called river blindness), because the black flies (vector) breed along fast running streams and rivers [188]. *O. ochengi* and *O. volvulus* show many parallels in their biology and share the black fly *Simulium damnosum s.l.* as a vector [26][27]. It has been proposed that *O. volvulus* arose from a host

switching event from cattle to their keepers as little as 10,000 years ago [24]. *O. ochengi* infection in cattle is not associated with any obvious pathology, and it was speculated that this is the consequence of a longer time of co-evolution between *O. ochengi* and cattle [191]. *O. volvulus* can render carriers completely blind. Two distinct strains of *O. volvulus* are known to exist in West Africa [24][26]: the Forest strain (which causes mild eye lesions) is endemic to the rainforest region, while the Savanna strain (causes severe eye lesions) is endemic in the savanna region [26].

The intradermal *O. ochengi* nodules are easily accessible. Their number increases over time because multiple sequential infections of the same cattle host are possible [188]. Because of this ease of accession and phylogenetic closeness, *O. ochengi* can serve as an experimentally more approachable animal model, for studying aspects of the *O. volvulus* biology [188], but not the pathology, since it causes little or no disease in cattle. Krüger et al. [24] isolated from black flies in Uganda iL3s of an undetermined *Onchocerca* spp., which they referred to as *Onchocerca* spp. variant Siisa. Based on very limited mitochondrial DNA sequence information, this variant appeared to be very closely related to *O. ochengi* and to *O. volvulus*, but the phylogenetic relationship between the taxa could not be resolved [24]. Eisenbarth et al. [190], using the partial sequences of the 12S, 16S and the *cox*1 mitochondrial genes as markers, identified microfilaria and adults of *Onchocerca* spp. Siisa also in black flies and cattle nodules in Cameroon. This demonstrated, that cattle is at least one of the definitive hosts of this variant. Recently, using nuclear and mitochondrial genetic markers, Hildebrandt et al. [191] has shown that *Onchocerca* spp. Siisa is interbreeding with *O. ochengi*. There are no obvious morphological differences between *Onchocerca* spp. Siisa and *O. ochengi* [24][190]. These findings suggest that *Onchocerca* spp. Siisa is a mitochondrial clade of *O. ochengi*. However, also in this study the phylogenetic relationship of *O. ochengi*, *Onchocerca* spp. Siisa and *O. volvulus* could not be resolved due to insufficient sequence information. To clarify this I determined the whole mitochondrial genomes of nine *O. ochengi* and two *Onchocerca* spp. Siisa individuals isolated from cattle.

1.4 Aims of this thesis

The unifying themes of this thesis are host specificity and host switching. Host switching at the level of current species that can use multiple hosts, is a very important process in the context of zoonotic transmission of pathogens to humans. Host switching in evolutionary time frames may be an important determinant for the formation of new parasite species. To address these questions I performed laboratory experiments and field studies. The aims of each of the four projects followed are outlined below.

1.4.1 Parasitological and transcriptomics comparison of *S. ratti* infections in rats and gerbils

In order to evaluate the consequences of living in a different, presumably suboptimal host, I decided to compare experimental *S. ratti* infections in rats (which are the preferred natural hosts) and in gerbils (which are permissive but presumably suboptimal hosts). I wanted to measure firstly parasite performance with respect to infection success, survival and reproduction and secondly, to compare the global gene expression profiles in order to identify genes that are differentially expressed between the parasitic adults isolated from both hosts.

1.4.2 Comparative transcriptomics analyses of different developmental stages of *S. papillosus*

Recently Hunt et al. [11] reported the genomes of four species of *Strongyloides*. While the genomic sequence of *S. papillosus* was included in this work, however the transcriptomic studies were not reported for this species. I sequenced the transcriptomes of different developmental stages of S. *papillosus* and compared them among themselves and with the published data of *S. ratti*. Special attention was given to Astacin and CAP protein families, which are very expanded in *Strongyloides* spp.

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1.4.3 Genetic variations and reproduction in human parasitic nematode, *S. stercoralis*

S. stercoralis is a human parasitic roundworm but natural infections have also been reported in dogs, cats and several non-human primates. The aim of this project was to determine to what extent *S. stercoralis* is transmitted from dogs to humans and vice versa. By determining the nuclear 18S rDNA and mitochondrial *cox*1 sequences of *S. stercoralis* individuals isolated from humans and dogs in the same households in rural Cambodia, I addressed the question of whether *S. stercoralis* in dogs and humans form single or separate populations.

1.4.4 Genetic and phylogenetic analyses of *O. ochengi* isolates using complete mitochondrial genome sequences

It is hypothesized that *O. volvulus*, the causative agent of human river blindness, evolved from the cattle parasite *O. ochengi* through a host switch from cattle to their keepers, as recently as 10,000 years ago. Based on very limited sequence information, it had been proposed that with respect to their mitochondrial genome, two different clades exist within the species *O. ochengi*, referred to as type "ochengi" and type "Siisa". Phylogenetically, these two-mitochondrial types appeared to be equally distant from each other, than either of them is from *O. volvulus*, raising some questions about the species status of *O. volvulus*. I set up to re-evaluate the phylogenetic relationship of *O. ochengi* type "ochengi", *O. ochengi* type "Siisa" and *O. volvulus* by including full mitochondrial sequence information.

2. Results and Discussion

2.1 Parasitological and transcriptomic comparison of *S. ratti* infections in rat and gerbil hosts

2.1.1 *S. ratti* infections: duration, worm output, sex ratios and worm burdens

In two independent experiments, groups of four male rats and four male gerbils were infected with 500 iL3s per animal. The worm output was measured starting at day six after infection as described in materials and methods. The nightly total worm counts are shown in Figure 6 A-B. The total worm count was higher in gerbils compared with rats, but the worm output was higher in rats during the first two weeks of infection. However, it is important to note, that the absolute numbers of worms counted are not really comparable between the two different hosts because they might pass different proportions of their daily output during the sampling period. The mean *S. ratti* total worm output count was highest around the 15th day post infection in rats and about one day later in gerbils and then declined in both hosts. However, while the decline in rats was fast and after one month, no worms were detected, in gerbils, substantial worm output was observed for more than four months and only after 6 months were no larvae found anymore.
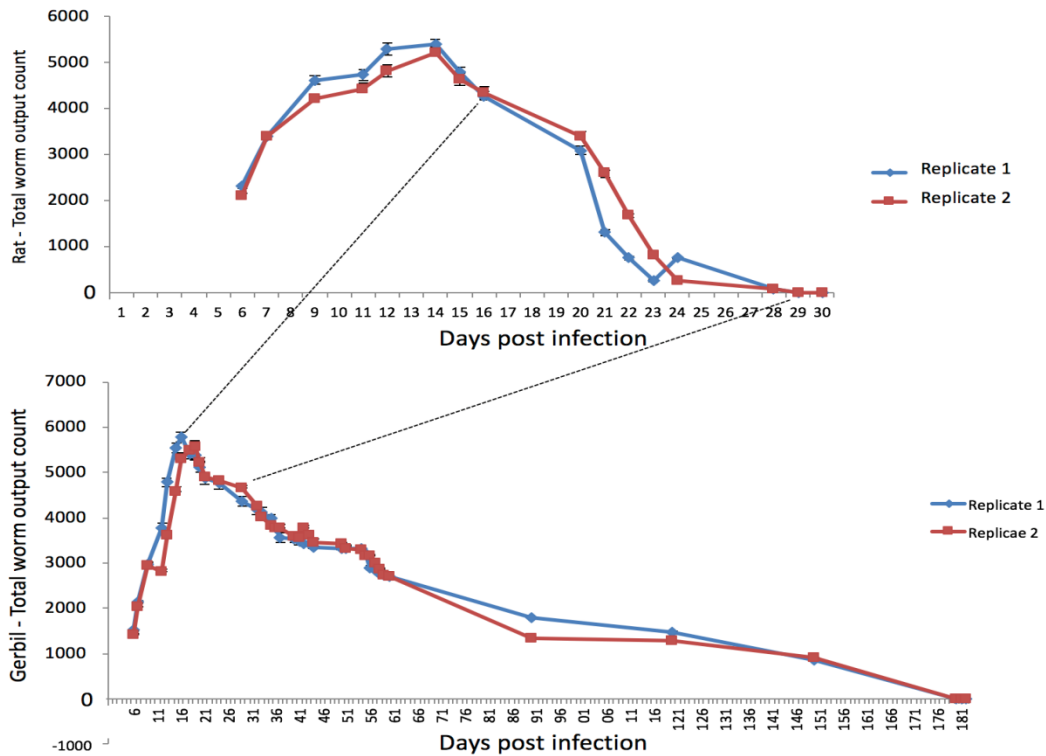
Figure 6. Nightly mean total worm count per host individual from rats (A) and gerbils (B) infected with 500 iL3 per animal. Error bars are the standard deviation between the six parallel cultures made from each collection cache. PI days = post infection days; TWC = total worm count; R1 = replicate 1; R2 = replicate 2. The TWC over the lifetime was significantly higher for the worms in gerbils (p =0.001768).

These results are in line with previous reports on other *Strongyloides* spp. raised in permissive but presumably suboptimal hosts. In *S. venezuelensis*, persistent infections of gerbils that lasted 450 days [164] and 570 days [21] were reported. In the second, the limiting factor was actually the life span of the gerbil hosts, which are short-lived animals. In these experiments, the gerbils were able to clear the *S. ratti* infections after about six months. This might be due to the low dose of iL3s (500) used in this experiment or because gerbils are indeed able to eventually clear *S. ratti* but not *S. venezuelensis* infections [164]. The quick clearance within about a month of *S. ratti* infections from rats is due to a strong anti-*S. ratti* immune response [155][156]. Khan et al. [192] claimed that the inability of Mongolian gerbils to expel *S. venezuelensis* is not due to a failure to recognize the parasite antigens, but due to defects in effectors/regulatory cells such as mast cells. Nolan et al. [22]

also reported prolonged *S. stercoralis* infection in gerbils of up to 130 days without using immunosuppressant drugs. *S. stercoralis* which is very closely related to *S. ratti,* has the special ability to maintain long lasting infections in humans and dogs through an auto-infective cycle [37]. Autoinfection has also been observed in experimental infections of gerbils with *S. stercoralis* [193]. However, the prolonged duration of *S. venezuelensis* [21][164] and *S. ratti* [19] appear not to be associated with autoinfection.

There was a significant difference in total male to female ratios between worms derived from gerbils and rats feces (Figure 7 A-B). While the sex ratio remained stable with a slight excess of females in cultures from rats, in cultures from gerbils, an excess (3.8 fold) of males was observed during the first two weeks, which then declined to approximately equal numbers of males and females after one month of infection. An elevated proportion of males has also been observed for *S. papillosus* (natural host sheep) in rabbits, which act as permissive hosts [46] and in cattle [173] but not for *S. ratti* in mice [61]. As expected, based on earlier literature [194], culture temperature had no effect on the sex ratio.
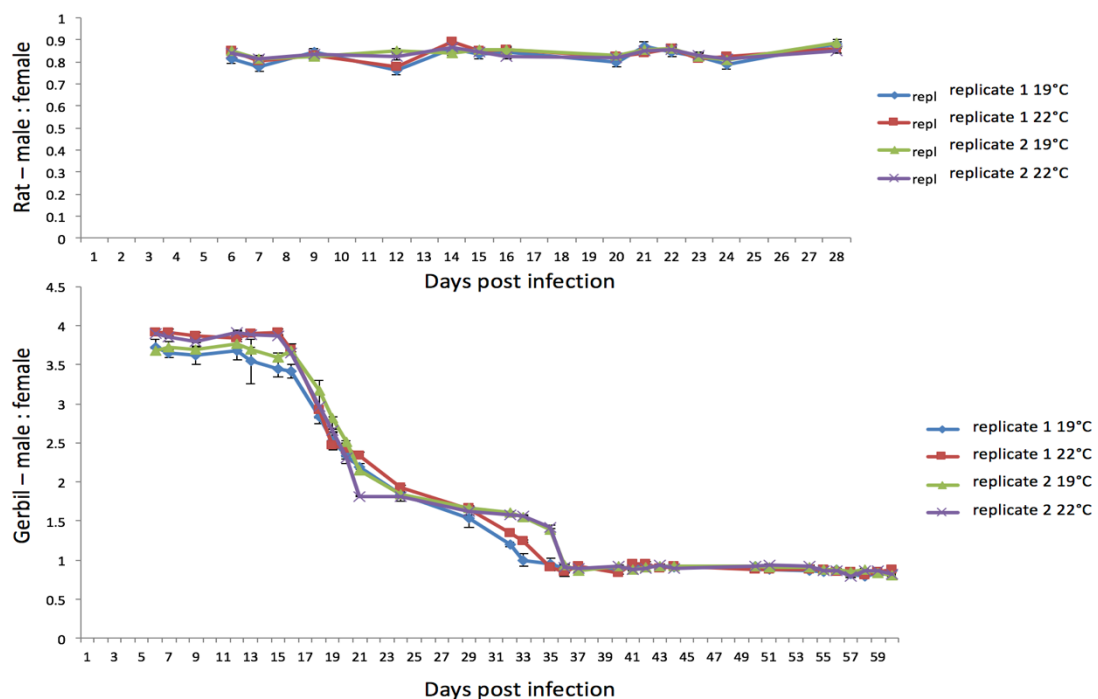
Figure 7. Total male to female ratios of worms collected from rats (A) and gerbils (B) feces cultured at 19°C and 22°C. Error bars are the standard deviations between the six parallel cultures made from each collection cache. PI days = post infection days; R1 = replicate 1; R2 = replicate 2. The difference in sex ratio over the lifetime is highly significant (p<0.00001).

In two separate experiments, groups of four rats and gerbils were infected with 500 iL3s per animal and 12 days after infection, the animals were sacrificed and the adult parasitic worms were isolated from the small intestine and counted. The worm burden in rats (288.25±6.6 and 290±9.93 in the two replicates, respectively) was significantly higher compared to gerbils (65±8.1 and 70±6.32) (Figure 8). There were no visible phenotypic differences between parasitic adults isolated from rats and gerbils. These results clearly showed that in gerbils the infection success is lower than in rats, but those worms that are successful in gerbils, survive longer and produce more progeny than in rats. The high worm output from gerbils when compared with rats might lead to the impression that gerbils are even better hosts than rats. But this cannot be concluded for various reasons. Firstly, the absolute numbers of worms observed cannot be directly compared between the two hosts, because the determined worm output was over night and not over the full day. The two host species have different circadian rhythms and it is therefore likely that they shed a different proportion of the total larvae produced during the collection window. For animal welfare reasons it was not acceptable to maintain the animals permanently in collection caches. Secondly, experimental infections were done by subcutaneous injection of infective larvae, thereby bypassing the natural host recognition and skin penetration. Thirdly, in gerbils a high, probably suboptimal, proportion of males were produced.
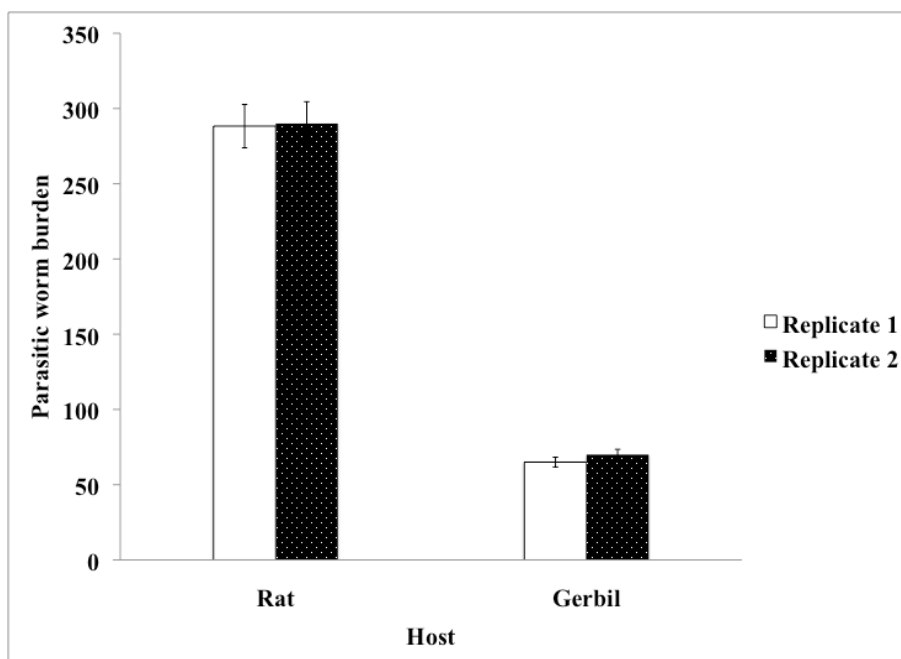
Figure 8. Mean number of parasitic worms isolated from sacrificed rats and gerbils. The error bars are standard deviations. The difference is highly significant (p<0.00001)

2.1.2 Transcriptomes of parasitic *S. ratti* adults isolated from rats and gerbils

The parasitic worms isolated from the small intestines of the four animals of a group were pooled resulting in two biological replicates. I sequenced poly (A) plus transcriptomes of parasitic worms isolated from both hosts. In all four samples between 10,000 and 11,000 transcripts were covered. The two biological replicates for both groups correlated very well (Figure 9). The expression profiles in the two hosts were very similar with only relatively few genes showing significantly different expressions. In the first replicate, we identified 259 transcripts, which were differentially expressed, of which 39 and 220 were higher in parasitic worms isolated from rats and gerbils, respectively. In the second dataset, we found 204 transcripts to be differentially expressed, 84 up-regulated in worms from rats and 120 up-regulated in worms isolated from gerbils. In most instances the differences were very close to the significance borders and only 23 transcripts, which

were differentially expressed in both RNA-Seq datasets were identified (Table 1). One transcript, predicted to encode a cuticular collagen, was differentially expressed in opposite direction in the two replicates. Among the other 22 transcripts, three were derived from putative astacin-like metalloendopeptidase genes and four from genes predicted to encode acetylcholinesterases.
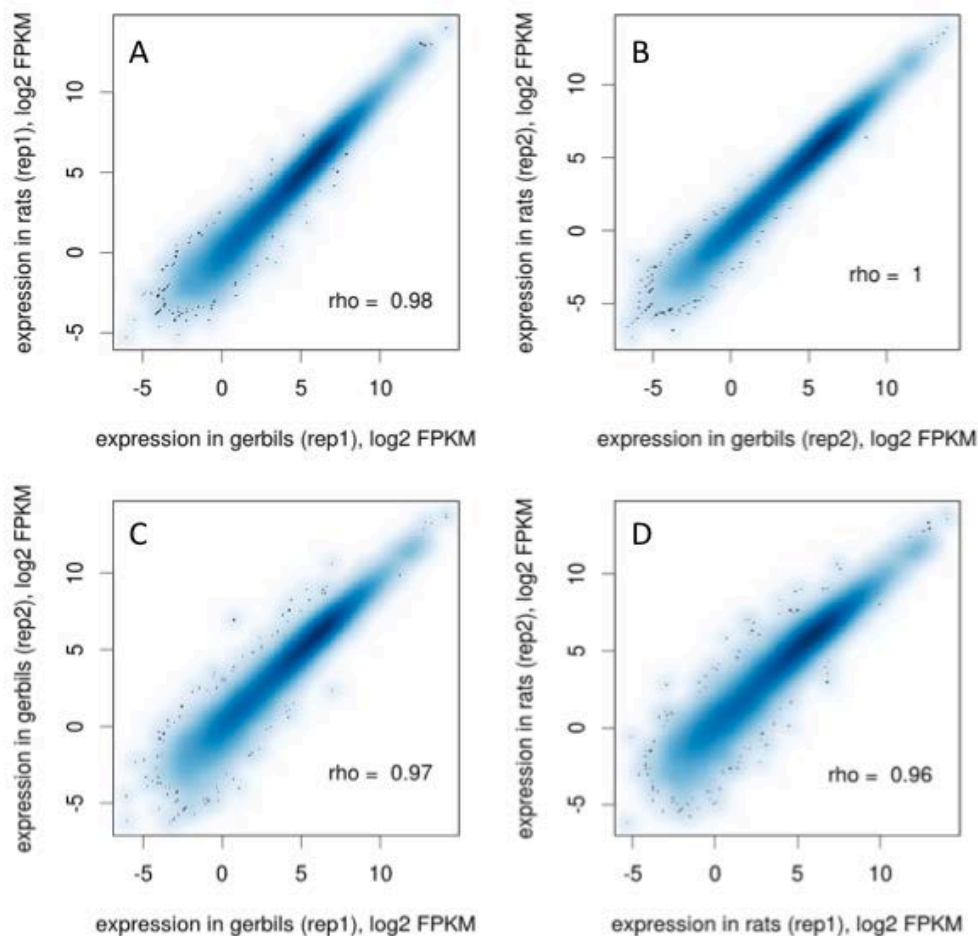
Figure 9. Correlation of the different parasitic adults RNA-Seq experiments. A) Comparison between rats and gerbils, replicate 1; B) comparison between rats and gerbils, replicate 2; C) Comparison between the two replicates in gerbils; D) Comparison between the two replicates in rats.

Despite the different hosts and the clear *S. ratti* performance in rats and gerbils, there were very little differences between the gene expression patterns. Similarly, an earlier study comparing parasitic adult *S. ratti* in rats

with varying immunological status, found only very little difference in over all gene expression in spite of considerable phenotypic differences [64]. In the present study, even among those genes that were significantly differentially regulated in both experiments, the fold changes were generally rather low. Only seven genes, among them two astacin-like and one acetylcholinesterase gene, were on average over the two experiments, up-regulated in gerbils more than two fold (maximum 3.4 fold) and only two genes, both Acetylcholinesterases, were reduced to less than half in gerbils. Astacin-like and acetylcholinesterase family genes were very prominently represented among the genes that were significantly differentially expressed in both experiments (three and four genes respectively among a total of 23). Both of these gene families are greatly expanded in Strongyloididae [11]. Different members of these families show different developmental stage specific expression patterns. All three astacin-like genes identified, belong to the fraction of this family described to be high in parasitic adults compared with free-living and infective stages [11], and all three were further up-regulated in worms from gerbils compared to worms from rats. In fact, the two most strongly up-regulated genes in gerbils were astacin-like genes. Of the four-acetylcholinesterase genes, one was up regulated in gerbils, while three were down regulated in this host. The two most strongly down-regulated genes in gerbils were acetylcholinesterase genes. Interestingly, both these genes were found by Hunt et al. [11] to be down regulated in parasitic adults when compared with iL3s. The significance of these observations remains open for the moment and further functional analyses will be needed to investigate the role of these genes during *S. ratti* infections in the two hosts.

Table 1. Expression values and comparison for all differentially expressed *S. ratti* transcripts*.*

No.	WormBase ParaSite Transcript ID	Fold change (R/G) RNA-Seq 1	P value	Fold change (R/G) RNA-Seq 2	P value	NCBI and InterPro blast (Spp. and gene prediction)
1	SRAE_X000195300	1.594	0.000573	1.488	7.34E-07	*H. polygyrus bakeri*, acetylcholinesterase-2
2	SRAE_X000061300	3.248	2.36E-10	2.510	1.76E-13	*B. malayi*, acetylcholinesterase
3	SRAE_X000062300	4.718	0	2.90	0	*D. viviparus* Secretory acetylcholinesterase variant 2
4	SRAE_X000147200	0.0952	3.67E-06	0.485	5.02E-06	*S. stercoralis*, Metalloproteinase proprotein
5	SRAE_X000147300	0.1322	0	0.580	3.59E-12	*S. stercoralis*, Metalloproteinase proprotein
6	SRAE_X000064500	0.6615	0.040236	0.774	0.0358	*A. suum*, Innexin-3
7	SRAE_0000057800	0.3606	1.08E-05	0.510	1.51E-07	Hypothetical protein
8	SRAE_X000012700	0.632	0.01564	0.736	0.026	*A.suum* Elongation of very long chain fatty acids
9	SRAE_X000030400	0.420	8.40E-08	0.556	5.80E-09	*C.remanei*, Fatty acid desaturase family member (fat 3)
10	SRAE_X000062000	0.202	0	0.5546	0	*B. malayi*, Neuronal acetylcholinesterase
11	SRAE_1000035700	0.48	0.030204	0.525	3.23E-05	*B. malayi*, Sugar transporter family protein
12	SRAE_10000101500	0.551	0.020092	0.695	0.026434	Hypothetical protein transcript evidence in iL3 larvae
13	SRAE_1000110000	0.499	3.12E-05	0.588	1.76E-06	*C. brenneri*, oxidoreductase, zinc binding dehydrogenase
14	SRAE_2000131800	0.526	0.016051	0.50	5.15E-08	Hypothetical protein, transcript evidence in iL3 larvae
15	SRAE_2000326000	0.618	0.007719	0.74	0.0172	*C. elegans*, Zinc metalloproteinase (nas)
16	SRAE_2000377500	1.52	0.01212	0.351	0	Nematode cuticle collagen
17	SRAE_2000456100	0.420	0.023931	0.508	0.0073	*A. suum*, DeHydrogenases, Short chain family member
18	SRAE_2000499920	1.418	0.029391	1.414	4.23E-05	Hypothetical protein transcript evidence in iL3 larvae
19	SRAE_2000498800	1.757	7.50E-05	1.298	0.0349	Hypothetical protein transcript evidence in iL3 larvae
20	SRAE_X000146200	0.201	0.000497	0.545	0.0416632	Hypothetical protein, no blast, no interpro
21	SRAE_X000177900	0.5819	0.024504	0.606	0.0004061	Glutathione peroxidase domain containing protein
22	SRAE_X000201400	0.564	9.88E-05	0.784	0.016248	*C. briggsae*, Argonaute Like Gene family member
23	SRAE_X000238600	1.66	0.0047	1.387	0.0091248	*C. briggsae*, Non specific lipid transfer protein

In summary, upon experimental infection by subcutaneous injection of infective larvae, *S. ratti* can establish patent infections that last for several months in Mongolian gerbils. The portion of infective larvae that successfully colonize the host is smaller than in the natural host (rats), but the ones that are successful live longer and reproduce well. However, they produce a high proportion of male progeny. These prolonged and stable infections make gerbil good laboratory host for the long-term maintenance of *S. ratti*. The

transcriptomes of parasitic *S. ratti* in rats and gerbils are very similar. Among the few differentially expressed genes, astacin-like and acetylcholinesterase family genes are prominent.

2.1.3 Contributions

I designed this study with Dr Adrian Streit. I performed all experiments in this study. I did subcutaneous infection of rats and gerbils, collection and culture of feces and counting worm output from the culture. I isolated parasitic adults from rats and gerbils and extracted total RNA. I prepared transcriptome libraries for sequencing for in all biological and technical replicates. The sequencing was done in the in house sequencing service facility Bioinformatic analyses were done by Dr. Christian Rödelsperger. I wrote the manuscript with Dr Streit. This work has been submitted to Experimental Parasitology.

2.2 Comparative transcriptomics analyses of different developmental stages of *S. papillosus*

2.2.1 Overview of the differentially expressed genes

In order to compare the gene expression profiles, ten transcriptome libraries from six developmental stages of *S. papillosus* were prepared and analyzed. Principal component analyses of the estimated expression levels quantified as FPKM (Fragments per kilobase transcript per million mapped reads) were estimated using Cufflinks [229]. The principal component analysis indicates that 55% of the variations can be explained by the first two principal components (Figure 10). The ten transcriptomes basically form three clusters, a cluster with young larvae and males, a cluster with parasitic and free-living females, and a cluster with infective larvae. The samples within a cluster are highly correlated in terms of gene expression values when compared with samples from other clusters. The median Spearman's correlation coefficients

for within-clusters comparison and between-clusters comparison were 0.92 (Q1: 0.82 and Q3: 0.99) and 0.73 (Q1: 0.68 and Q3:0.82) respectively (Figure 10). Variations between different developmental stages are considerably larger than the variation between biological replicates. Therefore, the identified transcriptome profiles robustly capture gene expression differences during the development of *S. papillosus*. Pairwise differential gene expression analysis showed that the number of differentially expressed genes vary widely from 0.2% (L1/L2 stage from parasitic females in comparison with L1/L2 from free-living) to 45% (iL3s in comparison with parasitic females stage) (Figure 11). Comparison between free-living and parasitic females showed only 10% of genes were significantly differentially expressed and of that, only 4.4% (917) of genes were up-regulated in parasitic females. Overall, 73% of *S. papillosus* genes are found as significantly differentially expressed (FDR< 0.05) in at least one comparison. This observation indicates that the evolution of small set of genes is enough to acquire parasitism. This relatively small set of infection-associated genes is consistent with previous studies, which found that 909 and 1188 genes were up-regulated in parasitic females in *S. ratti* and *S. stercoralis* respectively [11]. Other studies in *Strongyloides* spp. identified much smaller candidate sets for parasitism-associated genes[72][195].
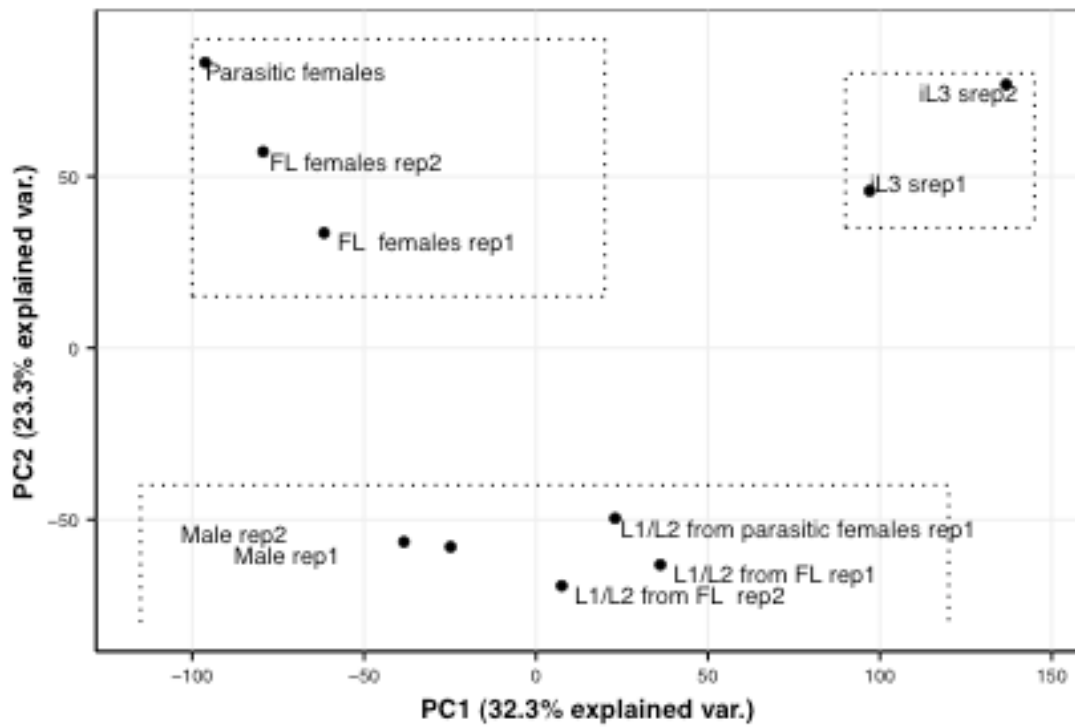
Figure 10. Principal component analysis of *S. papillosus* different stages transcriptomes replcates. The expression values show a subdivision of transcriptomes into distinct clusters that are defined by developmental stages and sex from different batches of RNA-Seq dataset.
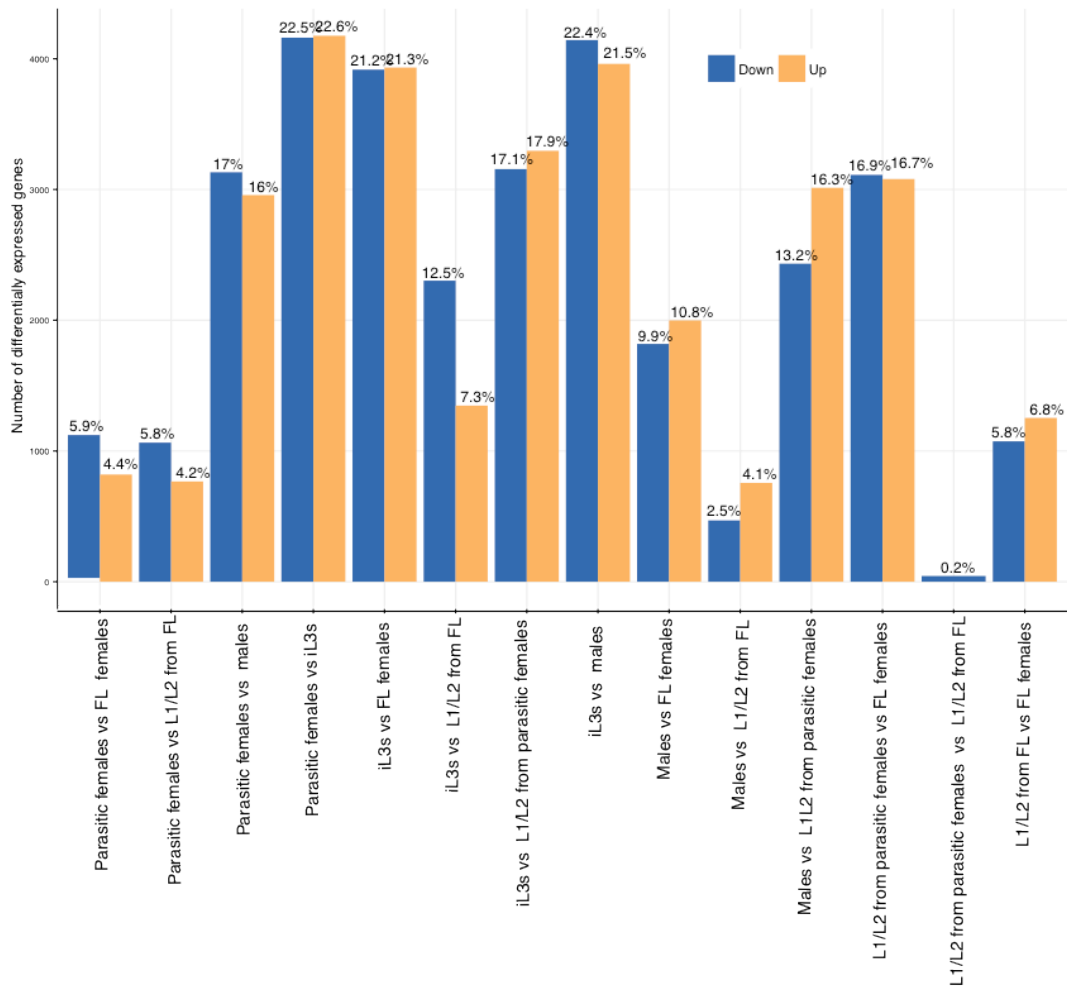
Figure 11. Numbers of significantly differentially expressed genes between different developmental stages of *S. papillosus* transcriptomes. Dark blue and orange colors indicate significant enrichment (FDR <0.05 and enrichment value > 1) and no enrichment respectively.

In order to better understand the evolution of gene expression in different developmental stages, the genes were classified into three groups based on sequence similarity to *S. ratti* using Orthomcl [196]. Homologous genes maintained as single copy in both species were treated as one-to-one orthologs, and paralogous gene clusters were classified as ancient and recent duplications depending on the presence and absence of *S. ratti* genes in the clusters respectively. Based on this definition, 9302 one-to-one orthologs, 2952 ancient duplicated genes, 3443 recently duplicated genes and 2759 orphan singletons with no homolog in either *S. papillosus* or in *S. ratti* were found. The enrichment of these different orthology classes among

differentially expressed genes was also tested. In the majority of the cases, one-to-one orthologs were highly enriched among genes differentially expressed between different developmental stages (Figure 12). One of the interesting findings is the strong depletion of recent duplicates in all comparison, except two female parasitic stage comparisons. Enrichment of duplicates among female parasitic stage-specific genes suggests a role of gene duplication in the evolution of parasitism in *Strongyloides*. In phylogenetic analyses of Neurotransmitter-gated ion channel (NGIC) genes (one-to-one orthologous) from *S. papillosus* and *S. ratti* were grouped together (Figure 13). This indicates the robustness of the computational approach used for assigning orthologous classes. The majority of one-to-one orthologs are up regulated in iL3 stage in comparison with free-living and parasitic females in both species. In general, one-to-one orthologs have similar expression patterns in *S. papillosus* and *S. ratti*, which implies a high degree of conservation.
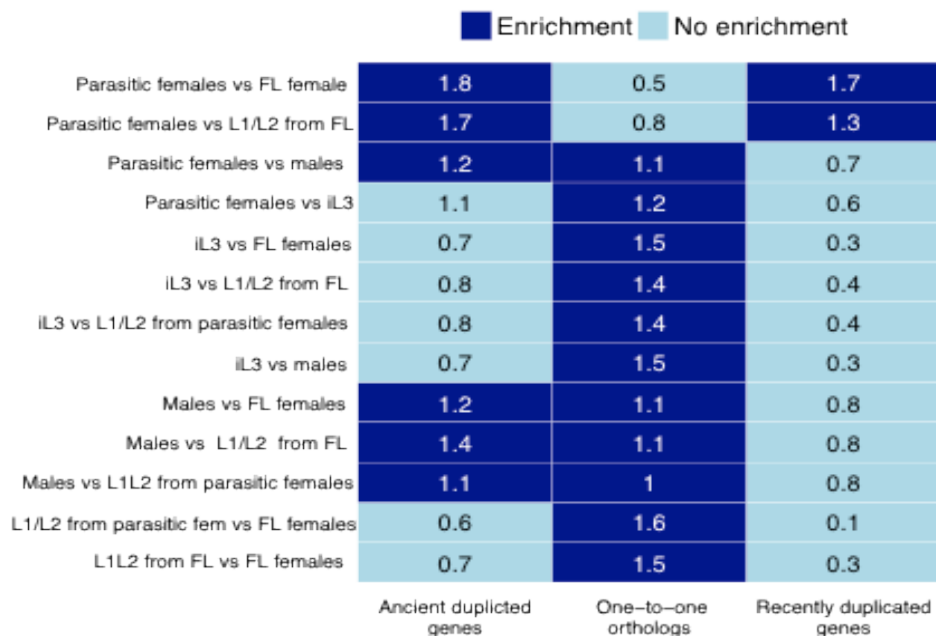
Figure 12. Pairwise comparison of *S. papillosus* transcriptome based on their expression. FL= Free-living; iL3= infective larvae 3; L1/L2=stage 1 and 2 larvae. This analysis reveals that most comparisons are enriched in one-to-one orthologs indicating that developmentally regulated genes tend to be conserved at the sequence level.
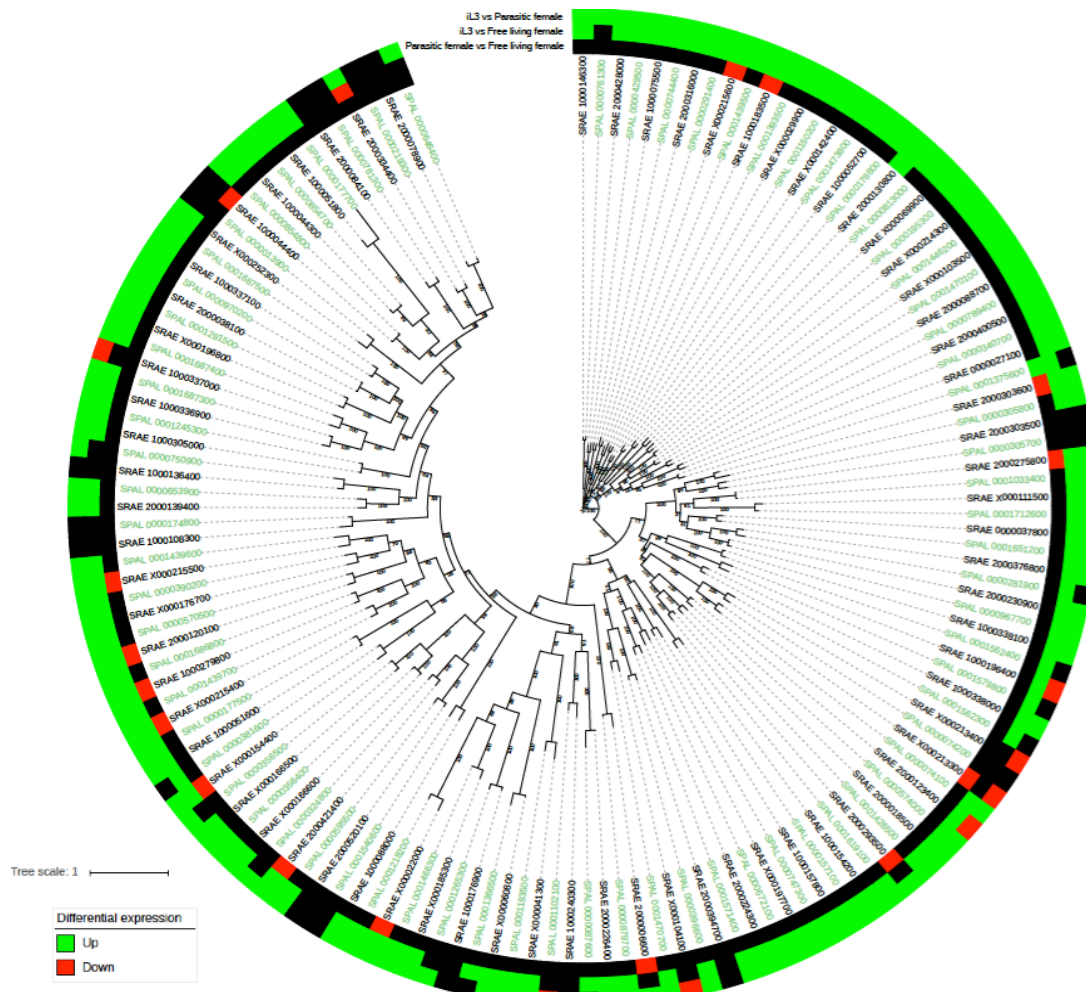
Figure 13. Gene expression, sequence evolution and phylogenetic tree of *S. papillosus* (green) and *S. ratti* (black) NGIC gene family. The heatmap around the phylogenetic tree indicates differential expression pattern of each gene in three different comparisons. In the heatmap, up-regulation, down-regulation and no change are shown in green, red and black respectively. This tree shows that the majority of one-to-one orthologs in the NGIC gene family have similar expression pattern in *S. papillosus* and *S. ratti*.

2.2.2 Expressions of Astacin and CAP gene family members

Protein domain enrichment analyses were performed to identify the functional component of the differentially expressed genes (Figure 14 A). 221 gene families show enrichment in at least 2 different comparisons, with Pkinase, HSP20, motile sperm, collagens, different sub families of GPCR and few other families in more than 5 comparisons. One of the interesting findings is the enrichment of astacin and CAP gene families among genes up-regulated in parasitic females compared with free-living females (Figure 14 B). This is consistent with the recent observation of astacin and CAPs enrichment in the parasitic females of *S. ratti* and *S. stercoralis* [11], and further supports the hypothesis proposed by Hunt et al. [11], that the expansions of astacins and CAPs are associated with the evolution of parasitism in *Strongyloides* spp. Apart from the enrichment of these two gene family members in the parasitic female stage, a considerable number of astacin and CAP genes were also up-regulated in iL3 stage. In these analyses, more than 200 genes with astacin domains show significant differential expression, of which 131 genes show up-regulation in parasitic females and 67 in iL3 stage in one or more comparisons. Similarly, 94 and 49 CAP domain genes show up regulation in parasitic females and iL3 in at least one comparison respectively.
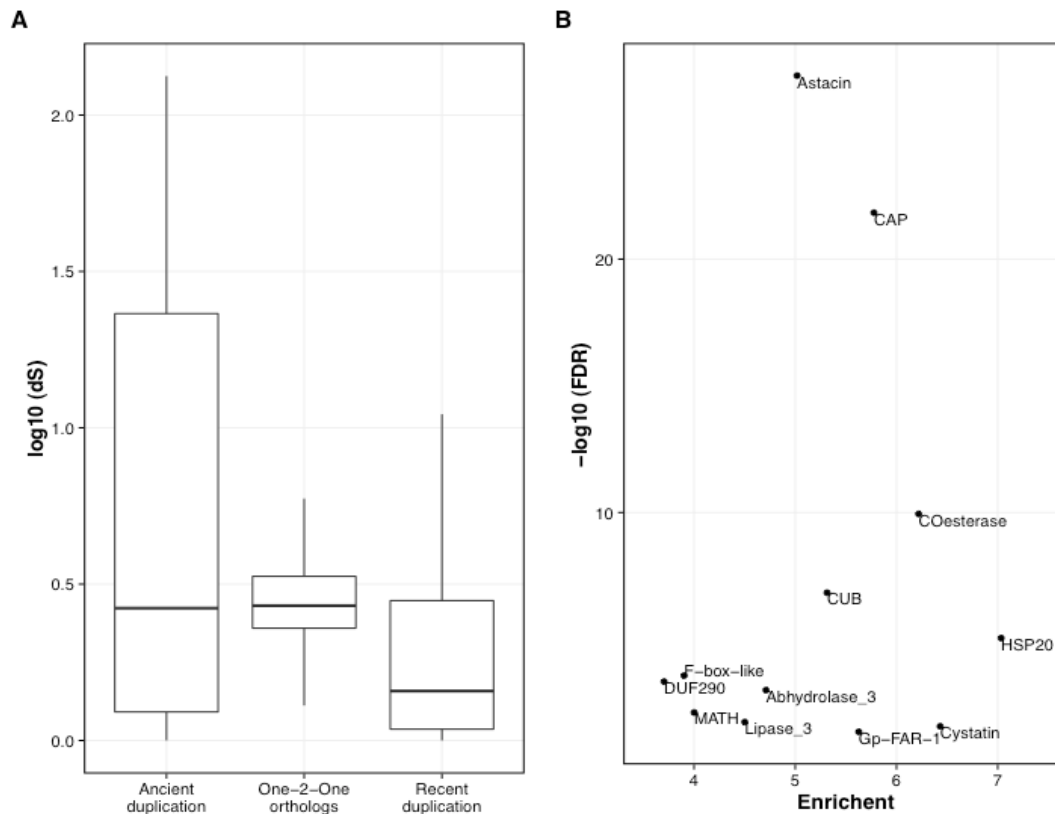
Figure 14. Age classification and domain enrichement of the *S. papillosus* transcriptomes. A) Box plot representation of the age of genes as measured in dS (synonymous substitutions rate). The median age of recently duplicated genes is lower than the one of ancient duplicated genes and one-to-one orthologs; B) Enrichment of protein domains among genes up regulated in paraistic females compared with free-living females (FDR < 0.05 and enrichment value > 1).

Upregulation of members of the same gene families in different developmental stages raised a question of how variable expression patterns can arise within a single gene family. Recent work in the nematode *Pristionchus pacificus* showed evidence for subfunctionalization in terms of developmental regulation in the HSP20 gene family [197]. A Phylogenetic tree was reconstructed using *S. papillosus* CAP protein sequences and its differential expression matrix, and two different sets of paralogous gene clusters with interesting differential expression pattern, labeled as Cap-A and Cap-B were found (Figure 16). Genes in Cap-A was up-regulated specifically in the iL3 stage and showed no significant change in other comparisons (Figure 15 A). Similarly, genes in Cap-B are up-regulated predominantly in the female parasitic stage (Figure 15 B). This confirms that different members of

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the CAP gene family have different expression profiles and the expression pattern is not conserved within this family.

On the other hand, the Astacin tree indicates that more than half of the Astacin gene family members show stage specific differential expression in parasitic females and iL3 stages (Figure 17). Two major clusters (Asta-A and Asta-B) in the Astacin tree were found. Whereas genes in Asta-A show no shared expression pattern, individual subclusters in Asta-B show shared but heterogeneous patterns of differential expression. For easy understanding, Asta-B was subdivided into five distinct subclusters based on their differential expression pattern. Genes in paralogous cluster Asta-B1 and Asta-B5 showed up-regulation in parasitic females (Figure 18 A and D), and Asta-B2 and Asta-B3 genes in iL3 (Figure 18 B and C). Subcluster Asta-B4 shows strong trends of downregulation in various comparisons including iL3. Even though, a large number of Astacin genes have no specific expression profiles, genes in clusters Asta-B1, Asta-B5, Asta-B2 and Asta-B4 reflect the previous observation of different functions performed by members of this gene family (Figure 17). These indicate that despite a large over-representation of Astacin and CAP proteins in parasitic stages, distinct members of the same gene family have increased levels of expression in other developmental stages as well (Figure 15 and 17).
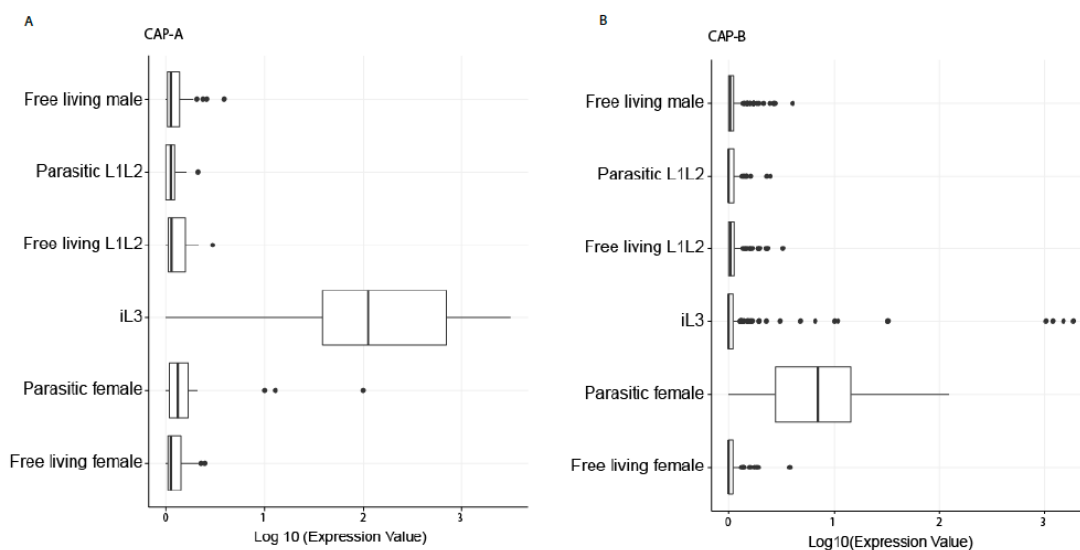
Figure 15. The expression values of different developmental satages *S. papillosus* CAP gene family. A) CAP-A and; B) CAP-B
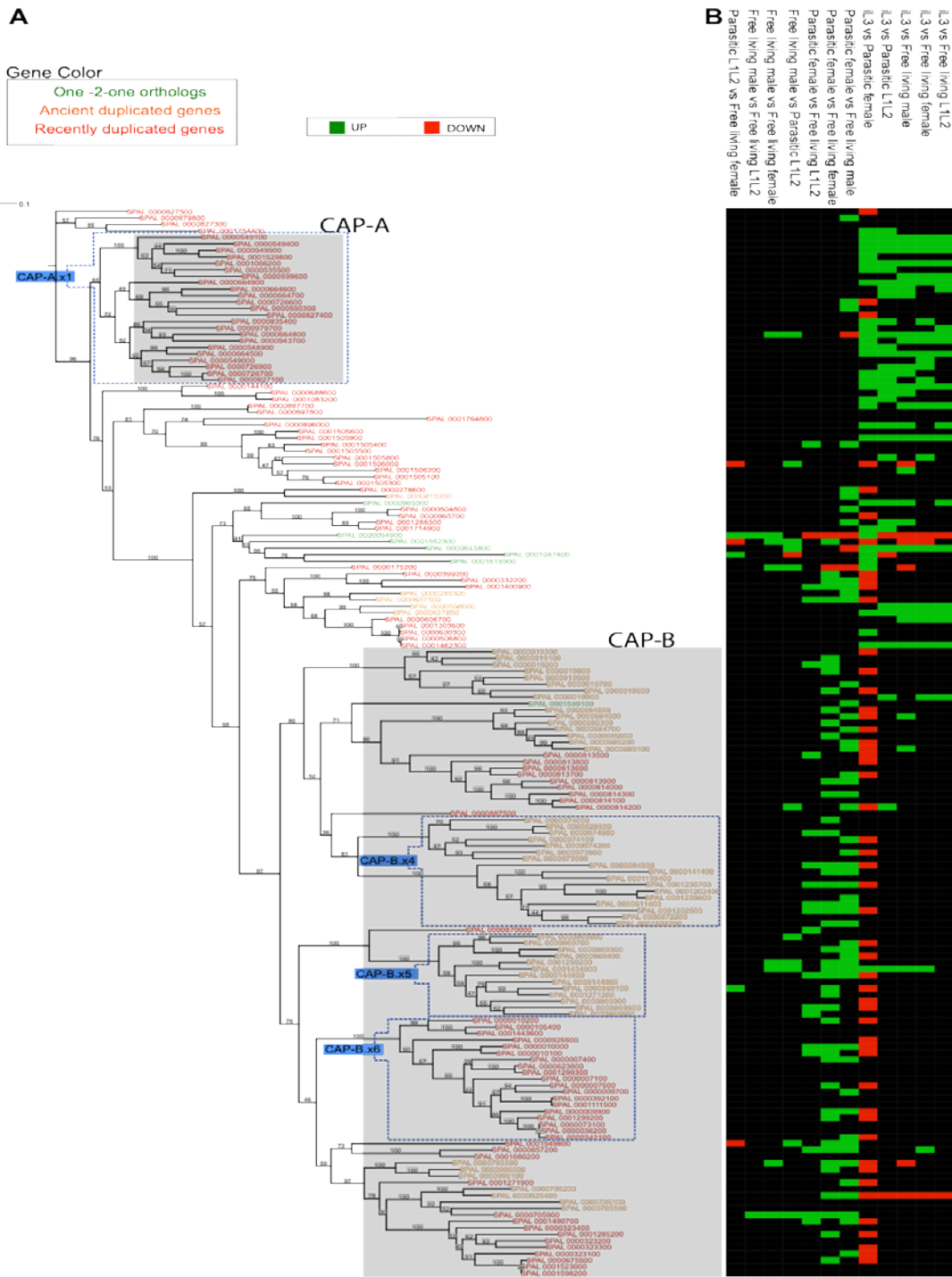
Figure 16. Phylogenetic tree and expression patterns of different developmental stages of *S. papillosus* CAP genes. A) Genes in the phylogenetic tree are colored based on gene class. Gray rectangles denote two arbitrarily defined paralogous clusters CAP-A and CAP-B, which represent relatively closely related gene sets with similar expression profiles. Blue doted lines indicate sub-clusters chosen for in-depth sequence analysis to test for positive selection; B) The heatmap shows the differential expression pattern for each gene across all pairwise comparison of *S. papillosus* developmental stages. Up-regulation, down-regulation and no change in gene expression are shown in green, red and black respectively in the heatmap.
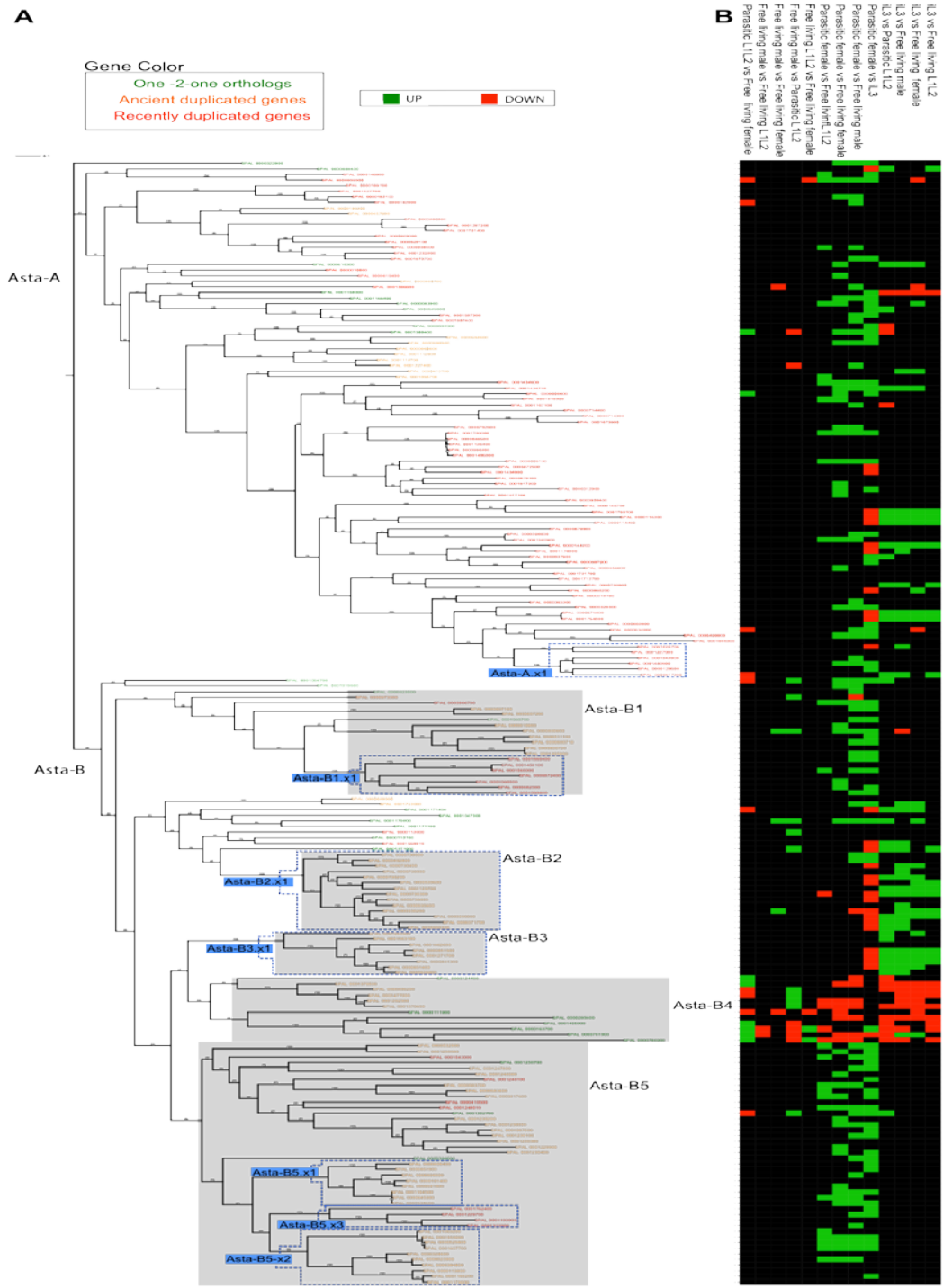
Figure 17. Phylogenetic tree and expression patterns of Astacin genes from different developmental stages of *S. papillsosus*. Leaves (genes) in the phylogenetic tree are colored based on gene class. Gray rectangles denote arbitrarily defined paralogous clusters Asta-B1, Asta-B2, Asta-B3, Asta-B4 and Asta-B5. Blue doted lines indicate subclusters chosen for in-depth sequence analysis to test for positive selection; B) The heatmap shows differential expression pattern for each gene in the tree across all pairwise comparison of *S. papillosus* developmental stages. Up-regulation, down-regulation and no change in gene expression are shown in green, red and black respectively.
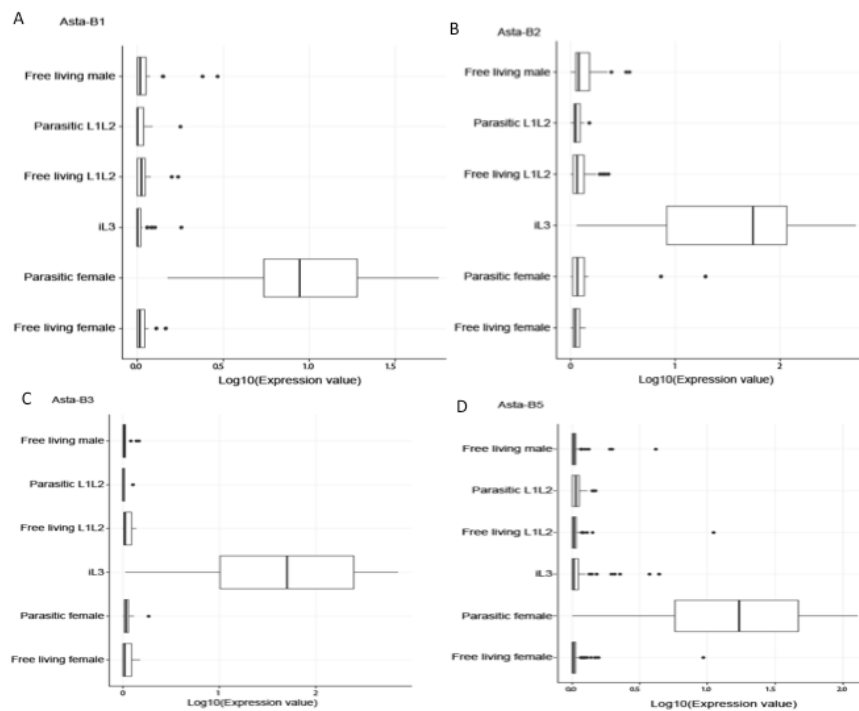
Figure 18. The expression values of *S. papillosus* different developmental stages Astacin genes families. A) Asta-B1; B) Asta-B2; C) Asta-B3 and D) Asta-B5 are shown as box plot.

This stage-specific expression of different sets of duplicated genes in parasitic females and iL3 stage indicates the possibility of sub-functionalization mechanism acting on Astacin and CAP gene families, where gene duplication is followed by partition of function or expression between duplicates. Similar patterns of sub-functionalization has been observed in the heat shock protein family (HSP20) of *P. pacificus*, where distinct paralogous gene clusters have higher expression levels in different developmental stages [197]. This shows that sub-functionalization is one of the dominant mechanisms in retention of duplicated genes in nematodes. Similar cases of sub-functionalization aided duplicated gene retention have been reported in different organism ranging from yeast [198] to insects [201][202]. Even though the expansion of CAP and Astacin gene families as identified in *Strongyloidides* cannot be proven to be the causative event leading to parasitism in this family, previous experimental studies have given first hints on what role these families may have during infection. CAP proteins are cystine rich secretary proteins, functioning as immunomodulatory molecules in parasitic nematodes [201]. They play a

crucial role in parasite establishment and immune modulation upon *Necator americanus* infection. Because of their inhibitory effect on neutrophill and platelet activity, these proteins are considered as a potential vaccine candidate against hookworms [205]. On the other hand, Astacins are metallopeptidase found in variety of different species [204]. Functional roles for Astacin proteases in parasitic nematodes include host tissue penetration by iL3s [205], cuticle formation and ecdysis [208][211] and tissue digestion, penetration and migration [210]. The identification of candidate genes associated with parasitism is of utmost importance for the development of potential treatments, especially in a family of nematodes such as *Strongyloidides,* of which has the potential to infect a wide range of vertebrate hosts including humans. The fact that gene duplications have previously been reported to be associated with parasitism in plants, arthropods, and trematodes [213][214] further support the nature of CAPs and Astacins as strong parasitism-associated candidate genes.

The current results confirm and extend previous findings on the evolution of parasitism-associated genes in *Strongyloides* nematodes. Astacin and CAP gene family evolution is a result of discrete and continuous changes in the environment, such as host switches and adaptation of the host immune response, but further studies in other systems are needed to bring these findings into the broader context of general evolution of parasitism in parasitic nematodes.

2.2.3 Contributions

Praveen Baskaran and I designed this study. I did all the parasitological works. I isolated total RNA and prepared transcriptome libraries for sequencing from all the six developmental stages in all replicates. The sequencing was done in the in house sequencing service facility. Praveen Baskaran and Dr. Christian Rödelsperger did all the bioinformatic analyses. I wrote the manuscript with Praveen, Dr. Christian Rödelsperger and Dr. Adrian Streit. This work has been submitted to Genome Biology and Evolution.

2.3 Genetic diversity and reproduction in the human parasitic nematode *Strongyloides stercoralis*

2.3.1 Different genotypes of *S. stercoralis* isolated from humans and dogs

Although some degree of host specialization was proposed in the literature, *S. stercoralis* is considered a species which lives in humans, dogs and other non-human primates [15][121][213]. To address the zoonotic importance and genetic diversity of *S. stercoralis,* the SSU region covering the HVR-I and HVR-IV were successfully sequenced for 340 and 99 single *Strongyloides* larvae isolated from humans and dogs respectively. All the *S. stercoralis* larvae isolated from humans, had the same nucleotide sequence at HVR-IV as the published *S. stercoralis* full length SSU sequence (AF279916)*,* which I used as a reference (human type genotype). From a total of 99 single *Strongyloides* larvae genotyped from dogs, only 14 had the human type genotype and the rest (85 larvae) differed at three positions (two indels, one base substitution, called dog type genotype) (Table 2 and Figure19).
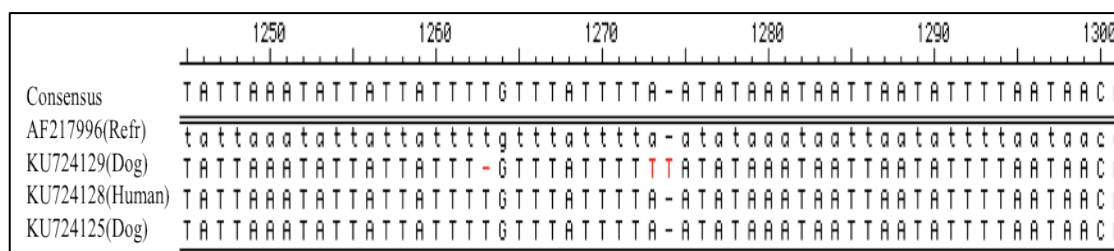
Figure 19. Nucleotide sequence of *S. stercoralis* 18S rDNA HVR-IV isolated from humans and dogs*.* Nucleotide differences at HVR-IV of *S. stercoralis* with dog type genotype are given in red. Deletion sites are given by (-) *S. stercoralis* with the human type genotypes from humans and dogs have similar nucleotide arrangement as reference sequence (AF279916).

Table 2. *S. stercoralis* larvae SSU HVR-I and HVR-IV genotypes from humans and dogs.

	SSU HVRs genotypes					
	HVR-I				HVR-IV	
Hosts	I	II	III	Total	Human type	Dog type
Humans	28	298	14	340	340	0
Dogs	88	10	1	99	14	85

Genotype I: 4T from position 176 to 179 and T at position 458 (KF926658); Genotype II: 5T from position 176 to 179 and T at position (KF926659); Genotype III: 5T from position 176 to 179 and A at position 458 (KF926660) [153]; Human type HVR-IV genotype: identical nucleotide arrangement at HVR-IV to AF279916 SSU reference sequence (KU724124); Dog type HVR-IV genotype: differed by two indels and one base substitution from the reference sequence (KU724129).

With respect to the HVR-I, three genotypes in humans as described by Schär et al. [153] were found and the same nomenclature for different HVR-I genotypes was used as described by Schär et al. [153] (Table 2). In humans, the highest proportions of *S. stercoralis* worms have genotype II (298) followed by genotype I (28) and genotype III (14) (Table 2). The majority of the *S. stercoralis* genotyped from dogs, had genotype I (88) followed by genotype II (10) and geneotype III (1) (Table 2). All 10 worms of HVR-I genotype II, three with HVR-I genotype I and one with HVR-I genotype III derived from dogs had the human type genotype at HVR-IV. Nine of the *Strongyloides* larvae sequenced from dogs were identical to *Strongyloides procyonis* (AB272234), a species known to occasionally occur in dogs [214]. In spite of the differences, the known *S. stercoralis* SSU sequence was the most closely related sequence in the databases, as judged by BLAST analysis to all variants except the *S. procyonis* ones, indicating that all these worms are very closely related with or belong to the species *S. stercoralis*. 67 *Strongyloides* larvae isolated and sequenced from pigs at HVR-I and HVR-IV

were different from *S. stercoralis* but similar to *Strongyloides ransomi* (AB453327).

Given that the larvae were sampled from people and dogs from the same households, and repeatedly worms with different genotypes were present in the same host individual, one would expect that there exists ample opportunity for worms of different genotypes to hybridize. In particular, the less frequent alleles would be expected to be present mostly in heterozygous state. However, like in a previous study [153], which was limited to human hosts, I did not find a single worm that was heterozygous for two different SSU alleles. There are two alternative explanations for these observations. Either, *S. stercoralis* in the current study area never reproduces sexually or productive mating occurs only within SSU genotypes. Should the latter be the case, the different SSU types would have to be considered different species. Absence of sexual reproduction may have two reasons. Firstly, the worms in this study area reproduce only through the homogonic cycle, which is non-sexual in all species of *Strongyloides* where it has been tested [48]. The second possible reason for the absence of sexual reproduction is that *S. stercoralis* may reproduce asexually also in the free-living generation.

2.3.2 Different mitochondrial *cox*1 haplotypes of *S. stercoralis* isolated from humans and dogs

Next, I asked if the different SSU genotypes of *S. stercoralis* isolates also vary at their *cox*1 mitochondrial gene sequences, which are only maternally inherited. I successfully sequenced 552 bp of the *cox*1 gene for a subset of 78 *S. stercoralis* larvae (57 from humans and 21 from dogs). The 57 larvae sequenced from humans were grouped into 7 haplotypes with pairwise nucleotides difference ranging from 0.18 to 2.71 % (Table 3). The 21 larvae sequenced from dogs were grouped into 11 haplotypes with pairwise nucleotides difference ranging from 0.54 to 6.16 %. Two of these haplotypes were identical with two of the haplotypes found in human derived worms (haplotype 2 and 3) and occurred exclusively in worms with a human type SSU haplotype. Also, all 4 dog-derived individuals with a human type SSU

haplotype had one of the two human type *cox*1 sequence variants. The pairwise nucleotides difference between the different human and dog isolates range from 0.18 to 7.61 %. The corellation between the SSU genotypes and *cox*1 haplotypes are given in Table 4. Next, I constructed a maximum likelihood phylogenetic tree using the different *S. stercoralis cox*1 haplotypes isolated from humans and dogs by MEGA6 [215]. The human and dog isolates with different haplotypes were grouped in separate clades and each clade forming two sub-clades with strong nodes (Figure 20). The dog isolates that have similar *cox*1 nucleotide sequences (haplotype) as in human isolates were grouped in the same clade, indicating these larvae with this haplotype could be shared between humans and dogs (zonootic). In general *S. stercoralis* form more diverse populations in both humans and dogs than previously thought. Also with respect to their mitochondrial genome *cox1*, the few worms from dogs with a "human-type" SSU haplotype were very close to human derived worms of the same SSU type. All these observations suggest that in the current study area there exists a dog-specific polulation (incapable of infecting humans) and a predominantly human specific one, which can also infect dogs.

Table 3. Pairwise nucleotide diversity between different *S. stercoralis cox*1 haplotypes from humans and dogs.

Haplotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Haplotype1																
Haplotype2	0.18															
Haplotype3	1.99	2.17														
Haplotype4	2.35	2.54	0.36													
Haplotype5	1.09	0.91	1.99	2.36												
Haplotype6	1.45	1.27	2.35	2.71	0.36											
Haplotype7	1.81	1.99	0.18	0.54	1.81	2.17										
Haplotype8	6.70	7.06	7.61	7.25	6.70	7.06	0.54									
Haplotype9	6.52	6.70	7.06	7.15	6.16	6.5	2.53	0.54								
Haplotype10	5.97	6.16	6.88	6.52	6.70	7.06	2.70	2.53	1.99							
Haplotype11	5.25	5.43	6.52	6.16	5.97	6.34	6.16	2.72	2.17	1.63						
Haplotype12	3.80	3.98	5.07	4.35	4.89	5.25	6.16	6.16	6.16	4.89	4.89					
Haplotype13	4.16	4.35	4.71	4.71	4.52	4.89	4.53	6.16	5.80	5.25	4.89	1.09				
Haplotype14	4.16	4.34	4.71	4.71	4.52	4.89	6.16	5.80	5.43	4.89	4.52	1.09	1.45			
Haplotype15	4.16	4.35	4.71	4.71	4.52	4.89	4.53	6.16	5.80	4.89	4.89	1.09	1.45	0.72		
Haplotype16	3.80	3.98	4.34	4.34	4.16	4.52	5.80	5.80	5.43	4.89	4.52	1.27	1.27	1.27	1.27	

The first column shows the accession number of different haplotypes of *S. stercoralis* sequences deposited in the NCBI nucleotide database from this study. Haplotypes 1 and 4-7 were isolated from human and Haplotypes 8-16 were isolated from dogs. Haplotypes 2 and 3 were found in both hosts.

Table 4. Correlation between *S. stercoralis* SSU genotypes and *cox*1 haplotypes isolated from humans and dogs.

*cox*1 haplotypes	No. of larvae	SSU genotypes					No. of larvae genotyped	
		HVR-I			HVR-IV			
		I	II	III	Human Type	Dog Type	Human	Dog
Haplotype1	19	2	17	0	19	0	19	0
Haplotype2	5	0	5	0	5	0	4	1
Haplotype3	20	1	16	3	20	0	17	3
Haplotype4	3	0	3	0	3	0	3	0
Haplotype5	2	0	2	0	2	0	2	0
Haplotype6	8	0	8	0	8	0	8	0
Haplotype7	4	0	4	0	4	0	4	0
Haplotype8	2	2	0	0	0	2	0	2
Haplotype9	1	1	0	0	0	1	0	1
Haplotype10	2	2	0	0	0	2	0	2
Haplotype11	2	2	0	0	0	2	0	2
Haplotype12	1	1	0	0	0	1	0	1
Haplotype13	2	2	0	0	0	2	0	2
Haplotype14	1	1	0	0	0	1	0	1
Haplotype15	3	3	0	0	0	3	0	3
Haplotype16	2	2	0	0	0	2	0	2
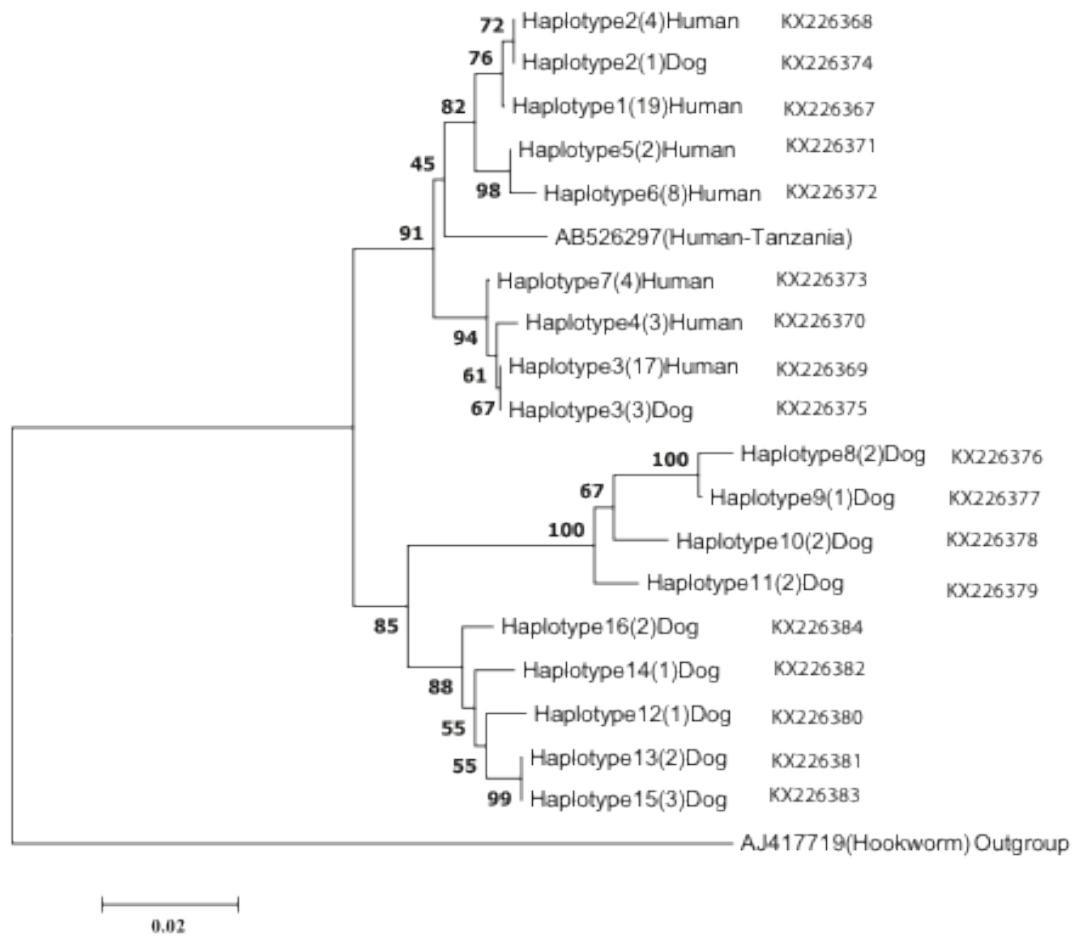
Figure 20. Maximum-likelihood reconstruction of phylogenetic tree based on partial sequence of *cox*1 *S. stercoralis* isolated from humans and dogs. *Necator americanus* (AJ417719) served as an outgroup. In brackets are the numbers of worms that have the indicated *cox*1 haplotype. The numbers with KX prefix are the GenBank accession numbers of the respective *S. stercoralis* haplotype deposited in the NCBI nucleotide database.

2.3.3 Reproduction infree-living generation of a laboratory isolate of *S. stercoralis*

Interestingly, as had been seen previously in Schär et al. [153], among the more than 450 larvae from humans and dogs genotyped, I did not find a single worm, which appeared heterozygous for two different SSU haplotypes. This indicates that the worms with different SSU haplotypes do not interbreed, either because productive mating occurs only within a haplotype group, or because *S. stercoralis* in this study area reproduces only non-sexually. Based on cytological observations, it was proposed for multiple species of *Strongyloides* [48], among them *S. stercoralis* [216]*,* that the free-living generations reproduce by sperm dependent parthenogenesis (pseudogamy). However, this hypothesis was disproved for some species of *Strongyloides* using molecular genetic approaches. Specifically, sexual reproduction was shown to occur in the free-living generations of *S. ratti* [45], *S. papillosus* [51]*,* and *S. vituli* [56]*,* but so far not for *S. stercoralis*. During the sampling for the field study, I did observe free-living adults of both sexes in appreciable numbers. The free-living generations of the laboratory isolate UPD strain of *S. stercoralis* is capable of reproducing through the heterogonic cycle [217]. I used this isolate to address the question if reproduction in free-living stages of *S. stercoralis* is sexual or not. First, I tested if males are required for successful reproduction. To this end, I isolated virgin young free-living females from one-day-old fecal cultures and cultured them individually at 22°C (n=192 females) or at 25°C (n=192 females) for 3 days. None of these 384 worms produced progeny. These results indicated that males/sperm are needed to produce viable progeny in *S. stercoralis*. However, this finding does not exclude reproduction by sperm dependent parthenogenesis (pseudogamy) as has been proposed [216]. In order to determine if males contribute to the genetic material of the progeny, I set up crosses of single virgin females and males from one-day-old fecal cultures as described in Materials and Methods. Of 18 pair crosses, I genotyped both parents and eight of their progenies using *ytP274* as a molecular genetic marker, which exists in two alleles (T or C) in the laboratory isolate (PV001) at position 231

of the marker. The genotyping results (Table 5) showed the inheritance of paternal alleles by the progenies with no indication for a deviation from Mendelian segregation, and with this they were inconsistent with clonal reproduction. These preliminary results, based on only a single marker, suggest that the reproduction in the free-living stages of the laboratory strain of *S. stercoralis* is sexual but we do not know yet if this is also the case for the wild isolates that I investigated. Determining if sexual reproduction occurs within SSU genotypes of wild isolates is therefore one of the most urgent questions to be addressed that arises from this study.

Table 5. Genotypes of *S. stercoralis* free-living parents and their progenies using *ytP274* as a molecular genetic marker.

Crosses	Female genotype	Male genotype	Progenies genotype
C1	C/T	C/T	4C/T, 1C, 3T
C2	C/T	C/T	4C/T, 2T, 2C
C3	C/T	C	4C, 4CT
C4	T	C/T	4C/T, 4T
C5	T	C/T	4C/T, 4T
C6	C	C/T	5C/T, 3C
C7	C/T	C	4C/T, 4C
C8	C/T	T	3C/T, 5T
C9	T	C/T	4C/T, 4T
C10	C	C/T	4C/T, 4C
C11	C/T	T	4C/T, 4T
C12	C/T	C/T	3C/T, 4C, 1T
C13	C/T	C/T	5C/T, 1C, 2T
C14	C/T	C/T	4C/T, 1C, 3T
C15	C/T	C	5C, 3C/T
C16	C/T	C/T	3C/T, 2C, 3T
C19	C	C/T	3C/T, 5C
C18	T	C/T	6C/T, 2T

The first column is number of the cross, the second and third column are genotypes of mother and father. The fourth column is genotypes of all the eight progenies genotyped per crosses. The *ytP274* marker consists of a single nucleotide polymorphism T/C at position 231.

This study reports the first large-scale genotyping of *S. stercoralis* from humans and dogs collected from the same locations at the same time. The rural communities in Cambodia mostly share their premises with their animals and the general hygiene and sanitation condition is ideal for human to animal and animal to human transmission of as soil transmitted helminthes including *S. stercoralis* [36]. Based on SSU and mitochondrial *cox*1 genotypes, the *S. stercoralis* worms in the current study area fall into multiple groups, which might be separate populations. These separate populations might represent closely related species or subspecies, which parasitize the same host but are not able to interbreed or a species complex of asexually reproducing worms. Although the *Strongyloides* spp. populations in humans and dogs are largely separate, dogs do carry at low numbers but fairly high prevalence *S. stercoralis* that is indistinguishable from *S. stercoralis* in humans. This suggests that *S. stercoralis* can be transmitted between humans and dogs.

2.3.4 Contributions

I, Dr. Adrian Streit and Pro. Dr. Peter Odermatt designed this study. Fabian Schär and I collected the worm samples from humans and dogs. I performed the PCR, sequencing and sequence analyses. I wrote the manuscript with Dr. Adrian Streit. A manuscript about this work is being prepared for submission.

2.4 Genetic and phylogenetic analyses of *O. ochengi* isolates using complete mitochondrial genome sequences

2.4.1 Mitochondrial genome size, content and organization

I conducted short read whole genome sequencing experiments with 11 adult single *O. ochengi* worms (3 males and 8 females). The mitochondrial sequences were retrieved from these data. For two individuals, one of the mitochondrial subtype "ochengi" and one of the variant "Siisa" (c.f. [191] [190]), I manually assembled and annotated the full mitochondrial genomes. The resulting reference genomes were deposited in GenBank under the accession numbers KX181289 (variant ochengi) and KX181290 (variant Siisa). Both sequences are 13,744 bp long (Figure 21) but differ at 158 single nucleotide positions. Compared to *O. volvulus* (AF015193) [218] and *O. flexuosa* (HQ214004) [219], the *O. ochengi* mitochondrial genome is slightly smaller due to shorter intergenic regions. The gene content and order is the same as in *O. volvulus* [218]. The mitochondrial genome of *O. ochengi* contains 12 protein-coding genes (*cox*1–*cox*3, *nad*1–*nad*6, *nad*4L, *atp*6 and *cob*), 22 transfer RNA (tRNA) genes, a small subunit ribosomal RNA gene (*rrnS*) also called *12S*, and a large subunit ribosomal RNA gene (*rrnL*) also called *16S*, and an AT-rich 318 bp non coding region. Like all other nematodes species whose mitochondrial genomes were sequenced (except for *T. spiralis* [220]), *O. ochengi* also lacks the protein-coding gene *atp*8. The nucleotide compositions of the entire mitochondrial genome of *O. ochengi* are very A-T rich (73.22%) and there is a bias for the nucleotide T being on the coding strand. Of G-C base pairs, the G is preferentially on the coding strand. The nucleotide composition of the coding strand of the type "ochengi" reference sequence (KX181289) is: A=2607 (18.97%), T=7456 (54.25%), G=2765 (20.12%) and C=916 (6.66%) (Table 6).
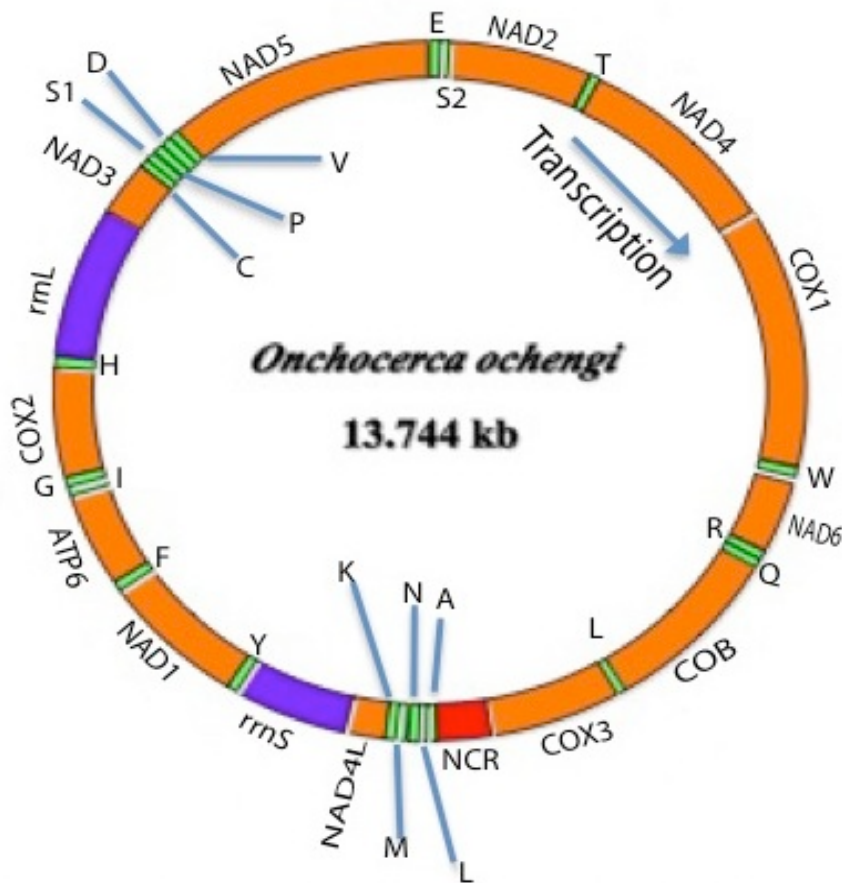
Figure 21. The mitochondrial genome of *O. ochengi* (KX181289). All genes are transcribed clockwise. Protein-coding and rRNA genes are indicated with the standard nomenclature. The tRNA genes are indicated with the one-letter code of their corresponding amino acids. There are two tRNA genes for Leucine: L1 for codons CUN and L2 for UUR; and two tRNA genes for Serine: S1 for codons UCN and S2 for AGN. "NCR" refers to the non-coding region.

Table 6. Positions and nucleotide sequence lengths of mitochondrial genes in *O. ochengi* (KX181289 and KX181290).

Genes	Positions	Lengths	Start codons	Stop codons	Anticodons
cox1	1-647	1647 bp	ATT	TAA	
tRNA(W)	1656-1711	56 bp			TCA
nad6	1758-2207	450 bp	ATT	TAG	
tRNA(R)	2214-2269	56 bp			ACG
tRNA(Q)	2272-2325	54 bp			TTG
cob	2329-3411	1083 bp	ATT	TAA	
tRNA(L:CUN)	3413-3468	56 bp			TAG
cox3	3468-4247	780 bp	ATT	TAA	
NCR	4248-4565	318 bp			
tRNA(A)	4566-4623	58 bp			TGC
tRNA(L:UUR)	4627-4682	56 bp			TAA
tRNA(N)	4676-4732	57 bp			GTT
tRNA(M)	4747-4810	64 bp			CAT
tRNA(K)	4810-4868	59 bp			CTT
nad4L	4868-5110	243 bp	TTG	TAA	
rrnS	5111-5794	684 bp			
tRNA(Y)	5788-5846	59 bp			GTA
nad1	5844-6743	900 bp	TTG	TAG	
tRNA(F)	6721-6779	59 bp			GAA
atp6	6780-7364	585 bp	ATT	TAG	
tRNA(I)	7378-7433	56 bp			GAT
tRNA(G)	7439-7496	58 bp			TCC
cox2	7499-8197	699 bp	ATT	TAA	
tRNA(H)	8196-8258	63 bp			GTG
rrnL	8256-9227	972 bp			
nad3	9228-9566	339 bp	CTT	TAG	
tRNA(C)	9568-9624	57 bp			GCA
tRNA(S:UCN)	9624-9678	55 bp			TGA
tRNA(P)	9689-9747	59 bp			AGG
tRNA(D)	9751-9814	64 bp			GTC
tRNA(V)	9816-9871	56 bp			TAC
nad5	9872-11467	1596 bp	TTG	TAG	
tRNA(E)	11480-11536	57 bp			TTC
tRNA(S-AGN)	11536-11592	57 bp			TCT
nad2	11588-12439	852 bp	ATT	TAG	
tRNA(T)	12447-12505	59 bp			TGT
nad4	12505-13737	1233 bp	TTG	TAA	

For protein coding genes, the initiation and the termination codons and for tRNA genes the anticodons are indicated.

Table 7. Codon usages of the 12 protein-coding genes in the mitochondrial genome of *O. ochengi* (KX181289).

Amino Acid	Codon	Number	Codon Frequency
Ala	GCG	6	0.173
	GCA	3	0.086
	GCT	75	2.16
Cys	TGT	98	2.82
	TGC	3	0.086
Asp	GAT	83	2.40
	GAC	3	0.086
Glu	GAG	41	1.20
	GAA	23	0.66
Phe	TTT	634	18.3
	TTC	4	0.115
Gly	GGG	34	0.98
	GGA	17	0.49
	GGT	191	5.50
	GGC	6	0.173
His	CAT	50	1.44
	CAC	3	0.086
Ile	ATT	176	5.07
	ATC	5	0.144
Lys	AAG	67	1.93
	AAA	9	0.26
Leu	TTG	311	8.96
	TTA	137	3.95
	CTG	3	0.086
	CTA	1	0.03
	CTT	23	0.66
	CTC	1	0.03
Met	ATG	111	13.71
	ATA	30	3.20
Asn	AAT	79	2.30
	AAC	4	0.115
Pro	CCG	6	0.173
	CCA	2	0.0576
	CCT	68	1.96
Gln	CAG	38	1.095
	CAA	13	0.37
Arg	CGG	10	0.228
	CGA	1	0.03
	CGT	42	1.21
	CGC	1	0.03
Ser	AGG	23	0.66
	AGA	14	0.40
	AGT	113	3.26
	AGC	4	0.11
	TCG	5	0.14
	TCA	3	0.086
	TCT	159	4.58
Thr	ACG	2	0.0576
	ACA	2	0.0576
	ACT	83	2.40
Val	GTG	44	1.30
	GTA	24	0.69
	GTT	263	7.58
	GTC	2	0.0576
Trp	TGG	45	1.30
	TGA	31	0.89
Tyr	TAT	222	6.40
	TAC	4	0.11
Stop	TAG	6	0.176
	TAA	7	0.20

2.4.1.1 Protein-coding genes

The *O. ochengi* mitochondrial genome encodes 12 protein-coding genes. All *O. ochengi* mitochondrial genes are transcribed in the clockwise direction. The order of the protein coding genes of *O. ochengi* is: *cox*1->*nad*6->cob->*cox*3->*nad*4L->*nad*1->*atp6*->*cox2*->*nad*3->n*ad*5->*nad*2->*nad*4 (Figure 18 and Table 6). The predicted translation initiation and termination codons for the 12 protein coding genes of *O. ochengi* are given in Table 6. The most common initiation codon for *O. ochengi* was ATT (7 of 12 protein genes), followed by TTG (4 of 12 protein genes) and CTT (1 of 12 protein genes). The termination codons TAA and TAG were used each six times. A total of 3,457 amino acids are encoded by the *O. ochengi* mitochondrial genome. The A+T percentage of protein coding genes of *O. ochengi* ranges from 66.85 % to 79.01 % (Table 8).

All codons are used except for Ala (GCC), Pro (CCC), Ser (TCC), and Thr (ACC), which is consistent with the strong bias against the C nucleotide. Codons composed of A and T nucleotides are predominantly used, reflecting the very strong bias toward A+T in the mitochondrial genome of *O. ochengi.* The codon usages in the mitochondrial genome of *O. ochengi* (KX181289) are given in Table 7. The most frequently used codons are Phe (TTT: 18.03%), followed by Leu (TTG: 8.96%), Val (GTT: 7.58%), Tyr (TAT: 6.40%), Gly (GGT: 5.50%), Ile (ATT: 5.07%) and Ser (TCT: 4.58%). The least utilized codons are Leu (CTA and CTC) and Arg (CGA and CGC), each codon used only once.

Table 8. Nucleotide compositions, SNPs and pairwise nucleotide differences in the whole mitochondrial genome of *O. ochengi* (KX181289) and (KX181290).

Gene/ Region	*O. ochengi* (KX181289)					*O. ochengi* (KX181290)					SNPs	Nucleotide Difference %
	%A	%C	%T	%G	%A+T	%A	%C	%T	%G	%A+T		
*cox*1	18.86	11.07	48.05	22.02	66.91	18.86	11.19	47.99	21.96	66.85	17	1.03
*nad*6	14.67	2.89	63.33	19.11	78.00	14.22	3.11	63.11	19.56	77.33	7	1.56
cob	18.93	8.13	54.02	18.93	72.95	18.84	7.94	54.20	19.02	73.04	15	1.39
*cox*3	20.51	6.54	51.54	21.41	72.05	20.13	6.41	51.67	21.79	71.79	14	1.80
*nad*4L	13.58	2.88	64.61	18.93	78.19	13.99	2.47	65.02	18.52	79.01	2	0.82
*nad*1	12.67	7.78	57.00	22.56	69.67	13.00	7.89	56.89	22.22	69.89	14	1.56
*atp*6	14.70	5.98	57.09	22.22	71.79	15.56	6.32	56.58	21.54	72.14	10	1.71
*cox*2	20.60	8.15	47.21	24.03	67.81	20.60	8.30	47.35	23.75	67.95	8	1.44
*nad*3	15.04	3.54	61.95	19.47	76.99	14.16	3.54	61.95	20.35	76.11	3	0.88
*nad*5	13.22	6.08	59.71	20.99	72.93	13.47	6.14	59.59	20.80	73.06	21	1.32
*nad*2	14.44	4.11	60.45	21.01	74.88	14.08	4.23	60.33	21.36	74.41	8	0.94
*nad*4	15.09	5.60	58.72	20.60	73.80	15.17	5.84	58.56	20.44	73.72	11	0.89
rrnS	25.73	7.31	48.54	18.42	74.27	25.58	7.31	48.68	18.42	74.27	6	0.87
rrnL	25.41	5.55	51.65	17.39	77.06	25.31	5.92	51.23	17.59	76.54	9	0.93
NCR	35.85	6.29	47.17	10.69	83.02	35.22	6.60	47.48	10.69	82.70	7	2.20
EMG	18.97	6.66	54.25	20.12	73.22	18.97	6.74	54.20	20.10	73.17	158	1.15

NCR and EMG stand for "non-coding regions" and "entire mitochondrial genome" respectively.

2.4.1.2 Ribosomal and transfer RNA genes

The *rrnS* and *rrnL* genes of *O. ochengi* were identified based on their sequence similarities with *O. volvulus.* The *rrnS* and *rrnL* are located between *nad*4L and tRNA (Y) and tRNA (H) and *nad*3 respectively. The length of the *rrnS* and *rrnL* genes of *O. ochengi* are 684 and 972 bp respectively (Table 6). The A+T content of *rrnS* and *rrnL* are 74.27 % and 77.06 % respectively (Table 8). A total of 22 tRNA sequences (vary in length from 55 to 64 bp) were identified in the mitochondrial genome of *O. ochengi.* The predicted secondary structures (not shown here) and locations of the *O. ochengi* tRNAs were the same as in *O. volvulus* [218]. Twenty of the 22 mitochondrial tRNA genes share a common secondary structure in which the TΨC arm and the variable loop are exchanged with a TV-replacement loops [221]. Two serine tRNA genes (S: UCN and S: AGN) contain typical D replacement loops [221]. In the mitochondrial genome of *O. ochengi,* some tRNA genes overlap with the adjacent genes. Five mitochondrial tRNA sequences overlap with the start of protein coding genes by 1–5 nucleotides (tRNA(L: CUN)-*cox*3, tRNA(K)-*nad*4L, tRNA(Y)-*nad*1, tRNA(S:AGN*)-nad*2, tRNA(T)-*nad*4). Two

mitochondrial tRNA sequences overlap with the end of protein coding genes by 2–23 nucleotides (*nad*1-tRNA(F) and *cox*2- tRNA(H)). Two tRNAs, tRNA(Y) and tRNA(H) overlap with *rrnS* and *rrnL* by 7 and 3 nucleotides respectively. Three pairs of *O. ochengi* tRNAs share 1-7 nucleotides overlap with each other (tRNA(L:UUR)-tRNA(N), tRNA(C)-tRNA(S:UCN) and tRNA(E)- tRNA(S:AGN)).

2.4.1.3 Non-coding sequences

A 318 bp long AT rich (83.02%) non-coding region is located between the *cox*3 and the tRNA(A) genes. There are also 19 short intergenic regions varying in length from one to 46 bp. The longest of them (46 bp) is located between tRNA(E) and *nad*6.

2.4.2 Sequence comparsion of mitochoderial genes

First, I performed a pairwise sequence comparison between the reference sequences for the two-mitochondrial clades. The results for the entire mitochondrial genome as well as separate comparisons of the protein coding genes, ribosomal RNA genes and the NCR are presented in Table 8. Overall; the two sequences differ at 158 positions (1.15%). Next, I conducted pairwise sequence comparisons between the 11 *Onchocerca* individuals from this study and the published sequences of *O. volvulus* (AF015193), *O. gutturosa* (unpublished) and O*. flexuosa* (HQ214004) (Table 9). Since for some of these no fully assembled mitochondrial genomes are available, this comparison was limited to protein coding genes. The pairwise nucleotide diversities within the species *O. ochengi* range from 0.029% to 1.36%. The samples fall into two groups confirming the existence of the two mitochondrial clades "ochengi" and "Siisa".

Among the 9 "ochengi" type individuals, the pairwise nucleotide diversities were between 0.029% and 0.211%. The pairwise nucleotide diversity between the two type "Siisa" individuals was 0.144%. The pairwise nucleotide diversity between the two-mitochondrial clades ranges between 1.25% and 1.29%.

Among the protein-coding genes, *cox*3, *nad*1, *nad*6 and *cox*2 would be suitable candidate loci for future population genetics studies, as they were the most variable between the *Onchocerca* isolates in the current study. The pairwise nucleotide diversities between the protein coding genes of *O. ochengi* and *O. volvulus, O. gutturosa* and *O. flexuosa* are around 3.3%, 7.1% and 10.3% respectively (Table 9).

Table 9. Pairwise nucleotides diversity between the 12 protein coding mitochonderial genes of *Onchocerca* spp.

Onchocerca sp.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
O. ochengi F1														
O. ochengi F2	0.096													
O. ochengi F3	0.048	0.048												
O. ochengi F4	0.096	0.048	0.038											
O. ochengi F6	0.086	0.067	0.057	0.076										
O. ochengi F7	0.067	0.086	0.038	0.096	0.096									
O. ochengi F8	0.105	0.029	0.057	0.057	0.076	0.096								
O. ochengi M2	0.211	0.182	0.172	0.190	0.172	0.192	0.192							
O. ochengi M3	0.086	0.029	0.057	0.038	0.076	0.076	0.038	0.172						
O. ochengi F5 (Siisa type)	1.29	1.27	1.24	1.29	1.28	1.28	1.28	1.36	1.28					
O. ochengi M1 (Siisa type)	1.28	1.26	1.25	1.27	1.27	1.27	1.28	1.34	1.23	0.144				
O. volvulus	3.29	3.28	3.30	3.30	3.32	3.32	3.27	3.37	3.285	3.34	3.33			
O. gutturosa	7.133	7.114	7.133	7.114	7.114	7.15	7.114	7.21	7.114	7.133	7.133	7.31		
O. flexuosa	10.28	10.26	10.27	10.28	10.29	10.26	10.24	10.32	10.24	10.25	10.27	10.17	10.07	

In the first column *are: 11 O. ochengi* isolates from this study, *O. volvulus* (AF015193) and *O. gutturosa* (unpublished sequence obtained from M. Blaxter, University of Edinbourgh) and *O. flexuosa* (HQ214004).The numbers are pairwise percentage nucleotide differences.

2.4.3 Phylogenetic analysis

From previous molecular genetic data based on nuclear SNP markers it was concluded that the two mitochondrial types interbreed freely and therefore belong to the same species [191]. On the other hand, *O. ochnegi* and *O. volvulus* are well-accepted different species as they are most probably not able to interbreed due to different chromosome numbers. *O. ochengi* has 2n=5 chromosomes and *O. volvulus* has 2n=4 chromosomes, possibly after loosing one pair of its chromosome due to chromosome fusion [25][222]. Somewhat puzzlingly, earlier studies [190][191] which were based on rather little sequence information, found the mitochondrial types "ochengi" and "Siisa" to be about equally distant from each other as either of them is from *O.*

volvulus. To resolve this, I conducted phylogenetic analyses of all the 11 *O. ochengi* individuals and 11 other Spirurida nematodes using Maximum Likelihood (ML) based on concatenated mitochondrial amino acid sequences of 12 protein-coding genes (Figure 22). This phylogenetic analysis clearly confirms the presence of the two mitochondrial clades "ochengi" and "Siisa", which are much more closely related to each other than either of them is to *O. volvulus.*
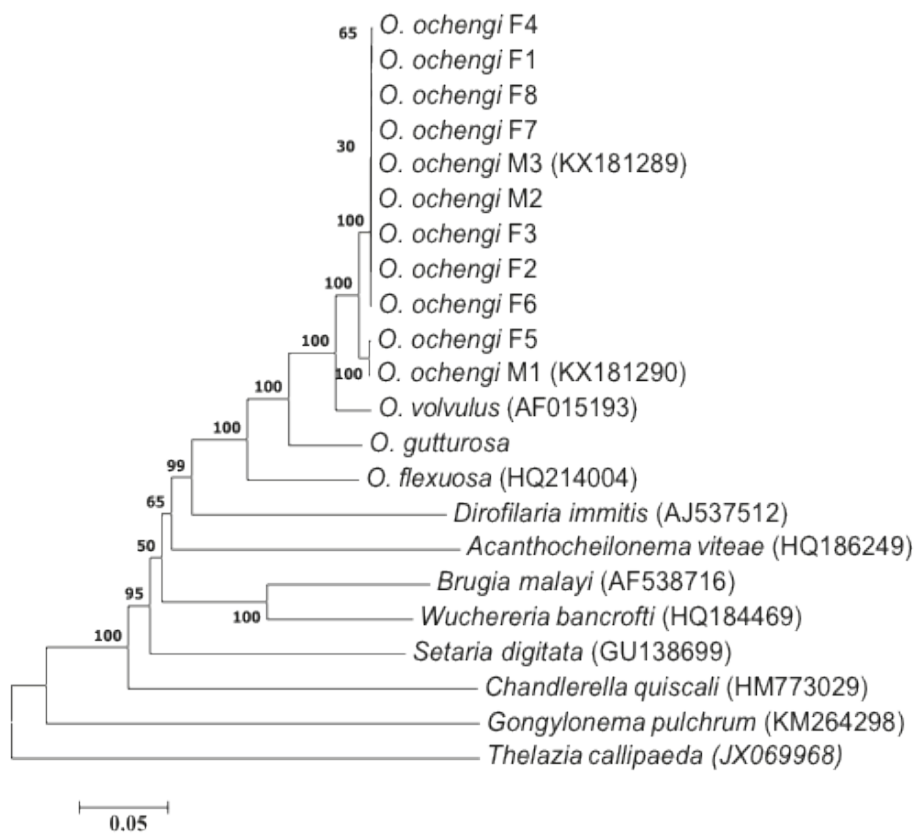
Figure 22. Maximum-likelihood reconstruction of a phylogenetic tree based on the amino acid sequences of 12 protein-coding mitochondrial genes. Included are the sequences of the 11 adult *O. ochengi* from this study and the sequences of three other species of *Onchocerca* and of 8 other Spirurida nematodes of variable phylogenetic distance to *O. ochengi*. F and M stands for *O. ochengi* females and males, respectively.

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It was hypothesized that *O. volvulus* and O*. ochengi* evolved from a common ancestor after humans acquired this infection from their cows upon cattle domestication in Africa about 10,000 years ago [24]. Usually, the pairwise nucleotide difference between the mitochondrial genomes of two interbreeding strains of the same species of nematodes is up to 6%, whereas between different species it is usually more than 10% [223]. The pairwise nucleotide difference of about 1.3% between the two-mitochondrial clades of *O. ochengi* is well in the range of expected mitochondrial genome sequence variation within the same species. Interestingly, the pairwise nucleotide difference between *O. volulus* and *O. ochengi* of only 3.3% is within the range of what would be expected for within-species variation in nematodes. This further supports the hypothesis that *O. ochengi* and *O. volvulus* are two closely related sister species, and therefore O*. ochengi* in cattle is a suitable animal model for many aspects of the biology, although not the pathology, of *O. volulus*, the causative agent of human onchocerciasis also called river blindness [188].

2.4.4 Contributions

I designed this study with input from Dr. Adrian Streit. Dr Alfons Renz provided adults *Onchocerca* worm samples in nodules. I isolated the adults' worms from the nodules by collagenase digestion. I extracted genomic DNA from individual worms. I extracted genomic DNA from individual worms and prepared the genomic DNA libraries for sequencing. The sequencing was done in the in house sequencing service facility. Dr. Christian Rödelsperger did all the bioinformatic analyses. I and Dr Adrian Streit wrote the manuscript. A manuscript about this work is in preparation for submission.

3. Materials and Methods

3.1 Culture and isolation of the parasites

3.1.1 *Strongyloides ratti*

Strongyloides ratti strain ED321 was used in these experiments [224]. For the characterization of the infection dynamics, four weeks old male wistar rats and gerbils were purchased from Charles River (Sulzfeld, Germany). Four rats and four gerbils were subcutaneously inoculated with about 500 iL3s as described [224]. The animals of the same species were housed together. Starting from six days after infection, feces were collected overnight from the animals by placing them in caches underneath lined with wet paper towels at room temperature for five days per week until no worms were detected in the feces in two consecutive samples (four weeks in the rats or two months in the gerbils). From gerbils, after two months, feces were collected once a month from the gerbils until no worms were detected anymore (infections in the gerbils lasted for about 6 months). During the first 4 weeks and 8 weeks of infections for rats and gerbils respectively, fecal samples were split in half and one part incubated at $19^°C$ and the other part at 22°C for 48 hours. In the gerbils 8 weeks post infection, the entire samples were incubated at 19°C. The worms were recovered from the cultures using Baermann technique [225]. Total worm outputs and total worm sex ratios (the number of iL3s (from the homogonic cycle) and free-living females and males) were determined from feces cultured at different temperature for each group of animals. The same experiment, as described above was repeated with different host animals purchased at a different time, infected with different batch of iL3s resulting in two independent biological replicates.

For the determination of worm burdens, other groups of male rats and gerbils each with four animals were infected with 500 iL3 as described above. The total worm output was determined on days 6, 8 and 11 after infections. The

animals were sacrificed on the 12th day after infection. The small intestines were dissected from the rats, slit longitudinally and then carefully washed with Phosphate-buffered saline (PBS). The cleaned intestinal tissues were spread inside down over wire mesh placed on top of Bearmann funnel filled with prewarmed PBS and incubated for 3 hours at 37°C according to method described by Harvey et al. [195]. Parasitic females were then handpicked from the sediment with a worm pick and washed in fresh PBS at least two times, before being counted and then stored at -80 °C in TRIzol (Ambion, USA) for total RNA extraction used in RNA-Seq experiments. The same experiment as described above was repeated with host animals purchased at a different time and with a different batch of iL3s. The worms from the four animals of each group were pooled for RNA isolation resulting in two biological replicates. The rats were kept in Techniplast type 4 cages with elevated tops in an in-house animal facility, which is subject to regular inspections by the veterinary authorities of Tübingen (Veterinäramt Tübingen). All experiments with animals were in accordance with national and international animal wellfare legislation and guidelines. The permits were granted by the Regierungspräsidium Tübingen (AZ35/9185.82-5).

3.1.2 *Strongyloides papillosus*

The *S. papillosus* isolate LIN, originally isolated from sheep and maintained in rabbits [51] was used this experiment. Pathogen-free female rabbits (New Zealand White) purchased from Charles River Laboratories (Sulzfeld, Germany) were used to raise the parasite. The rabbits were subcutaneously inoculated with about 2000 infective larvae (iL3) [51]. Starting ten days post infection (p.i), the feces were collected overnight, mixed with sawdust and cultured at 25°C as described [51]. Worms of different developmental stages were isolated after the times specified below using the Baermann technique as described [51]. Stage 1 and 2 larvae (L1/L2) derived from parasitic adults females were isolated after 8 hours of culture. Free-living males and females were collected after 28 hours of culture. L1/L2, which were progenies of free living generations were collected after 48 hours of culture. To isolate iL3s, the culture dishes were placed in larger dishes with water as described [51] and after 7 days of culture the iL3s that had crawled into the water were collected.

These larvae were a mixture of iL3s from the direct and the indirect cycle. The isolate LIN, when kept in rabbits, has a strong tendency towards the indirect cycle [51] such that the majority of the iL3s were the progeny of free-living animals. At 22 days p.i, the rabbits were sacrificed to harvest the parasitic adults. The small intestines were dissected from the rabbits, its contents removed, slit longitudinally and carefully washed with PBS. The cleaned intestinal tissues were spread inside down over wire mesh placed on top of Bearmann funnel, filled with PBS and incubated at 37 °C for 3 hours according to method described by Harvey et al. [195]. The worms were then handpicked from the sediment of Baermann funnel, washed with PBS, counted and stored at -80 °C in TRIzol reagent (Ambion, CA, USA). All animal experiments were in accordance with national and international animal wellfare guidelines and legislation. The rabbits were kept in an in-house animal facility, which is regularly inspected by the local authorities (Veterinäramt Tübingen). The permits were granted by the Regierungspräsidium Tübingen (AZ35/9185.82-5).

3.1.3 *Strongyloides stercoralis*

This project was performed in collaboration with Prof. Dr. Peter Odermatt and his group from the Swiss Tropical and Public Health Institute at Basel, Switzerland and the group of Dr. Sinuon Muth from the National Center for Parasitology, Enthomology and Malaria Control at Phnom Penh, Cambodia. Stool samples were collected from humans and dogs in the same household in Anlong Svay and Chom Long villages, which are rural communities in Northern Cambodia. *S. stercoralis* larvae were isolated according to methods described by Schär et al. [153]. Briefly, stool samples were collected for two consecutive days from each member of the household who agreed to participate in the study. All the fecal samples collected from humans were analyzed within 3 hours after collection using Baermann and Kato-Katz methods [153]. The sediment from each sample was checked with a microscope and samples positive for *S. stercoralis* larvae were preserved in 70% alcohol in eppendorf tubes. Fecal samples were collected from dogs, pigs and cats from *S. stercoralis* positive households with the help of owners and field workers. On the day of collection, about 3 g of feces was placed on a

freshly prepared agar plates from each sample and incubated for 36 hours at 28°C to allow *S. stercoralis* larvae to develop to adults and migrate onto the agar. Then the plates were examined under the microscope for the presence of moving larvae or free-living adults. Positive plates were washed with 70 % alcohol and the worms were collected in 1.5 ml Eppendorf tubes. All the samples collected from humans and animals were transported to the Max Planck Institute for Developmental Biology at Tübingen, Germany for molecular taxonomy. This experiment was conducted by the approval of 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 on the study procedures and provided written informed consent prior to enrolment. All data handled was strictly confidential. Individuals positive for *S. stercoralis* were treated with Ivermectin (200 µg/kg BW over two days).

3.1.4 *Onchocerca ochengi*

Several skin nodules containing adult *Onchocerca* worms were collected from different cattle in Ngaoundéré abattoir, Adamawa Region, Cameroon [27]. The nodules were stored in 80% ethanol and shipped to the Max Planck Institute for Developmental Biology in Tübingen for molecular analyses. 3 adult males and 8 adult females (9 *O. ochengi* and 2 *Onchocerca* spp*.* Siisa) were used in the current analyses.

3.2 RNA isolations and RNA-Seq library preparation

S ratti and *S. papillosus* frozen in TRIzol (see above) were thawed, vortexed and refrozen in liquid Nitrogen at least three times. Then total RNA was isolated using standard TRIzol extraction method according to the manufacturer's instructions (Ambion Inc., USA). The integrity of the RNA was verified by gel electrophoresis and the concentration was quantified using Qubit fluorimeter measurement (Invitrogen Inc., USA). RNA-Seq libraries were prepared from one µg of total RNA using TruSeq RNA Library preparation kit v2 (Illumina Inc., USA) according to manufacturer's

instructions. The libraries were quantified with Qubit® 2.0 (Invitrogen Inc., USA) and Bionalyzer (Agilent Inc., USA) and then normalized to 5 nM. Paired end sequencing was done in one multiplexed lane using HiSeq2000 platforms (Illumina Inc., USA) at the in-house genome center.

3.3 DNA isolation and DNA-Seq libraries preparations

Adult *O. ochngi* worms were isolated from the nodule tissue by collagenase digestion as described by Kläger et al. [187]. Briefly, nodules were incubated at 37 °C overnight in 0.2% collagenase with PBS solution and then washed several times with PBS. DNA was extracted from single worms using the Epicenter DNA extraction kit (Epicenter, USA) according to the manufacturer's instructions. The DNA was quantified using Qubit fluorimeter measurement (Invitrogen Life Technologies, USA). Before library preparation, partial *cox1*, *12S*, and *16S* were amplified using the primer pairs reported earlier [191] and sequenced using one of the primers used for the amplification. The partial *cox*1, *12S* and *16S* were blast-aligned against the NCBI nucleotide database to determine if the worms were *O. ochengi* or *Onchocerca* spp. Siisa according to the published in the sense of the older literature [24][190][191]. The DNA libraries were prepared using the Low Input DNA library preparation kit (Rubicon Genomics, USA) according to the manufacturer's instructions from 50 ng of genomic DNA. The libraries were quantified using Qubit and Bioanalyzer (Agilent Technologies, USA) and then normalized to 2.5 nM. The samples were sequenced as paired ends in one multiplexed lane using HiSeq2000 platform (Illumina Inc, USA) at the Max Planck Institute for Developmental Biology in-house genome facility.

3.4 Molecular genotyping of *S. stercoralis* single larvae

Single worm crude DNA preparations from ethanol-fixed samples were performed in PCR tubes [51] as follows: Single larvae were picked and washed two times with PBS and then incubated in 10 µl 1X 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 2hrs, followed by incubation at 95 °C for 15 min. 25 µl reaction volume PCR were prepared using: 2.5 µl of 10X ThermoPol Reaction buffer (New England BioLabs), 0.5 µl of 2mM dNTPS, 0.5 µl of 10 mM of each reverse and forward primers (specified below), 1.25 units Taq DNA polymerase (New England BioLabs), 2 µl of worm lysate as template and 20.7 µl of nuclease free water. The thermocycling conditions were: initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 55°C for 15 sec, 72 °C for 2 min, and a post amplification final extension at 72°C for 10 min followed by cooling to 4 °C. Five µl of the PCR products were used for 2% agarose gel electrophoresis and visualized with ethidium bromide to analyze the PCR fragments. 0.5 µl of PCR product were used for sequencing with the primers indicated below. The sequencing reactions were done using the BigDye® Terminator v3.1 Cycle sequencing Kit according to the manufacture's protocol (Applied Biosystems, USA) and sequenced at the in house sequencing facility.

The HVR-I of the SSU was amplified using primers RH5401 and RH5402. The PCR products were sequenced using primer RH5403. A 420 bp fragment of the sequence was used for the analyses because this region is usually invariable in the same species of nematodes and was used for molecular taxonomy in other studies [226][227]. However, there wer reports of polymorphisms within *S. stercoralis* at two positions: stretch of Ts starting at position 176 consisted of either 4Ts or 5Ts and at position 458 a T or an A compared to the reference sequence AF279916 [121][153]. The HVR-IV of the SSU was amplified using the primer pair 18SP4F and 18SPCR and sequenced using primer 18SP4F. A 620 bp fragment of the sequences was used for the analysis. In *Strongyloides* spp. HVR-IV had been described to vary between species of *Strongyloides*, but not normally within the same species [121][153] and it was recommended for species diagnosis [121][153]. A portion of the mitochondrial gene *cox*1 was amplified using the primer pair TJ5207 and TJ5208 and sequenced using TJ5207. A 522 bp of the sequences was used for the analysis. All primer sequences are given in

appendix 1. The sequences of the nuclear and mitochondrial markers used are given in Appendix 2-4.

3.5 Crossing free-living stages of *S. stercoralis*

This experiment was done in the laboratory of Prof. Dr. James B. Lok at the School of Veterinary Medicine, University of Pennsylvania, USA. For this study the *S. stercoralis* laboratory isolate UPD and the isofemale line PV001, which is derived from UPD were used. Both lines were maintained in prednisolone treated (immuno-suppressed) dogs at the School of Veterinary Medicine, University of Pennsylvania, USA in accordance with protocols 702342, 801905, and 802593 from the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Feces were collected overnight and mixed with charcoal and cultured as previously described [226] and incubated at 22 ^{o}C unless specified differently.

To determine if free-living females are able to produce viable progeny without males the worms were harvested from one-day-old fecal cultures using Baermann funnels and 192 (virgin) L3-L4 females were placed individually in 24 well plates containing NGM agar spotted with 30 µl OP50 incubated at 22 ^{o}C for 72 hours and the rest at 25 ^{o}C for 72 hours. Then the cultures were checked for the presence of viable progeny produced by the unmated females. Another 192 females were isolated from fecal cultures that had been incubated at 25 °C and treated the same except that they were incubated at 25 °C throughout the experiment.

For the crossing experiment, L3-L4 male and female larvae were isolated by the Bearmann method. Single (virgin) females and males were handpicked and transferred in male-female pairs on 6 cm NGM plates spotted with 50 µl of OP50 and 20 µl of water taken from a bermann funnel and incubated at 22 ^{o}C for 24 hrs. From pairs, where the female contained developing embryos in the uterus, the males were transferred into PCR tube containing 10 µl of lysis buffer (see above) and frozen and stored at -20 ^{o}C for single worm crude DNA extraction as described above. The females were left on the agar plates until

all the eggs were laid and then the females were picked into a separate PCR tube with lyses buffer and frozen and stored at -20 °C. After all the eggs had hatched, all the L1/L2s were transferred individually into separate PCR tubes with lysis buffer and frozen and stored at -20 °C. Single worm crude DNA extractions and single worm PCR was performed as described above [51]. Genotyping was done for 36 parents from 18 crosses and eight progenies (144 larvae) from each cross, using *ytP274 as* molecular genetic marker (see appendix5).

3.6 Data and sequences analyses

3.6.1 Strongyloides ratti infections and transcriptomics

The nightly worm output count and parasitic worm burden in both hosts were expressed as mean per animal ± one standard deviation. The male to female ratio was calculated by dividing total number of males by the total number of females (free-living and iL3s) per culture. The differences in overall worm output and overall proportion of males produced were evaluated statistically using a Student t-test in Microsoft Excel. Comparative analyses of *S. ratti* parasitic adults RNA-Seq datasets were done by aligning raw reads to the *S. ratti* reference genome (version 4, which was obtained together with gene annotations from collaborators at the Welcome Trust Sanger Institute [11] using Tophat (version 2.0.3). Estimation of expression levels and identification of differentially expressed genes was done by Cufflinks and Cuffdiff (version 2.0.1) [228]. Both biological replicates were analyzed separately and for the final analysis, differentially expressed genes that showed significant differences (FDR corrected P-value <0.05) in both replicates were considered.

3.6.2 Strongyloides papillosus transcriptomics

Sequencing reads were preprocessed using Trimmomatic [229] to remove adapters and trim low quality reads. Preprocessed reads were aligned to the *S. papillosus* reference genome (PRJEB525) using Tophat version 2 [230], followed by expression level estimation using Cufflinks [229]. Differentially expressed genes were identified by all possible pairwise comparisons of the

samples using Cuffdiff. Genes with logFC greater than 1 or less than -1 with FDR (false discovery rate) less than 0.05 were treated as differentially expressed genes. Protein domain annotation of *S. papillosus* protein sequences was carried out using the hmmsearch program and the PFAM domain database obtained from the HMMER package (Version 3.0). Domain enrichment analysis of differentially expressed genes was done in R version 3.2.5 and statistical significance (FDR < 0.05) was determined using a Fisher exact test. Multiple sequence alignments were generated using Clustal omega [231]. Ambiguously aligned sequences were removed and the multiple sequence alignment of the two protein families was corrected manually. In order to determine the homology relationship of *S. papillosus* genes, the *S. ratti* gene annotation (PRJEB125) was downloaded from WormBase (WBPS4) and classification of genes into different homology classes were preformed using orthomcl [196].

3.6.3 *Onchocerca ochengi* mitochondrial genome analyses

The mitochondrial DNA (mtDNA) was extracted from the whole genome sequences for all the eleven *Onchocerca* worm samples [62]. The extracted sequences were assembled manually and aligned to the complete mitochondrial genome sequence of *O. volvulus* (AF015193) [218] using MUSCLE [232] and assembled using SeqBuilder (DNASTAR, Inc). The protein coding genes and the two ribosomal RNA (rRNA) of the *O. ochengi* mtDNA were identified based on the *O. volvulus* mitochondrialprotein coding genes [218]. The codon usages of 12 protein genes were examined using the invertebrate mitochondrial genetic code [215] and the nucleotide composition of codon families and amino acid occurrence frequencies were computed [233]. The tRNA genes were identified by ARWEN v1.2 [221] using the metazoan mitochondrial tRNA sequences as source. The analyses of the pairwise nucleotide difference of 12 protein-coding genes of the eleven *Onchocerca* isolates from cattle (this study), *O. volvulus* (AF015193), *O. gutturosa* (unpublished) and *O. flexuosa* (HQ214004) were done by MEGA6 [215]. Nucleotide sequences alignment containing all the eleven *Onchocerca* isolates were generated using the MUSCLE algorithm [232]. The nucleotide alignment was checked for SNPs and the Indels were removed from the

dataset. Protein coding genes pairwise nucleotide diversity was calculated between the isolates using Mega6 [215].

3.6.4 *Strongyloides stercoralis* single worm sequence analyses

The sequence data obtained from single worm genotyping of *S. stercoralis* from humans and dogs were blasted against the NCBI nucleotide database to confirm the species identification. The sequences were aligned using SeqMan Pro version 12 (DNAStar Inc., USA) and compared to the SSU reference sequence (AF279916) to find sequence differences (SNPs and Indels). The sequences of *ytP274* of parent and progenies in the crossing experiment were analyzed mannually by visualy inspecting each chromatograph at position 231. If only a signal for T or only for C was present the worm was scored as homozygous for the respective nucleotide, if two ovelapping signals were present the worm was scored as heterozygous.

3.6.5 Phylogenetic analyses

3.6.5.1 *Strongyloides papillosus* gene family trees

Multiple sequence alignments were generated using Clustal omega [231]. Ambiguously aligned sequences were removed and the multiple sequence alignment of the two protein families was corrected manually. Prottest [234] was used to identify the best substitution model for tree reconstruction using corrected alignment and phylogenetic trees were generated using Phangron R package [235]. The interactive tree of life web server [236] was used to display the differential gene expression information of all comparisons for Astacin and Cap genes with the phylogenetic tree. The Codeml tool from phylogenetic analysis by maximum likelihood (PAML) analysis was used to test for selection and to detect positively selected sites (PSS) [237]. In order to determine the homology relationship of *S.papillosus* genes, we downloaded the *S.ratti* gene annotation (PRJEB125) from WormBase (WBPS4) and the classification of genes into different homology classes was preformed using orthomcl [196].

3.6.5.2 *Strongyloides stercoralis cox*1 tree

The different *S. stercoralis* partial *cox*1 nucleotide sequences and the corresponding *N. americanus* (AJ417719) out-group sequence [240] were aligned using the MUSCLE [234]. A phylogenetic tree was reconstructed using the maximum likelihood method from MEGA6 [216].

3.6.5.3 Onchocerca ochengi mitochondrial genes tree

Considering the high degree of intraspecific variation in nucleotide sequences of mitochondrial genes of nematodes [239], the protein coding mitochondrial genes were used for phylogenetic analyses. The mitochondrial sequences of the samples from this study (nine of the mitochondrial type Ochengi and two of the type Siisa) and ten published and one unpublished Spirurida nematode sequence [*Acanthocheilonema viteae* (HQ186249), *Brugia malayi* (AF538716), *Chandlerella quiscali* (HM773029), *Dirofilaria immitis*: (AJ537512), *Gongylonema pulchrum*, *Setaria digitata* (GU138699), *Thelazia callipaeda* (JX069968), *Wuchereria bancrofti* (HQ184469), *Onchocerca volvulus*: (AF015193), *Onchocerca flexuosa* (HQ214004) and *O. gutturosa* (unpublished, provided By Prof. Dr. M. Blaxter, University of Edinbourg)] were used for the molecular phylogenetic analyses. The 12 protein coding genes of each nematode were aligned using MUSCLE [232]. The evolutionary history was inferred by using the Maximum Likelihood method based on the Poisson correction model [215]. The tree with the highest log likelihood (-40799.8035) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches (bootstrapping). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis involved 22 amino acid sequences. The coding data was translated assuming Invertebrate Mitochondrial genetic code table. Evolutionary analyses were conducted in MEGA6 [215].

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Appendix

1. Nuclear and mitochonderial PCR primers used in *S. stercoralis* genotyping

Primer ID	Sequence (5'-3')
HVR-I_F (RH5401)	AAAGATTAAGCCATGCATG [7][226]
HVR-I_R (RH5402)	CATTCTTGGCAAATGCTTTCG [7][226]
HVR-IV_F (18SP4F)	GCGAAAGCATTTGCCAA [121]
HVR-IV_R (18SPCR)	ACGGGCGGTGTGTRC [121]
*cox*1_F (TJ5207)	TTTGATTGTTACCTGCTTCTATTTT [This study]
*cox*1_R (TJ5208)	TTTTACACCAGTAGGAACAGCAA [This study]
ytP274_F (TJ6026)	CAGGACCACCTGGACAAGTT [This study]
ytP274_R (TJ6027)	CTTTCCATCCTGATGCCACT [This study]

2. *S. stercoralis* SSU HVR-IV sequence (AF279916)

AGTTAGAGGTTCGAAGGCGATCAGATACCGCCCTAGTTCTAACCGTAAA
CTATGCCTACTAGATGTATGAATTATTAGTTATAATAATTTATGCATCTTCT
CGGAAACGAAAGTCTTTCGGTTCCGGGGGGAAGTATGGTTGCAAAGCTGA
AACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGG
CTTAATTTGACTCAACACGGGAAAACTCACCCGGGCCGGACACTATAAG
GATTGACAGATTGATAGCTCTTTCATGATTTAGTGGTTGGTGGTGCATGG
CCGTTCTTAGTTCGTGGATATGATTTGTCTGGTTGATTCCGATAACGAGC

GAGACTTTTATGTTATATTAAATATTATTATTTTGTTTATTTTAATATAAATA
ATTAATATTTTAATAACAGATTAATAGTGTTTAACTATTTGAGAGAGAGCG
ATAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCTGCACGCGCGCTAC
AATGTAGTGATCATTATGTTCCTGTTTAGAGATAAATGGGTAAACATTGAA
AACATTACGTAACTGGGAATGAAAATTGCAATTATTTTTCATGAACGAGG
AATTCCAAGTAAACGTAAGTCATTAGC

3. *S. stercoralis* SSU HVR-I sequence (AF279916)

AAACCGCGGAAAGCTCATTATAACAGCTATAGACTACACGGTAAATATTT
AGTTGGATAACTGAGGTAATTCTTGAGCTAATACACGCTATTTATACCAC
ATTAGTGGTGCGTTTATTTGATTAAACCATTTTTATATTGGTTGACTCAAA
ATATCCTCGCTGATTTTGTTACTAAACATACCGTATGTGTATCTGGTTTA
TCAACTTTCGATGGTAGGGTATTGGCCTACCATGGTTGTGACGGATAAC
GGAGAATTAGGGTTCGACTCCGGAGAGGGAGCCTGAGAAACGGCTACC
ACATCCAAGGAAGGCAGCAGGCGCGAAAATTACCCAATTTTAGTTAAAA
GAGGTAGTGACGAAAATGACAACCAAATATTATTATTAATATTTGGATTG
AAAATCTTCAAGTTTAAAT

4. *S. stercoralis cox*1 sequence ((LC050212))

TTTGATTGTTACCTGCTTCTATTTTTTTGGTTTTTTTGGCTTGTTTTGTTGA
TAATGGTTTGGGTACTAGTTGAACAATTTATCCTCCTCTATCTACTTCAGG
TCATCCTGGTTCTAGTGTTGATTTGGCTATCTTCAGTTTACATCTTTCTGG
TATTAGCTCTATTTTAGGTGGTATTAATTTTATGTGTACTGTTAAAAATTTG
CGTTCTAGTTCTGTTTCTCTTAATAATATGAGTTTATTTATTTGAACTATTT
TTGTTACTGTTTTTTTATTGGTTTTGTCTCTACCTGTTTTAGCTGGTGCTAT
TACTATGTTGTTAATTGATCGTAATTTTAATGGTTCTTTTTTTGATCCTAGT
TTTGGTGGTAATCCTTTGATTTATCAGCATTTGTTTTGGTTTTTTGGTCAT
CCAGAAGTTTATATTTTAATTTTACCTGCTTTCGGTATTATTAGTCAATGTA
CTTTGTATTTGACTGGTAAAAAGAAGTTTTTGGTTATTTGGGTATGGTTT
ATGCTATTTTAAGTATTGGTTTAATTGGTTGTGTAGTTTGAGCTCATCATA
TGTATACTGTTGGTATAGATTTTGATTCTCGTGCTTACTTTACTGCTGCTA
CTATGGTGATTGCTGTTCCTACTGGTGTAAAA

5. *S. stercoralis ytP274* sequence (This study)

CAGGACCACCTGGACAAGTTGTATATGTAGAACCGGATGCTAATAGAATT
GAACCAATATCAGGGCCACCAGGACCACAAGGCCCCCCAGGACCTCAA
GGACCACCAGGAATTCAAGGACCTAGAGGTGAACCAGGAATAGGAATGC
CAGGACCGCCGGGATTATTTACAGGTTTAACAGAAATGGATCTTGCAAG
GATAGCTGCATATCCAGGAATCAAAGGTGAATGTGTTGAAAGAAAGGGA
GTGGATGATTATAGCAATGATTCAGAAGAGCTTCCTATATATGATAGAAA
GATTCACAGACAAACAGTAAAGGAGAAAAGGAGATAAAGGTGATCCT
GGACCACAAGGACCACCAGGTCTACCAGGACTTTCAGGAACAACAAAAA
CATCTGCTACCTACACAGCTCCAGGAGGCGTAGAAGTTTACCAAACAAC
ACAAGAATTATTGGGAGCTACAAATTCTTTTAGACAAGGTGCATTGGCTT
TCAGTATTTCATCCCAACAATTAATGATCAGAGTGGCATCAGGATGGAAA
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