Genetic and genomic studies of the parasitic nematode *Strongyloides* spp. – the human parasite *S. stercoralis* and the laboratory model species *S. papillosus*

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Summary

The parasitic nematode genus *Strongyloides* contains an important human pathogen and many animal parasitic species, which can serve as models for basic and applied research. *Strongyloides* spp. undergo a unique and complex lifecycle, which includes easily accessible sexual free-living generations in between asexual parasitic generations. In this thesis I present two projects, one with a more applied focus on the human parasite *S. stercoralis*, and the other on basic biological questions within the sheep parasite *S. papillosus*.

1) To investigate whether dogs are a source for zoonotic *S. stercoralis* transmission, I isolated *S. stercoralis* from humans and dogs living in the same households in northern Cambodia. I analyzed individual worms genetically using the nuclear 18S rDNA, the mitochondrial gene *cox1* and, for a subset, the whole genome sequence as molecular markers. I found that dogs carry two populations, possibly species/sub-species of *S. stercoralis*, which are distinguishable by their 18S hyper variable region IV (HVR-IV). One population is dog specific but the other one is zoonotic and could be shared with humans. This suggests that dogs are a reservoir for zoonotic strongyloidiasis and need to be considered in *S. stercoralis* treatment and prevention. Interestingly, I never detected any hybrids between the different HVR-I haplotypes, although they sometimes co-occurred in the same host individuals. I also demonstrated that free-living *S. stercoralis* do reproduce sexually. However, both mitochondrial and nuclear genome comparisons argue against the hypothesis that the different HVR-I haplotypes represent reproductively isolated populations. To further investigate this paradox, I isolated and analyzed *S. stercoralis* from humans in southern China. Strikingly, I failed to find sexual stages and almost all worms were hybrids of different HVR haplotypes, with high levels of genomic heterozygosity, indicating that this population predominantly (if not exclusively) undergoes asexual life-cycles.

2) In *S. papillosus* the sex chromatin is a portion of a chromosome, which undergoes chromatin diminution in males and is evolutionarily derived from an X chromosome. I tested the hypothesis that meiotic recombination is absent in the diminished region even in females. As the rather fragmented *S. papillosus* genome assembly was a hindrance to this project, I therefore sequenced an iso-female isolate using Illumina and PacBio platforms, resulting in a significantly improved genome assembly. The new assembly consists of 79 contigs as opposed to the 4353 scaffolds in the previously published one. To then test the hypothesis, I firstly crossed single males and females, examined their progeny and determined the recombination frequency between molecular markers at different genomic loci. As a second independent approach, I sequenced the whole genomes of individual *S. papillosus* isolated from the field and analyzed the linkage disequilibrium. Both approaches revealed that recombination is virtually absent in the sex chromatin, while it is present in the non-sex chromatin in both sexes of *S. papillosus*.

Zusammenfassung

Die parasitische Nematodengattung *Strongloides* umfasst eine wichtige humanpathogene und viele tierparasitische Arten, die als Modellorganismen für die Grundlagen- und angewandte Forschung dienen können. *Strongyloides* spp. durchlaufen einen einzigartigen komplexen Lebenszyklus, in dem sich einfach zugängliche freilebende geschlechtliche Generationen mit parasitischen ungeschlechtlichen Generationen abwechseln. In dieser Doktorarbeit präsentiere ich zwei Projekte. Das erste über den Humanparasiten *S. stercoralis* ist eher anwendungsorientiert, das zweite beschäftigt sich mit grundlagenbiologischen Fragen im Schafparasiten *S. papillosus*.

Zu 1) Um herauszufinden, ob Hunde eine Quelle für zoonotische *S. stercoralis* Übertragung sind, habe ich diese Würmer in einem Feldversuch in Nordkambodscha aus dem Kot von Hunden und Menschen isoliert. Ich habe einzelne Würmer genetisch analysiert und dabei die nukleäre 18S rDNA, das mitochondrielle Gen *cox1* und für einen Teil der Proben sogar das ganze Genom als Marker benutzt. Das Ergebnis zeigte, dass es in Hunden zwei Populationen von Würmern gibt, welche sich in ihrer 18S hypervariablen Region (HVR) IV unterscheiden und bei denen es sich möglicherweise um nah verwandte Arten oder Unterarten von *S. stercoralis* handelt. Eine Population ist hundespezifisch, die andere ist die gleiche, die auch im Menschen vorkommt. Dies legt nahe, dass Hunde ein Reservoir für zoonotische Strongyloidiasis darstellen und bei der *S. stercoralis* Behandlung und Prävention berücksichtigt werden müssen. Interessanterweise habe ich nie Hybride zwischen den verschiedenen HVR I Haplotypen gefunden, obwohl solche im gleichen Wirt vorkommen können und ich auch zeigen konnte, dass sich freilebende *S. stercoralis* geschlechtlich fortpflanzen. Andererseits sprechen Vergleiche der mitochondriellen und der nukleären Genome gegen die Hypothese, dass die verschiedenen HVR I Haplotypen unterschiedliche, reproduktiv isolierte Populationen repräsentieren. Um dieses Paradox weiter zu untersuchen, habe ich *S. stercoralis* von Menschen in Südchina isoliert und untersucht. Hier waren fast alle Hybride zwischen verschiedenen HVR I Haplotypen. Zudem habe ich praktisch keine geschlechtlichen Tiere gefunden, was nahelegt, dass diese Population überwiegend, wenn nicht ausschließlich, die ungeschlechtlichen Lebenszyklen durchläuft. In Übereinstimmung damit habe ich genomische Hinweise auf Asexualität, wie z.B. eine hohe Heterozygotie, gefunden.

Zu 2) Das Geschlechtschromatin in *S. papillosus* ist ein Teil eines Chromosoms, der in Männchen eine Chromatin-Diminution durchmacht und evolutionär von einem X abstammt. Ich habe die Hypothese, dass es in dieser Region auch in Weibchen keine meiotische Rekombination gibt, getestet. Die starke Fragmentierung der *S. papillosus* Genomsequenz stellte ein Hindernis für dieses Projekt dar. Deshalb sequenzierte ich eine Inzuchtlinie mit den Illumina und PacBio Plattformen. Daraus resultierte eine aus 79 Contigs bestehende Genomsequenz, was verglichen mit den 4353 Scaffolds der publizierten Sequenz eine deutliche Verbesserung darstellte. Danach kreuzte ich zuerst einzelne Männchen und Weibchen, analysierte deren Nachkommen und berechnete die Rekombinationsfrequenzen zwischen verschiedenen molekularen Markern. In einem zweiten, unabhängigen Ansatz sequenzierte ich die Genome von einzelnen *S. papillosus* Individuen aus einer wilden Population und analysierte das Kopplungsungleichgewicht. Beide Ansätze bestätigten, dass es in den beiden Geschlechtern von *S. papillosus* praktisch keine Rekombination im Geschlechtschromatin, wohl aber in den autosomalen Bereichen, gibt.

List of publications

Different but overlapping populations of Strongyloides stercoralis in dogs and humans - Dogs as a possible source for zoonotic strongyloidiasis

Siyu Zhou*, Tegegn G. Jaleta*, Felix M. Bemm, Fabian Schär, Virak Khieu, Sinuon Muth, Peter Odermatt, James B. Lok, Adrian Streit.

PLOS neglected tropical diseases.

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*Shared first authorship, published as Jaleta, Zhou et al.

Characterization of a non-sexual population of Strongyloides stercoralis with hybrid 18S rDNA haplotypes in Guangxi, Southern China

Siyu Zhou, Xiaoyin Fu, Pei Pei, Marek Kucka, Jing Liu, Lili Tang, Tingzheng Zhan, Shanshan He, Yingguang Frank Chan, Christian Rödelsperger, Dengyu Liu and Adrian Streit.

PLOS neglected tropical diseases.

Published: May 6, 2019.

DOI: 10.1371/journal.pntd.0007396.

1. Introduction

1.1 The phylum Nematoda

The phylum Nematoda, is one of the largest phyla in the animal kingdom. It is diverse and it is estimated to comprises over a million species (Lambshead 1993). Until now, around 25 000 nematode (also known as roundworm) species have been described, more than half of which are parasitic nematodes (Zhang 2013).

Nematodes inhabit a wide variety of ecosystems, including soil, fresh water and marine, from tropics to polar, and from the lowest to highest altitude. Nematodes are either free-living or parasites of plants, animals and humans, and they can be found in extreme ecological environments such as deserts, oceanic trenches, mountains and volcanos. The ubiquity of nematodes, their dominating in population and species numbers and their interaction with a variety of organisms, makes nematodes play a very critical role in ecosystems (Hsueh, Leighton et al. 2014) (https://en.wikipedia.org/wiki/Nematode).

Based on morphological and ecological traits, taxonomic classification of free-living and parasitic nematodes has been attempted in the 1930s (Chitwood and Chitwood 1933, Filipjev 1934), and was later revised and improved by many taxonomists (Chitwood 1937, Chitwood 1958, Andrássy 1976, Lorenzen 1994). Five major clades have been proposed, based on the sequences of the small subunit (*SSU* or 18S) of the ribosomal DNA (rDNA) from 53 taxa covering the major free-living and parasitic taxa (Blaxter, De Ley et al. 1998). Parasitic species can be found in all 5 clades, suggesting parasitism has evolved independently multiple times in the history (Blaxter, De Ley et al. 1998, Dorris, De Ley et al. 1999). Later a sub-division into 12 clades has been suggested based on the *SSU* sequences of 339 and later 1215 taxa covering a wide range of nematode species (Holterman, van der Wurff et al. 2006, van Megen, van den Elsen et al. 2009). Nematodes species with similar morphological and ecological characteristics can be found in all the clades, indicating that extensive convergent evolution has occurred (van Megen, van den Elsen et al. 2009). However, a more detailed systematization is still in process and the 959 Nematode Genome initiative will bring significant insight into the phylogenetic framework (Bert, Karssen et al. 2011, Kumar, Koutsovoulos et al. 2012, Blaxter and Koutsovoulos 2015). The phylogenetic structure of the phylum Nematoda is presented in the figure 1 below.

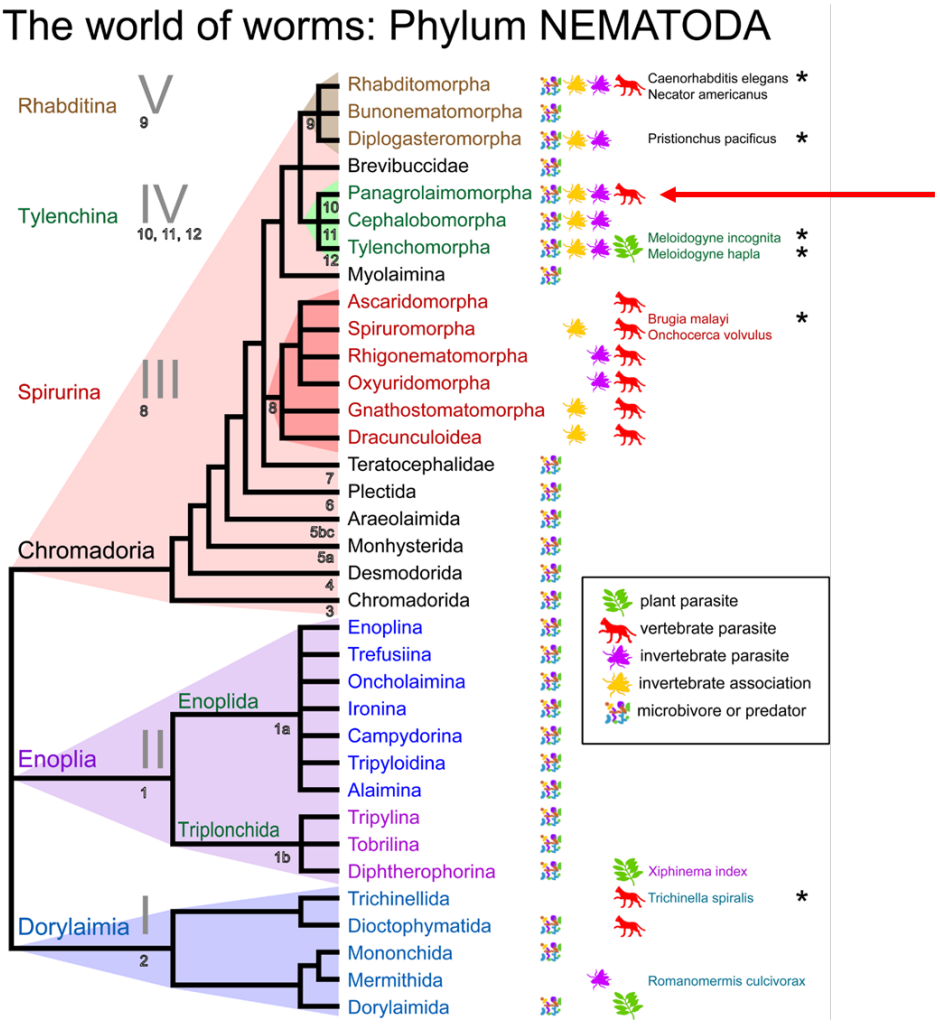

Figure 1. The overview phylogenetic structure of the Nematoda based on *SSU* sequences, modified from (Blaxter 2011). The red arrow indicates the position of Genus *Strongyloides* in the phylogeny.

1.2 The genus *Strongyloides*

According to the classification by (Blaxter, De Ley et al. 1998) and later modified by (Dorris, De Ley et al. 1999) and (Blaxter 2011), the genus *Strongyloides* belongs to family *Strongyloididae* and its phylogenetic position is shown in figure 1. This genus contains more than 50 species, which are gastrointestinal obligate parasites of various vertebrates, including birds, amphibians, reptiles and mammals including humans (Speare 1989). Most of *Strongyloides* species are fairly host-specific, which is common in animal parasitic nematodes (Dorris, Viney et al. 2002, Viney and Lok 2015).

1.2.1 Phylogenetic relationship of different *Strongyloides* species

Strongyloides species, in particular the easily accessible outside-of-the-host developmental stages, usually share many morphological characteristics. The parasitic stage is fairly helpful for species identification. It has been shown that the shape of stoma and ovaries of parasitic females varies among different species but such features might not be sufficient for separating closely related species (Little 1966). Also, obtaining parasitic worms is not easy because it normally requires sacrificing the host. Therefore in general, the differentiation of *Strongyloides* spp. based on morphological observation is rather difficult, and some species were defined based on the hosts where they were found (Speare 1989).

In 2002 the phylogenetic relationship of 10 species of *Strongyloides* has been analyzed based on *SSU* sequences (Dorris, Viney et al. 2002). More recently, more precise phylogenetic analyses have been completed based on 18S and 28S rDNA sequences (Hino, Tanaka et al. 2014), amino acid sequences of conserved single-copy orthologs and mitochondrial genes (Hunt, Tsai et al. 2016). The phylogeny of representative *Strongyloides* species is presented in the figure 2 below.

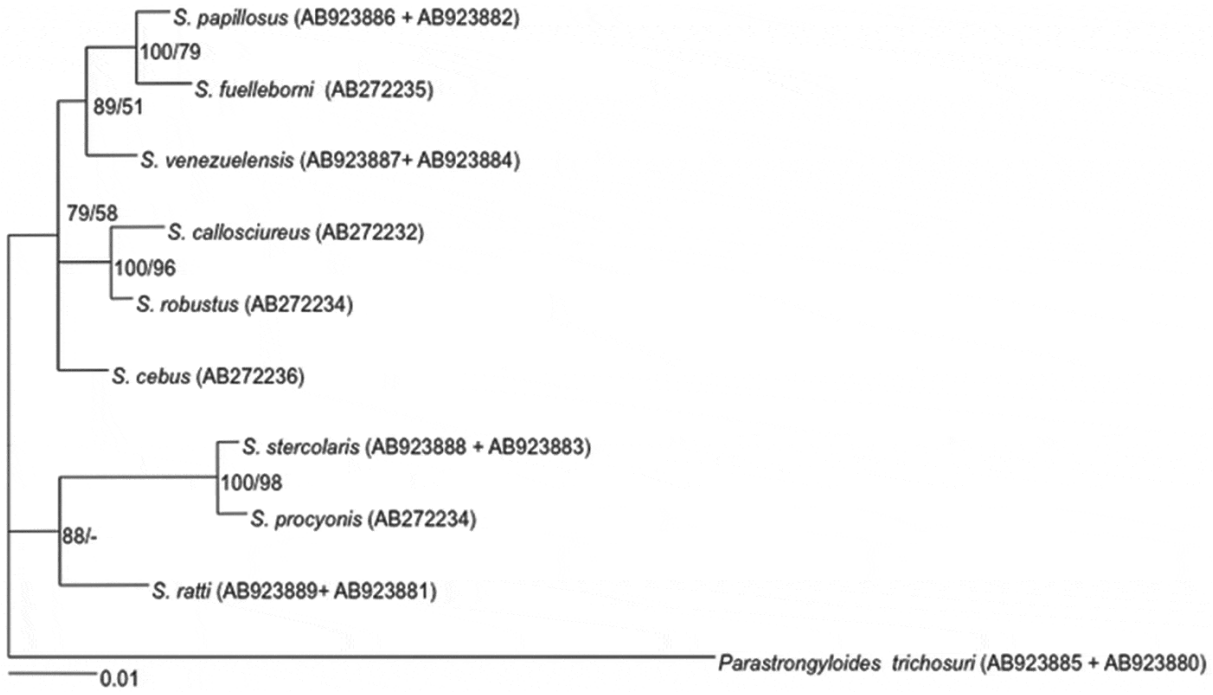

Figure 2. The phylogenetic relationship between representative *Strongyloides* species based on 18S and 28S rDNA sequences (Hino, Tanaka et al. 2014).

1.2.2 Morphology, life cycle and reproduction

Strongyloides spp. have rather unique and complicate life cycles, including parasitic and free-living generations (Figure 3). Depending on their developmental stages in the life cycle, the morphology is quite distinctive, such that *Strongyloides* spp. can easily be recognized.

Infective third stage larvae (iL3s) *Strongyloides* spp. are all females, with a filariform oesophagus occupying almost half of the body length. The filariform iL3s are approximately 0.4-0.8 mm in length and 14-19 μm in width with a trifurcated tail, which is a distinct character for *Strongyloides* spp. (Little 1966, Viney and Lok 2015). iL3s infect hosts by skin penetration, and migrate in the host body through circulatory system. When larvae reach the lung or naso-frontal region, they break through and are coughed up and swallowed into the digestive system. During the migration to the small intestine, they molt via L4 stage and become parasitic adult females (Tindall and Wilson 1988, Schad, Aikens et al. 1989).

The parasitic females are approximately 2-4 mm in length and 30-60 μm in width, with a blunt end tail and filariform oesophagus occupying about one third of the body length. The vulva is at around two thirds body length from the anterior end and didelphic ovaries spiral around or parallel the intestine, depending on the species (Little 1966, Speare 1989). Cytological observation, as well as genetic analysis, have shown that parasitic females undergo mitotic parthenogenesis. Additionally, the absence of parasitic males or sperm in parasitic females and the possibility of establishing productive infections with a single iL3 also agrees with parthenogenesis (Zaffagnini 1973, Viney 1994, Streit 2008).

Depending on the species, eggs or hatched first stage larvae (L1) produced by the parasitic females are released to the environment from the host by defecation. Eggs are 40-85 μm in length with a thin wall (Viney and Lok 2015). Larvae are either female or male, and have different developmental choices: (1) Female larvae may develop via L2 into filariform iL3, which can infect a new host (direct cycle/homogonic cycle). (2) Alternatively, via L2-L4 stages female and male larvae can develop into free-living female and male adults (indirect cycle/heterogonic cycle) (Streit 2008, Viney and Lok 2015).

Free-living female and male adults are rhabditiform, approximately 0.8-1.2 mm in length and 40-70 μm in width, while females are generally 1.1-1.3 times larger than males. The female has a didelphic ovary and the vulva opens at the middle of the body. The male has one testis, equal spicules, and a ventral curve to the tail (Little 1966). There was a debate on the reproduction model of the free-living generation. For several *Strongyloides* species, it has

been shown that females and males are both required for successful reproduction. Male dependent meiotic parthenogenesis, also termed as pseudogamy (sperm is required to trigger the egg development), has been proposed based on cytological studies, e.g. *S. papillosus* (Zaffagnini 1973, Triantaphyllou and Moncol 1977), *S. ransomi* (Triantaphyllou and Moncol 1977) and *S. stercoralis* (Hammond and Robinson 1994). Nevertheless, this was disproven by several genetic studies. The sexual reproduction of free-living females and males has been confirmed for *S. ratti* (Viney, Matthews et al. 1993), *S. papillosus* (Eberhardt, Mayer et al. 2007), *S. vituli* (Kulkarni, Dyka et al. 2013) and more recently for *S. stercoralis* (Jaleta, Zhou et al. 2017) (part of this thesis). In most *Strongyloides* species, only one free-living generation occurs between parasitic generations, and their progeny are all females, which will develop into filariform iL3 and have to survive in environment until they find a suitable host (Streit 2008, Viney and Lok 2015).

The representative life cycle of *Strongyloides* species is presented in figure 3. Most *Strongyloides* spp. follow this life cycle but with few exceptions: *S. planiceps* can undergo up to 9 successive free-living generations outside of the host (Yamada, Matsuda et al. 1991). *S. stercoralis* and *S. felis* have an additional auto-infection life cycle, where larvae produced by parasitic females develop into iL3 while still within the host, resulting in a re-infection of the same host. With this unique feature, *S. stercoralis* and *S. felis* infection can be a lifelong burden for the hosts unless effectively treated (Stern and Joshpe 1971, Speare and Tinsley 1987, Viney and Lok 2015).

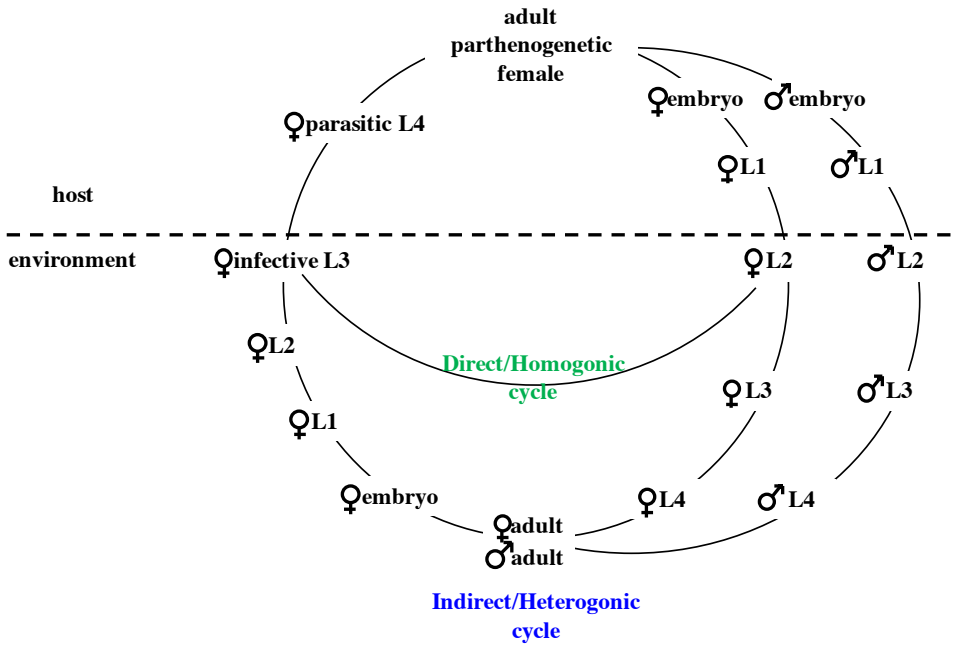

Figure 3. Representative life cycle of *Strongyloides* spp., modified from (Streit 2008). Notice that, dependent on the species, embryonated eggs or already hatched L2 larvae are passed to the environment.

1.2.3 Sex determination and karyotype

Some *Strongyloides* species, such as *S. ratti* and *S. stercoralis* have two pairs of autosomes, and the females have two X chromosomes while males have only one, thus employing an XX/XO sex determination system (female: 2n=6; male: 2n=5). Nevertheless, other *Strongyloides* species such as *S. papillosus*, *S. ransomi* , *S. venezuelensis* and *S. vituli* carry only two pairs of chromosomes, including a large (L) and a medium (M) pair (2n=4) (Streit 2008).

In 1977, Triantaphyllou and Moncol have proposed that the large chromosome was formed by the fusion of the X chromosome and one autosome, based on the cytological observation of *S. papillosus* and *S. ransomi* (Triantaphyllou and Moncol 1977). Later, it was observed that in some oocytes in parasitic females during the single mitotic maturation division, a large portion of each of the two large chromosomes appears beaded. While one chromosome recovers, from the other the beaded portion gets eliminated, resulting in a 1L3M1S (1 large, 3 medium-sized and 1 small chromosome, with one M and the S being the remnants of the second large chromosome) karyotype. In the oocytes, where beaded chromosomes are not formed, the karyotype remains 2L2M (2 large and 2 medium-sized chromosome). The 2L2M and 1L3M1S eggs will develop into females and males, respectively (Albertson, Nwaorgu et al. 1979). The male and female karyotypes with “beaded” and “un-beaded” large chromosomes are shown in figure 4.

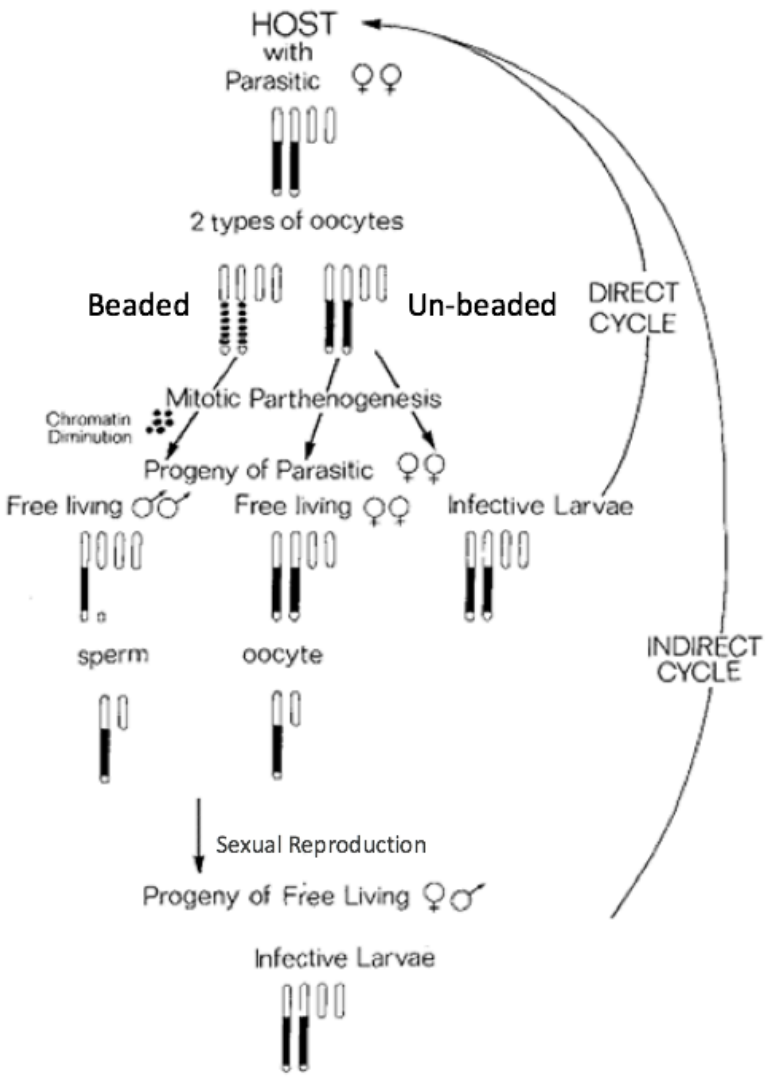

Figure 4. The karyotypes of different stages of *S. papillosus*, the oocytes with "beaded" and "un-beaded" large chromosome are indicated, modified from (Albertson, Nwaorgu et al. 1979).

With molecular approaches, it has been shown that the large chromosome in *S. papillosus* consists of genetic material homologous to the X chromosome and to autosome number I of *S. ratti* and the hemizygous region in males corresponds to the X chromosome in *S. ratti* (Nemetschke, Eberhardt et al. 2010). Additionally, *Parastrongyloides trichosuri*, a closely related species to *Strongyloides* spp., also has three pairs of chromosomes and employs XX/XO sex determination, suggesting that two pairs autosomes and two X chromosome in females and one X chromosome in males is the ancestral state, and the large chromosome occurred during the evolution by the fusion of the X and one autosome (Kulkarni, Dyka et al. 2013, Streit 2014). The karyotypes and evolutionary history of *Strongyloides* spp. and the chromosome relationship between *S. papillosus* and *S. ratti* are shown in figure 5.

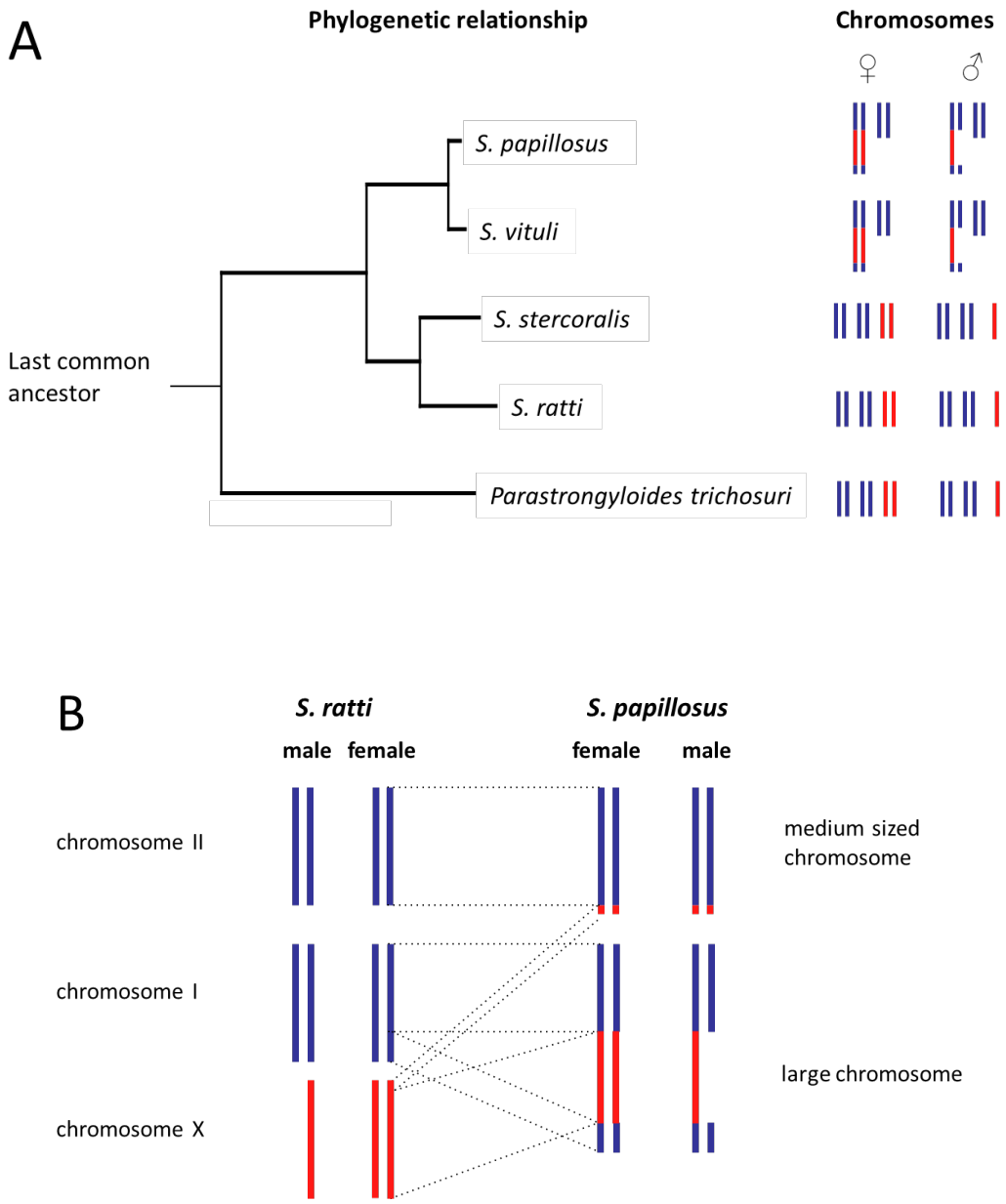

Figure 5. (A) Karyotypes and evolutionary history of *Strongyloides* spp. Modified from (Kulkarni, Dyka et al. 2013). (B) Chromosome relationship between *S. papillosus* (2n=4 in females, 2n=5 in males) and *S. ratti* (2n=6 in females, 2n=5 in males). Modified from (Nemetschke, Eberhardt et al. 2010). Blue: autosome (in *S. ratti*) or homologous to an *S. ratti* autosome (in *S. papillosus*); Red: X chromosome (in *S. ratti*) or homologous to the *S. ratti* X (in *S. papillosus*). A few translocations exist, only one is shown to symbolize this fact.

It is very interesting that parasitic females parthenogenetically produce progeny of both sexes, but the mechanism is largely unknown. Environmental factors, particularly the immune status of hosts, play a crucial role in sex determination. In *S. ratti*, it has been reported that in the hosts with a strong immune response, the larvae produced by parasitic females are more male-

biased, while in the hosts receiving immuno suppressive treatment, the sex ratio is more female-biased (Harvey, Gemmill et al. 2000, Crook and Viney 2005).

1.2.4 Chromatin diminution

As discussed above, during the sex determination of *S. papillosus* males, from one large chromosome the portion homologous to the X chromosome of *S. ratti* is removed by programmed DNA elimination (PDE). This is a case of sex specific chromatin diminution (Streit, Wang et al. 2016). It appears to be random which of the two large chromosomes undergoes diminution (Nemetschke, Eberhardt et al. 2010). The region undergoing chromatin diminution is flanked by non-eliminated chromatin. Consequently, two breakpoints appear between the eliminated and non-eliminated region of one large chromosome in males. This male-specific chromatin diminution is very unique in *S. papillosus* and probably a few other species of *Strongyloides* and has not been found in any other nematode (Streit, Wang et al. 2016).

Interestingly, the free-living males of *S. papillosus* only pass the intact large chromosome but never the diminished one to their offspring, while females randomly "choose" one of the two large chromosomes, hence the free-living generation only produces female progeny. (Nemetschke, Eberhardt et al. 2010). However, the details of this female-only-ensuring mechanism are still unclear.

Apart from *Strongyloides* spp., cases of chromatin diminution with different functions have been found in a few other animals and are best studied the nematodes ascarids. Ascarids belong to the order *Ascaridida* and are phylogenetically distant from genus *Strongyloides*. Studies on *Parascaris* spp. and *Ascaris* spp. have described that chromatin diminution happens in the somatic cells during the first several cell divisions generating a genetic difference between the germline and the soma (Muller, Bernard et al. 1996, Muller and Tobler 2000). This differs from the chromatin diminution of *Strongyloides* spp. where PDE occurs in germline during mitotic parthenogenesis and is related to sex determination.

The DNA elimination leads to a loss of about 80-90% or 13% of the genome in the soma in *Parascaris equorum* and *Ascaris suum*, respectively. Most of the lost genome are repetitive sequences: in *Parascaris* they are 5 and 10 bp repeats and in *Ascaris* they are mostly 121 bp repetitive sequences (Streit, Wang et al. 2016). Nevertheless, in *Ascaris* 685 genes that are lost during PDE were identified and these genes are mainly expressed in germline and early

embryogenesis. From this it was concluded that the chromatin diminution in *Ascaris* is a mechanism of gene regulation by eliminating genes involved in gametogenesis and early embryogenesis (Wang, Mitreva et al. 2012).

There are fundamental differences between chromatin diminution in ascarids and *S. papillosus*. In ascarids the process generates a difference between the germline and soma, in *S. papillosus* it leads to a difference between the sexes. While in ascarids both homologous copies are either eliminated or retained, in *S. papillosus* only one of the two copies is removed. The diminished region in *S. papillosus* contains a large number of genes, many of which have *C. elegans* orthologs with known functions (Nemetschke, Eberhardt et al. 2010, Nemetschke, Eberhardt et al. 2010, Hunt, Tsai et al. 2016). All these evidences and the phylogenetic distance indicate that the phenomenon of chromatin diminution in ascarids and *Strongyloides* spp. arose independently in evolution.

1.3 Chromosome recombination

In eukaryotes, chromosome recombination naturally takes place during meiosis, allowing the genetic material exchange between homologous chromosomes, thereby generating a novel chromosome which will be passed to the offspring. Therefore, with sexual reproduction, an individual can inherit recombined chromosomes from both parents thus increases genetic diversity. In mitosis, recombination between sister chromosomes may also occur, but no novel chromosome can be formed since the sister chromosomes are identical. Here only meiotic recombination will be mainly discussed.

1.3.1 Meiotic recombination in *C. elegans*

In nematodes, mechanisms and pathways of meiotic recombination have been extensively studied in the model organism *C. elegans* and systematically reviewed in 2007 and 2013 (Garcia-Muse and Boulton 2007, Lui and Colaiacovo 2013). A dissected gonad from *C. elegans* hermaphrodite stained with DAPI (4',6-diamidino-2-phenylindole) is shown below in figure 6.

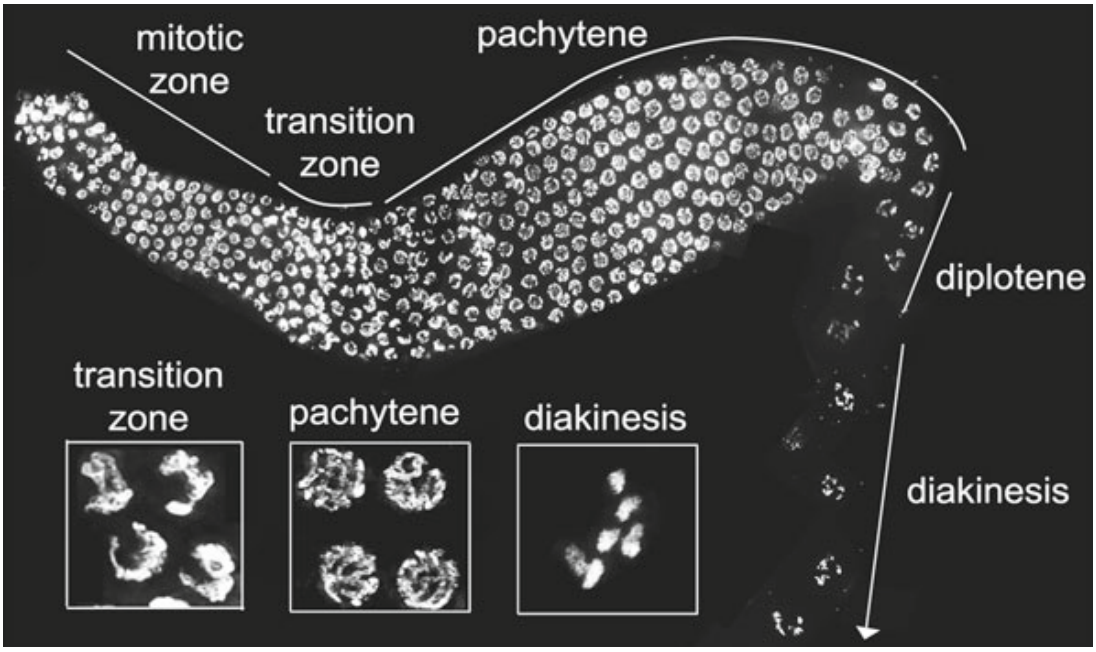

Figure 6. Dissected and DAPI-stained gonad of *C. elegans* hermaphrodite (Lui and Colaiacovo 2013).

In the distal tip is the **mitotic zone**, also called proliferation zone. It contains mitotic cell nuclei undergoing DNA replication and nuclear divisions (Kimble and Crittenden 2005, Crittenden, Leonhard et al. 2006).

Moving proximally, in the **transition zone** the nuclei, which have a duplicated genome and therefore each chromosome consists of two sister chromatids, enter meiotic prophase I. In the transition zone, cohesin complexes hold together the sister chromatids, homologous chromosome pairing takes place, synaptonemal complex (SC) begins to assemble and recombination is initiated by the formation of double strand breaks (DSBs). The "crescent"-shaped appearance of the nuclei is due to the chromosomes polarization towards one side of nuclei (Dernburg, McDonald et al. 1998, Lui and Colaiacovo 2013, Hillers, Jantsch et al. 2017).

Cells enter **pachytene** when homologous chromosomes are fully synapsed and the SC is completely assembled. From early- to late-pachytene, obligate crossovers (COs) occur, resulting in DNA exchange between homologous chromosomes. In *C. elegans*, synapsis happens only once and independently for each pair homologous chromosomes. Also, although initially many DSBs are generated, usually only one of them eventually results in a CO on each pair of homologous chromosomes. The pachytene zone of *C. elegans* is relatively long, and the "spaghetti"-shaped nuclei can be clearly observed (Hillers, Jantsch et al. 2017).

In the **diplotene** stage, synapsis and SC disassemble, homologous chromosomes are then held together by chiasmata instead of SC. Chromosomes begin to condense, and bivalents which are the paired and duplicated homologous chromosomes containing four chromatids, become visible (Nabeshima, Villeneuve et al. 2005).

In the most proximal region of *C. elegans* germline, oocytes are arrested at **diakinesis** waiting for fertilization. In this stage chromosomes are held together by chiasmata and highly condensed, therefore the six bivalents can be observed easily (Lui and Colaiacovo 2013, Hillers, Jantsch et al. 2017).

1.3.2 Recombination hotspots and pattern

Recombination hotspots refer to the small chromosomal regions (a few kb or less) with a high tendency of meiotic recombination. In many eukaryotes, COs are not randomly distributed, their occurrences are highly concentrated in hotspots, and the flanking regions are often called coldspots with a meiotic exchange frequency lower than average. This variation of recombination frequency is normally quantified by comparing to the physical distance and measured as centi Morgans per kilobase (cM/kb) (Lichten and Goldman 1995).

Hotspots have been described in many well-studied organisms such as *S. cerevisiae* (Gerton, DeRisi et al. 2000), *Arabidopsis* (Kim, Plagnol et al. 2007), mouse (Smagulova, Gregoretti et al. 2011) and human (Myers, Bottolo et al. 2005). Nevertheless, true recombination hotspots have not been found in *C. elegans* (Kaur and Rockman 2014), although COs are also not randomly distributed on the chromosomes. On central regions of *C. elegans* chromosomes (less pronounced on X chromosome) there are so called “central gene clusters” with high gene density, where the recombination rates are lower. Conversely, on chromosome arms the gene density is lower and the recombination frequency is higher (Barnes, Kohara et al. 1995).

It has been noticed that, in these organisms where recombination hotspots can be found, such as yeast and mammals, DSBs and homologous recombination are needed for SC formation. In *C. elegans* and *Drosophila*, where recombination hotspots have not been identified, SC formation occurs before and independent of DSBs and homologous recombination (Zickler and Kleckner 1999, MacQueen, Colaiacovo et al. 2002, McKim, Jang et al. 2002, Hunter 2003). Therefore, there has been a hypothesis that recombination plays an important role in chromosome alignment in many but not all organisms, and the hotspots are byproducts in the process of recombination (Hey 2004).

To date, there are only a few studies of recombination patterns in other nematode species apart from *C. elegans*. For instance, *Pristionchus pacificus* is a well-studied clade V nematode species associated with beetles, and it has 6 chromosomes which are largely macro-syntenic to their *C. elegans* counterparts. But unlike *C. elegans*, the recombination pattern in *P. pacificus* do not show distinction between chromosome central and arm regions (Srinivasan, Sinz et al. 2003, Rodelsperger, Neher et al. 2014). Also, plentiful evidence suggested that recombination hotspots evolve rather quickly. Even between very closely related species such as human and chimpanzees, hotspots are not shared (Ptak, Roeder et al. 2004, Ptak, Hinds et al. 2005). Moreover, even though a general recombination pattern exists within the species, the recombination distributions and rates can differ within populations. For instance, in *C. elegans*, the CO frequencies and positions are affected by sex, parental age, temperature and other environment factors (Lim, Stine et al. 2008, Hillers, Jantsch et al. 2017). On the other hand, examples of conserved recombination patterns in closely related species have been reported. Therefore, recombination patterns cannot be described as generally conserved or as generally divergent (Smukowski and Noor 2011).

1.3.3 Detection of recombination

Recombination frequency and its distribution along chromosomes can be measured using traditional phenotypic markers or DNA sequence polymorphisms such as SNPs markers. The former allows studying large number of individuals while the later allows assessing many markers in one experiment (Hillers and Villeneuve 2009). Cytologically, crossovers can be directly detected by observing the bivalents at the diakinesis stage (Hillers and Villeneuve 2009) and the crossover sites can be visualized by COSA-1 (CrossOver Site Associated-1) immuno staining foci in pachytene oocytes in *C. elegans* (Yokoo, Zawadzki et al. 2012).

Next-generation sequencing provides a powerful technique for analyzing the recombination sites with much higher resolution. In plants, recombination patterns have been finely mapped by measurement of linkage disequilibrium (LD) in *Arabidopsis thaliana* (Kim, Plagnol et al. 2007, Horton, Hancock et al. 2012). In mammals, recombination rates and hotspots across the whole human genome have been reported for the first time in 2005, by studying the LD of around 1.6 million SNPs in three human populations (Myers, Bottolo et al. 2005). A few years later, the genome-wide distribution of recombination hotspots in mouse has been studied by applying chromatin immune-precipitation followed by high-throughput sequencing (ChIP-seq) (Smagulova, Gregoretti et al. 2011). As for nematodes, the genome-wide

recombination landscape of *C. elegans* has been characterized by examining the LD patterns of 125 wild isolates (Rockman and Kruglyak 2009).

1.3.4 Recombination and sexual reproduction

The origin of sexual reproduction and how it competes with asexual reproduction remains a mystery. Most high organisms reproduce sexually, despite the two-fold cost of sex: (1) the cost of males and (2) the cost of one individual only passing 50% of own genes to each of its offspring, implying the fitness of the offspring outcompetes the cost (Smith and Maynard-Smith 1978).

It is generally believed that genetic recombination plays an important role in overcoming this two-fold cost. Recombination exchanges the genetic material between homologous chromosomes, generating a novel allele combination, which is different from any chromosome in the parents and can be passed to gametes and eventually incorporated in the offspring. The process can separate negative/deleterious and positive/beneficial mutations at different loci and it can combine the beneficial mutations. This increases the efficiency of natural selection and is therefore favored by evolution. In the non-recombining organisms or populations, deleterious mutations tend to accumulate which might lead to the extinction of the population (Felsenstein 1974, Barton and Charlesworth 1998).

1.3.5 Absence of recombination

In sexual organisms, the absence of genetic recombination is often found in the sex specific chromosome. For several example, it has been shown that the sex chromosome (Y chromosome in XX/XY system and W chromosome in ZW/ZZ system) evolved from a normal homologous chromosome pair by reducing the recombination in particular chromosomal regions, frequently followed by genetic degeneration (gene loss and accumulation of repetitive sequences) and in extreme cases to the complete loss of the non-recombining portions, reviewed by (Graves 2006, Bergero and Charlesworth 2009).

In some, presumably relatively recently arisen Y and W chromosomes it has been shown that even the Y or W specific region is still highly homologous to the X or Z chromosome, respectively, indicating an origin of both sex chromosomes from a homologous chromosome pair (Yu, Hou et al. 2008). In other, presumably older cases the Y or W specific regions have diverged very much (Handley, Ceplitis et al. 2004). While the male specific region of Y

chromosomes (MSY) does not recombine with the X chromosome, other parts that do recombine are called pseudo autosomal regions (PAR) and are maintained. For instance, around 5% of the human Y is able to recombine with the X chromosome. It has been shown that, even the non-recombining regions of Y chromosome have some homologues on the X chromosome, which also supports XY homology (Skaletsky, Kuroda-Kawaguchi et al. 2003).

In addition to Y and W chromosomes, a few examples of non-recombining chromosomes in otherwise sexual organisms have been reported. For example, it has been shown that the dot chromosome, also referred as F elements of several *Drosophila* species hardly recombines. The dot chromosome is small in size (about 1-2 Mb) and shows some distinct features such as highly repetitive sequences, large gene size, high rates of gene rearrangement while retaining a similar gene density as in other autosomes (Leung, Shaffer et al. 2010, Riddle and Elgin 2018).

1.3.6 Genomic consequences of absence of recombination

In asexual reproduction, genetic recombination is absent in the whole genome because meiosis does not happen. This results in the accumulation of deleterious mutations known as Muller's ratchet (Muller 1964). The asexual reproduction may have short term benefits under favorable environmental conditions but this cannot confront the advantage of high genetic diversity and rapid adaption to environment in sexual reproduction. Therefore, strictly asexual reproduction is rather rare in multi-cellular eukaryotes (Dawson 1995). In sexual organisms, such effects also hold true for the genomic regions where recombination is absent.

In diploid organism, asexuality (whole or partial of the genome) will lead to the independent evolution of the two homologous chromosomes and consequently increase the divergence between them. This phenomenon was described by Meselson, where he found individual genome of bdelloid rotifers represent more than two distinct alleles. He also found highly divergent gene copies (up to 4), which is highly conserved and present as single copy in all other invertebrates. Such divergence was observed in asexual bdelloid rotifers but not in the sexual non-bdelloid rotifers where recombination and genetic exchange happens (Mark Welch and Meselson 2000). Therefore, the increasing of allelic divergence caused by the absence of recombination is commonly referred as Meselson effect (Butlin 2002).

The consequences of asexuality/absence of recombination can be examined at the genomic level, for instance by analyzing the heterozygosity and nucleotide substitutions. In an asexual

population, a new mutations can hardly become homozygous, therefore a high heterozygosity is expected compared with a sexual population. Also, the purifying selection is reduced and slightly deleterious mutations can accumulate, which result in an increase of the proportion of non-synonymous substitutions related to the synonymous substitutions. Such genomic consequences have been observed by comparing the sexual and asexual lineages of the same species, or between closely related sexual and asexual species, such as stick insects (Henry, Schwander et al. 2012), freshwater snails (Neiman, Hehman et al. 2010), ribbon worms (Ament-Velasquez, Figuet et al. 2016) and plants (Pellino, Hojsgaard et al. 2013, Hollister, Greiner et al. 2015).

For *S. stercoralis*, a recent study found a higher genomic heterozygosity of the samples from Japan than the samples from Myanmar (Kikuchi, Hino et al. 2016). The authors attributed this finding to the fact that in Japan *S. stercoralis* were isolated for patients who had presumably been infected years or decades ago and the infections had been maintained within the host through the inherently asexual auto-infection cycle for many generations. In Myanmar, on the other hand, where new infections are presumably rather frequent, *S. stercoralis* are likely to have reproduced sexually regularly through the indirect/heterogonic life cycle (Kikuchi, Hino et al. 2016).

1.3.7 Gene conversion

Gene conversion is the unidirectional and non-reciprocal transferring the genetic material between two highly homologous sequences, where one sequence is used as “template” (or “donor”) to repair the “acceptor” sequence. It can be allelic (interallelic) and non-allelic (interlocus). The non-allelic gene conversion occurs between different genomic loci, for example, between paralogous sequences on the same (in *cis*) or different (in *trans*) chromosomes. The allelic gene conversion occurs at the same genomic locus between the homologous chromosomes. During homologous recombination in meiosis, gene conversion can occur when non-crossover (NCO) instead of crossover (CO) is formed (Chen, Cooper et al. 2007, Chen, Ferec et al. 2010).

Gene conversion can happen in both mitosis and meiosis between homologous chromosomes rather than sister chromatids. Therefore, asexuality would stop homologous recombination but not gene conversion. The gene conversion generates new allelic combinations and may reduce the Meselson effect in asexual organisms (Tucker, Ackerman et al. 2013).

1.3.8 Recombination in *Strongyloides* spp.

As described in 1.2.2, the parasitic generation of *Strongyloides* spp. reproduces via mitotic parthenogenesis and the free-living generation reproduces sexually. Therefore, meiotic recombination can only happen in the free-living generations. Unsurprisingly, the recombination and meiotic progress in *Strongyloides* spp. remains largely unstudied. Also, the germline organization of *Strongyloides* spp. differs greatly from *C. elegans* (Kulkarni, Lightfoot et al. 2016), which may be associated with some characteristics in the meiotic mechanism.

In *S. ratti* (2n=6), a genetic linkage map with 74 molecular markers was generated and it has shown that recombination happens in all the three chromosomes. Compared to the autosomes, the recombination frequency may be relatively reduced between the X chromosomes (Nemetschke, Eberhardt et al. 2010).

In *S. papillosus* (2n=4) recombination was detected in the non-diminished regions in both sexes. Strikingly, it has been noticed that the, rather few, genetic markers analyzed in the diminished region were perfectly genetically linked, even in females, indicating little or no recombination occurs in this region (Nemetschke, Eberhardt et al. 2010). To my knowledge, such absence of recombination on a large chromosomal region (~20 Mb) while recombination occurring on other parts of the same chromosome has never been described in any organism so far. We know that the diminished region of *S. papillosus* is homologous to the X chromosome in *S. ratti*. (Nemetschke, Eberhardt et al. 2010, Hunt, Tsai et al. 2016). If this observation is true, it would indicate that one portion of a chromosome (sex chromatin region) reproduces essentially asexually while the rest of the chromosome undergoes normal sexual reproduction

However, this finding was preliminary, because firstly it was only based on a small number of genetic markers. Secondly, the published *S. papillosus* genome assembly is highly fragmented (Hunt, Tsai et al. 2016), and the physical distance between the analyzed markers was unknown. Testing the hypothesis that meiotic recombination is absent in the male diminished region of *S. papillosus* in females was one aim of this thesis.

1.4 Genomes of nematodes

As a model organism and the most extensively studied nematode, *C. elegans* was the first multicellular organism and thence the first nematode species with its whole genome sequenced after viruses, bacteria and yeast (Consortium 1998). Later the genomes of many nematodes species have been sequenced, including several parasitic nematodes, such as *Ascaris suum* (Jex, Liu et al. 2011), *Brugia malayi* (Ghedin, Wang et al. 2007), *Meloidogyne hapla* (Opperman, Bird et al. 2008), *Trichinella spiralis* (Mitreva, Jasmer et al. 2011), and *Necator americanus* (Tang, Gao et al. 2014). To date, the draft genomes of 78 nematode species/strains have been published or are public available, and this number is growing fast (959 nematode genomes, http://www.nematodes.org/nematodegenomes/) (Kumar, Koutsovoulos et al. 2012). A very recent study has compared the genomics among 81 nematode and platyhelminth species, many of which are parasites. It focused on the parasitic lineage-specific genomic differences, identified hundreds of gene families expanded in parasitic species and provided genomic references for understanding parasitism (International Helminth Genomes 2019).

The sizes of sequenced nematode genomes range from 20 Mb to 700 Mb, which is rather small compare with many other metazoans, especially vertebrates (Leroy, Duperray et al. 2003, Coghlan 2005, Burke, Scholl et al. 2015, Hunt, Tsai et al. 2016, International Helminth Genomes 2019). However, the estimated numbers of genes in nematodes are generally not quite different from humans (roughly 20 000) (Rödelsperger, Streit et al. 2013, Ezkurdia, Juan et al. 2014, Hunt, Tsai et al. 2016). Therefore, compared with vertebrates, the nematode genomes are rather compact, with little repetitive sequences, fewer and shorter introns and shorter intergenic sequences (Rödelsperger, Streit et al. 2013). However, the underlying reason is still unclear. The speculations are a high rate and large size of DNA deletion in nematodes (Witherspoon and Robertson 2003) and that they are more efficient in regulating gene expression or in controlling transposon activity (Rödelsperger, Streit et al. 2013).

1.4.1 Genomes of *Strongyloides* spp.

In the last century limited numbers of individual genes were characterized and studied in *Strongyloides* spp. (Fisher and Viney 1996, Moore, Ramachandran et al. 1996). The first large-scaled omics study of *Strongyloides* spp. was based on expressed sequence tags (ESTs) in 2004. 10,921 ESTs (3311 clusters) of *S. stercoralis* were generated from free-living L1s

and iL3s, and they were compared with *C. elegans* (Mitreva, McCarter et al. 2004). A year later, 14,761 ESTs (4152 clusters) were obtained from *S. ratti*, including free-living, infective and parasitic stages (Thompson, Mitreva et al. 2005). Further, microarray analyses were performed on *S. ratti* and *S. stercoralis*, to determine the temporal expression patterns of many genes over different developmental stages (Thompson, Barker et al. 2006, Thompson, Barker et al. 2008, Ramanathan, Varma et al. 2011).

Originally the whole genome sequencing of *S. ratti* was initiated based on traditional sequencing strategies. Later, the sequencing efforts were expanded to other species and methodology resulting in the publication of the genomes of four *Strongyloides* species, *S. stercoralis*, *S. ratti*, *S. papillosus*, *S. venezuelensis* and two fairly close relatives, the facultative parasite *Parastrongyloides trichosuri* and the free-living *Rhabditophanes* sp. KR3021 (Hunt, Tsai et al. 2016). The genome sequencing was also complemented with transcriptomic analyses of different developmental stages in *S. ratti* and *S. stercoralis* (Hunt, Tsai et al. 2016) and later *S. papillosus* (Baskaran, Jaleta et al. 2017). This work revealed interesting genomic and transcriptomic features of *Strongyloides* spp.. For example, compared to *C. elegans* and also other parasitic nematodes, the two key gene families encoding Astacins and SCP/TAPS are significantly expanded and many members are differentially expressed between parasitic, infective and free-living stages, indicating that they play roles in the transition between the life stages and might have been important for the evolution of parasitism (Hunt, Tsai et al. 2016, Baskaran, Jaleta et al. 2017).

The genome assemblies are shown in table 1. The assembly sizes of the different *Strongyloides* species vary between 42.6 to 60.2 Mb, which is rather small, even in comparison to the genomes of other nematodes, but their protein-coding sequences are similar in size of other nematodes. The GC contents in *Strongyloides* spp. are between 21%-26%, making them the most AT rich nematode genomes (Hunt, Tsai et al. 2016).

Table 1. Genome assemblies of *Strongyloides* spp. and related species (Hunt, Tsai et al. 2016).

	S. ratti	*S. stercoralis*	*S. papillosus*	*S. venezuelensis*	*P. trichosuri*	*Rhabditophanes* sp. KR3021	*C. elegans*
Clade	IV	IV	IV	IV	IV	IV	V
Number of chromosomes	3 (ref. 72)	3 (ref. 73)	2 (ref. 74)	2 (ref. 21)	3 (ref. 22)	5[a]	6 (ref. 16)
Assembly version	V5.0.4	V2.0.4	V2.1.4	V2.0.4	V2.0.4	V2.0.4	WS244
Assembly size (Mb)	43.1	42.6	60.2	52.1	42.2	47.2	100.2
Number of scaffolds	115[b]	675	4,353	520	1,391	380	6
N50 of scaffolds (kb)	11,700	431	86	715	837	537	17,500
N50 (number)	2	16	129	16	12	22	3
Maximum scaffold length (Mb)	16.8	5.0	1.7	5.9	6.2	7.3	20.9
GC content (%)	21	22	26	25	31	32	36
Number of genes	12,451	13,098	18,457	16,904	15,010	13,496	23,629
Number of exons	33,796	34,366	40,821	40,619	35,049	37,987	145,275
Exons, combined length (Mb)	17.5	17.9	22.4	20.3	20.8	17.8	30.1
Median exon length (bp)	263	265	304	261	348	276	146
Number of introns	21,345	21,268	22,364	23,715	20,039	24,491	169,506

The quality of the assemblies varies. Among *Strongyloides* spp., the *S. ratti* genome is the most contiguous: 93% of the genome were assigned to 12 scaffolds. The two autosomes were even assembled into single scaffolds. This made the *S. ratti* genome the second most contiguous assembled nematode genome after *C. elegans*. The single-scaffold chromosomes enabled the study of the chromosome organization of *S. ratti*. Other than in *C. elegans*, in *S. ratti* the difference between gene-rich centers and gene-poor arms was not found. Within *Strongyloides* species the chromosomal location of particular genes is highly conserved. Although within chromosome rearrangements are rather frequent, there are large blocks of synteny, in particular between the species which share the same karyotype: between *S. ratti* and *S. stercoralis* (2n=6), and between *S. papillosus* and *S. venezuelensis* (2n=4) (Hunt, Tsai et al. 2016).

The sex chromatin (X chromosome or diminished region) was identified by resequencing and quantitative comparison of the differential coverage of sequences in males and females (or iL3s, which are all females). The sex chromatin is clearly homologous in all species where it could be determined (the particular isolate of *S. venezuelensis* and *Rhabditophanes sp*. KR3021 do not form males).

The *S. papillosus* genome assembly remained rather unsatisfactory. Although probably equally complete like the others with respect to gene coverage, the genome is in over 4000 pieces of scaffolds/contigs, which is 6-38 times the number of the other three sequenced *Strongyloides* species. One possible reason is that the S. *papillosus* LIN isolate used for sequencing was neither an iso-female isolate, like *S. ratti* and *S. stercoralis*, nor an isolate that undergoes 100% clonal homogonic development, like *S. venezuelensis*. On the contrary, the LIN isolate was derived from a wild population of *S. papillosus* collected from naturally infected sheep and was maintained at an unknown but likely relatively large population size in rabbit hosts for about 20 generations (2-3 generations/year) largely via the sexual heterogonic

life-cycle. In other words, the LIN isolate was not inbred and highly genetically polymorphic (Eberhardt, Mayer et al. 2007). The other reason could be that the *S. papillosus* genome contains many long repetitive sequences, especially in the diminish region, which strongly impedes the assembly. For my project most importantly, there was no scaffold bridging the diminished and the non-diminished regions. Improving the quality of the *S. papillosus* genome assembly was therefore highly desirable and was one aim of this thesis.

1.4.2 Genome sequencing strategies

As mentioned above, the sequencing of the first complete nematode genome (*C. elegans*) was finished in 1998. It was achieved by traditional Sanger sequencing of 2527 cosmids, 257 yeast artificial clones (YACs), 113 fosmids, and 44 PCR products (Consortium 1998). Sanger sequencing was developed by Frederick Sanger in 1977. It commercialized in 1980s and remains widely used nowadays for sequencing at low throughput but with high accuracy and relatively long read lengths (around 1 kb). Sanger sequencing is based on the termination of DNA synthesis by the incorporation of ddNTPs (di-de-oxy NTPs) during DNA replication, therefore it is also called as chain termination method (Sanger, Nicklen et al. 1977).

The high-throughput sequencing methods have been developed in the late 1990s. To distinguish them from Sanger sequencing, they were called second generation, or next generation sequencing (NGS) methods. The principle behind NGS is massive parallel sequencing of short fragments which are randomly generated from the genome. Depending on the sequencing strategies applied, the NGS can be sub-divided into several categories, which were commercialized at different platforms such as Roche/454, Illumina/Solexa, ABI/SOLiD, Helicos/HeliScope, etc. (Shendure and Ji 2008, Ansorge 2009). The key advantage of NGS is the high-throughput which allows the whole genome being sequenced at the same time and within a short time. The challenges of NGS are the short read length and the complexity of computational analysis of the sequencing data (Niedringhaus, Milanova et al. 2011). The application of NGS was definitely a revolutionary progress for the genomic research. With NGS technology many nematode genomes including *Strongyloides* spp., could be completed.

More recently, the third generation sequencing has been developed. It is also called long-reads sequencing since this new technique is able to sequence very long fragments (usually 10-150 kb). This overcomes the limitation of NGS which produces only short reads (usually 50 to 300 bp) meanwhile keeps the advantage of high-throughput. Such long read sequencing significantly improves the genome research because it can span some regions of repetitive

sequences and produce much contiguous structure and accurate assembly (Lee, Gurtowski et al. 2016). The two most successful commercialized platforms are the Pacific Biosystems (PacBio) using single molecular real time (SMRT) sequencing (Eid, Fehr et al. 2009) and the Oxford Nanopore Techniques using nanopore-based device to detect the single molecular (Schneider and Dekker 2012). However, the main draw-back of third generation sequencing is the rather high error rates which is caused by the sequencing machinery.

Another technique commonly used in genome assembly is optical mapping, which was originally developed in 1990s (Schwartz, Li et al. 1993). The main principle of optical mapping is the producing of "nicks" on the single and stretched DNA molecules, using restriction enzyme sites or fluorescently labeled specific motif sequences (Lam, Hastie et al. 2012). The resulting high resolution "fingerprint" spectrum can be used to guide the assembly: order and orientate contigs/scaffolds, fill assembly gaps, correct the assembly errors such as false joins, inversions, translocations, therefore improve the assembly accuracy (Tang, Lyons et al. 2015). Conventionally, optical mapping relied on the restriction enzyme fragmentation of DNA molecules and visualized under microscope (Schwartz, Li et al. 1993). Later, automatic and high-throughput systems have been developed, using microarray, microfluidics and automatic fluorescence microscopy capture system and commercial platforms such as OpGen and BioNano became available (Niedringhaus, Milanova et al. 2011, Tang, Lyons et al. 2015).

Currently, a common strategy for *de novo* genome assembly is to integrate the sequencing methods from different platforms. The third generation sequencing produces long reads with a high-throughput for generating contigs. NGS generates accurate short reads for correcting the errors. Sanger sequences, which usually exist from the early project, can also be used for consensus validation with accurate and relatively long contiguous reads. Optical mapping provides genome-wide fine maps and guides the scaffolding. High quality assemblies by integrating various sequencing strategies were achieved in bacteria (Zhou, Kile et al. 2004) and in much more complex eukaryote genomes, such as rice (Kawahara, de la Bastide et al. 2013), maize (Zhou, Wei et al. 2009), grass (VanBuren, Bryant et al. 2015), parrot (Ganapathy, Howard et al. 2014) and goat (Bickhart, Rosen et al. 2017).

1.5 Human strongyloidiasis

1.5.1 Causative agents

Strongyloidiasis is one type of soil-transmitted helminthiasis (STHs), and one of the most neglected trophic diseases (NTDs) prioritized by the World Health Organization (WHO).

In human, strongyloidiasis is mainly caused by *S. stercoralis*, and to a much lesser extent by *S. fuelleborni fuelleborni* and *S. fuelleborni kelleyi* (Grove 1996). *S. stercoralis* has a cosmopolitan distribution (Viney and Lok 2015). *S. fuelleborni fuelleborni* has been found in Africa and Asia, parasitizing primarily primates but human infection cases have been reported in Africa (Pampiglione and Ricciardi 1971, Hira and Patel 1977, Evans, Markus et al. 1991, Hasegawa, Sato et al. 2010). *S. fuelleborni kelleyi* infection has been reported only in Papua New Guinea and it occurred exclusively in humans (Vince, Ashford et al. 1979, Ashford, Barnish et al. 1992). Molecular evidence suggested that *S. fuelleborni kelleyi* is not a subspecies of *S. fuelleborni*, but rather an independent species (as *S. kelleyi*) (Dorris, Viney et al. 2002). In this thesis, mainly human strongyloidiasis caused by *S. stercoralis* is discussed.

1.5.2 Global prevalence

In 1989, the global prevalence of human strongyloidiasis has been systematically reviewed for the first time. The infection of humans with *S. stercoralis* has been reported across five continents including the United States, Latin America, Europe, Asia, Africa and Pacific region. High infection rates have been found in parts of Latin America and sub-Saharan Africa (Genta 1989). More recent reviews on the world wide (Schar, Trostdorf et al. 2013, Puthiyakunnon, Boddu et al. 2014) and regional (Wang, Xu et al. 2013, Buonfrate, Mena et al. 2015, Schar, Giardina et al. 2016) distribution of *S. stercoralis* indicate that number of infected people is still increasing over the past years.

Generally, *S. stercoralis* is more prevalent in tropical and sub-tropical areas such as South America, central Africa and Southeast Asia. Locally, as many as 10% to 40% of the population in those epidemic regions are estimated to be infected (Schar, Trostdorf et al. 2013, Puthiyakunnon, Boddu et al. 2014). Also, it has been pointed out that high temperature and moisture, poverty, poor sanitary conditions such as walking with barefoot and open defecation are the risk factors over epidemic regions (Albonico, Bisoffi et al. 2018).

Noticeably, for many countries or regions where the environment and economic conditions are favorable for *S. stercoralis* transmission, no or only few studies were conducted. For instance, for 56.5% of the African countries there are no data available for *S. stercoralis* infection. Very few studies were available from China which has the largest population in the world and they were conducted in one province by the same research group (Steinmann, Zhou et al. 2007, Steinmann, Du et al. 2008, Yap, Du et al. 2013, Steinmann, Yap et al. 2015). Additionally, among the available studies more than 60% used diagnostic methods with low sensitivity for *S. stercoralis* (Schar, Trostdorf et al. 2013). Therefore, the infected population is probably largely underestimated. Recent estimates of the number of people infected with *S. stercoralis* range from 30-100 million (Bethony, Brooker et al. 2006, Nutman 2017) and "at least 370 million" (Bisoffi, Buonfrate et al. 2013). The global prevalence of *S. stercoralis* infection is shown in figure 7 below.

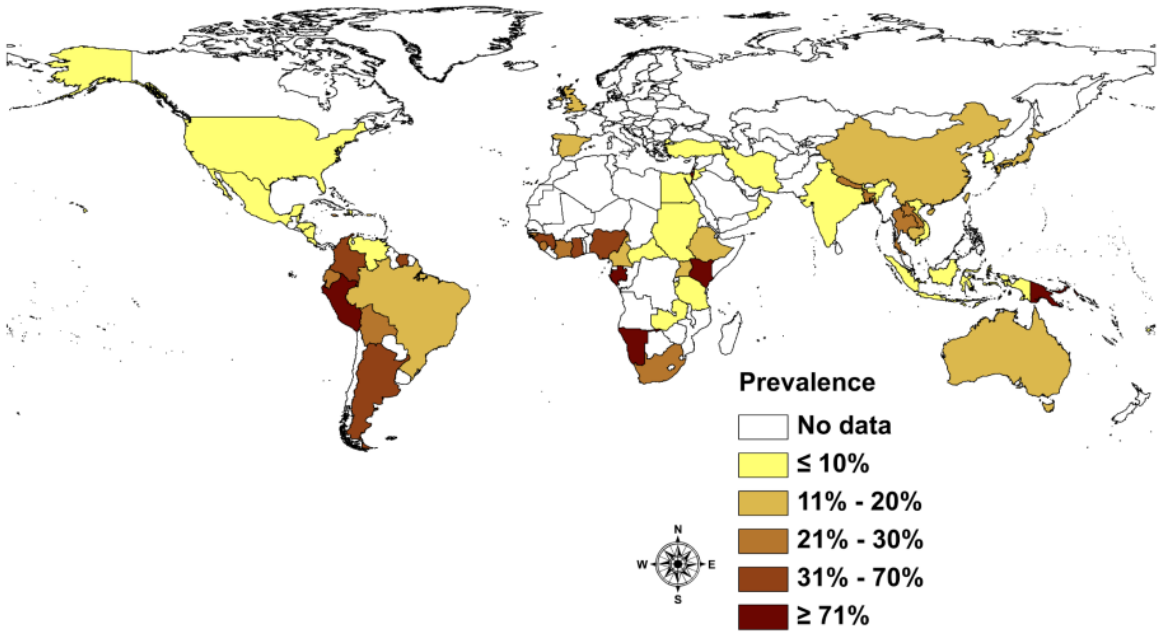

Figure 7. Global prevalence of *S. stercoralis* infection by community-based studies (Schar, Trostdorf et al. 2013). Notice in many countries, only one or a few local surveys were included, therefore in such cases the prevalence rates showing on the map are not representing the whole country but rather local areas. The details can be found in (Schar, Trostdorf et al. 2013).

1.5.3 Zoonotic aspects

Whether strongyloidiasis is a zoonotic disease or to what extent it is a zoonotic disease has been discussed for decades. Although humans are generally considered as the natural host for *S. stercoralis*, non-human primates, dogs and cats have been proposed to be suitable hosts (Sandground 1928, Faust and Kagy 1933, Riggio, Mannella et al. 2013, Thamsborg, Ketzis et al. 2017). Also it has been shown that Mongolian gerbils can be used as the experimental host for *S. stercoralis* (Nolan, Megyeri et al. 1993, Kerlin, Nolan et al. 1995, Nolan, Bhopale et al. 1999). However, natural infection with other *Strongyloides* species have been found in cats (*S. felis*, *S. tumefaciens* and *S. planiceps*) (Thamsborg, Ketzis et al. 2017) and primates (*S. fuelleborni*) (Hasegawa, Hayashida et al. 2009). And it has to be noticed that the study identifying *S. stercoralis* in cats were based merely on morphology (Abu-Madi, Al-Ahbabi et al. 2007), while in primates *S. stercoralis* was identified based on morphology (Penner 1981) but molecular evidence were also available (Hasegawa, Hayashida et al. 2009, Hasegawa, Sato et al. 2010). Additionally, natural infection of *S. stercoralis* in gerbils has not been found. Therefore, the doubt remains whether the *Strongyloides* spp. found in the animals were truly *S. stercoralis*.

Concerns about zoonotic strongyloidasis are mainly focused on dogs, whether dogs can harbor human *S. stercoralis* and if they play roles as reservoirs for human strongyloidiasis. In 1922, Brumpt proposed that *S. canis* is the natural *Strongyloides* sp. of dogs and is distinct from the human parasite *S. stercoralis*, although morphological differences have not been observed (Sandground 1925). This was supported by Augustine in 1939, when he failed to establish an infection in a human volunteer with dog derived *Strongyloides* sp. (Augustine and Davey 1939). On the other hand, dogs are commonly used as experimental hosts for human derived *S. stercoralis* (Grove and Northern 1982, Grove, Heenan et al. 1983, Lok 2007, Yuan, Lok et al. 2014), therefore many researchers suggested *Strongyloides* sp. in dogs and humans are the same species. However, the few studies based on molecular analysis of rather few individuals favored a separation of *Strongyloides* spp. in humans and dogs (Ramachandran, Gam et al. 1997, Hasegawa, Hayashida et al. 2009, Hasegawa, Sato et al. 2010). Recently, we (Jaleta, Zhou et al. 2017)（part of this thesis) and (Nagayasu, Aung et al. 2017) employing molecular analysis of large numbers of individual *Strongyloides* spp. from humans and dogs suggested that dogs naturally carry at least two different species of *Strongyloides*, one of which is the same *S. stercoralis* as found in humans.

1.5.4 Manifestations

The manifestations of *S. stercoralis* infection vary from asymptomatic to severe, often fatal, forms of strongyloidiasis. In most cases, it appears to be asymptomatic, but skin irritation at the site of larvae penetration and mild gastrointestinal symptoms could happen including abdominal pain, diarrhea and constipation (Grove 1989, Nutman 2017). However, in immune-compromised patients, the risk of life-threating strongyloidiasis is highly increased (Marcos, Terashima et al. 2008). When the host becomes immunosuppressed, symptomatic auto-infection occurs because the immune system is unable to control the number of larvae emerging from the auto-infective cycle, resulting in a complicated disease called hyper-infection. Additionally, disseminated strongyloidiasis can occur when larvae migrate to other organs beyond pulmonary and gastrointestinal systems. Eventually, this massive infection of larvae throughout the host body leads to various severe manifestations including respiratory, gastrointestinal, cardiopulmonary, dermatologic and central nervous system features (Grove 1989, Keiser and Nutman 2004, Vadlamudi, Chi et al. 2006, Marcos, Terashima et al. 2008, Olsen, van Lieshout et al. 2009, Nutman 2017).

Estimates of the mortality of disseminated strongyloidiasis vary but go up to 87%, which is a dramatic increase comparing to 15% in patients with hyper-infection syndrome (Marcos, Terashima et al. 2008). The high mortality is commonly accompanied with bacterial infection such as *Escherichia coli* (Ghoshal, Ghoshal et al. 2002) and *Streptococcus bovis* (Link and Orenstein 1999), as they are carried by larvae and disseminate in the host along larvae migration, and therefore lead to sepsis, bacteremia, meningitis and eventually multiple organ failure (Concha, Harrington et al. 2005) Once the state of full disseminated strongyloidasis is reached, prognosis is poor, even with treatment.

1.5.5 Diagnosis

Diagnosis of asymptomatic human strongyloidiasis is challenging because eggs are normally not present in the stool and the larvae output may be very low and irregular. Symptomatic infection of *S. stercoralis* is relatively easy to detect due to the high number of larvae in the stool or body fluids, however only if *S. stercoralis* is suspected and specifically looked for. Because the symptoms associated with strongyloidiasis are unspecific and usually have common other causes, *S. stercoralis* is frequently detected only rather late. Undoubtedly, early diagnosis and treatment is very crucial, for preventing hyper-infection and disseminated strongyloidiasis which could be fatal (Nutman 2017, Albonico, Bisoffi et al. 2018).

1.5.5.1 Morphological diagnosis

The conventional diagnosis is based on morphology and relies on the direct observation of larvae by microscopy, which is still considered as the "gold standard" until now. The direct diagnostic methods include stool smear and Kato-Katz (Santos, Cerqueira et al. 2005). Many indirect methods have been developed to improve the diagnostic sensitivity: formalin ethyl acetate concentration, agar plate culture, Harada-Mori filter paper culture, and Baermann technique (Sato, Kobayashi et al. 1995, Siddiqui and Berk 2001). Noticeably, these methods are not specifically used for detecting *S. stercoralis* but for helminths in general. Additionally, most surveys on STHs not specifically aim at strongyloidiasis and frequently use egg detection, therefore *S. stercoralis* infection are often missed and its prevalence is largely underestimated. Nevertheless, the sensitivity of the agar culture and Baermann methods is largely improved when multiple stool samples from several collecting days are examined (Siddiqui and Berk 2001, Khieu, Schar et al. 2013).

1.5.5.2 Molecular diagnosis

Molecular diagnostic methods are based on the detection of *S. stercoralis* DNA from stool samples. PCR, nested PCR and real-time PCR techniques have been used for detecting species-specific sequences, such as the 18S ribosomal RNA gene (18S rDNA, or *SSU*) and cytochrome c oxidase 1 (*cox1*) of the mitochondrial DNA (Verweij, Canales et al. 2009, Moghaddassani, Mirhendi et al. 2011, Sharifdini, Mirhendi et al. 2015). Four hyper-variable regions (HVRs) in the 18S rDNA were characterized by comparing 34 isolates of 15 *Strongyloides* species. The HVRs are corresponding to the small loop or stem-and-loop regions in the secondary structure of 18S rDNA, which allows accumulation of mutations. HVRs, especially HVR-IV, contain species-specific nucleotide arrangements and are therefore suitable for identifying different *Strongyloides* species (Hasegawa, Hayashida et al. 2009).

Molecular diagnostic techniques are highly specific, but the sensitivity varies from study to study. Biological samples like stool are always challenging for PCR based diagnoses. First of all, the larvae output is variable depending on the infection level. Less than 10^{-1} larvae/g stool might not be sufficient for detection. Secondly, the materials are highly heterogeneous because other organisms including a variety of bacteria are also present in stool. Thirdly, inhibitors such as nucleases, bacterial protease, acid and other debris may interfere with the PCR amplification. Also, the thick and complex cuticle of *S. stercoralis* increases the

difficulty of DNA extraction (Abu Al-Soud and Radstrom 2000, Repetto, Alba Soto et al. 2013, Sharifdini, Mirhendi et al. 2015). Therefore, the purification of DNA from such complex material is curial. Some researchers combined the conventional and molecular techniques, where they isolated DNA and perform PCR from worms isolated from stool by Baermann or agar plate cultures (Schar, Guo et al. 2014, Hasegawa, Kalousova et al. 2016, Laymanivong, Hangvanthong et al. 2016, Nagayasu, Aung et al. 2017). This requires the worms being found and isolated with conventional methods prior to the molecular identification. Compared to directly DNA extraction from stool, this method is more sensitive and specific, but it is labor intense and not suitable for large-scale studies. Also, expensive equipment and molecular techniques might not be accessible in some areas where *S. stercoralis* are found.

1.5.5.3 Immunological diagnosis

Several serological techniques have been developed for detecting *S. stercoralis*, such as ELISA (Enzyme-Linked Immunosorbent Assay), GPAT (Gelatin Particle Agglutination Test) and IFAT (Indirect Fluorescence Antibody Test), against crude larval antigens (Sato, Takara et al. 1985, Sato, Toma et al. 1991, Sithithaworn, Sithithaworn et al. 2005, Boscolo, Gobbo et al. 2007). Compared to conventional Baermann and Kato-Katz techniques, these serological diagnosis show higher sensitivity (56%-100%) (Requena-Mendez, Chiodini et al. 2013).

However, the specificity of serological tests has been a matter of large concern. A study of *S. stercoralis* infection of refugees in Canada in 1980s using ELISA shows only 29% specificity (Gyorkos, Genta et al. 1990). Cross reaction with other parasites has been reported, such as *Ascaris* and *Schistosoma* (Gam, Neva et al. 1987, Koosha, Fesharaki et al. 2004). Moreover, past infection may not be distinguishable from current infection because the antibodies persist in patient for rather long time. Therefore, it is generally believed that tests based on the direct observation of *S. stercoralis* worms or their DNA underestimate the infection prevalence while the immunological tests overestimate it (Siddiqui and Berk 2001).

Many efforts have been made to improve the specificity. It has been found that pre-incubation of sera with *Onchocerca gutturosa* extracts can largely remove the cross-reactivity with other helminths (Conway, Atkins et al. 1993, Lindo, Conway et al. 1994). Subsequently, a 31-kDa recombinant immunodiagnostic antigen (NIE) derived from L3 cDNA library has been developed (Ravi, Ramachandran et al. 2002). The serological assays based on NIE have better sensitivity (87% - 98%) and specificity (95% - 100%), and show no cross-reactivity with

filarial infections (Ravi, Ramachandran et al. 2002, Ramanathan, Burbelo et al. 2008, Krolewiecki, Ramanathan et al. 2010). Therefore ELISA with NIE was a significant improvement in serological diagnosis and was suggested as an ideal commercial product (Krolewiecki, Lammie et al. 2013, Pak, Vasquez-Camargo et al. 2014). Nevertheless, similar as molecular techniques, serological diagnosis requires certain lab facilities which are sometimes not available in endemic regions.

1.5.6 Treatment

The most commonly used drugs against strongyloidiasis are ivermectin and benzimidazoles (albendazole and thiabendazole) (Henriquez-Camacho, Gotuzzo et al. 2016). They are board-spectrum and effective medication against various parasitic infection including strongyloidiasis, onchocerciasis, lymphatic filariasis, ascariasis and trichuriasis (Datry, Hilmarsdottir et al. 1994, Marti, Haji et al. 1996, Taylor, Hoerauf et al. 2010). Ivermectin and albendazole are recommended intestinal anthelmintics on the WHO model list of essential medicines (EML) (WHO 2017).

Targets of such medicines are adult worms but not larvae, therefore repeat treatment is necessary to completely eradicate the infection. The recommended oral dosage is 200 µg/kg/day for two days for ivermectin, 400 mg/12 hours for 7 days for albendazole and 50 mg/kg/day for two days for thiabendazole. It has been suggested that ivermectin has higher efficiency compared with albendazole. Also, ivermectin has better performance than thiabendazole in terms of adverse effects. Therefore, ivermectin is the drug of choice for the treatment of strongyloidiasis (Concha, Harrington et al. 2005, Albonico, Becker et al. 2016, Henriquez-Camacho, Gotuzzo et al. 2016, Varatharajalu and Kakuturu 2016, Albonico, Bisoffi et al. 2018). However, it has to be noticed that in many countries, among them China, although multiple clinical trials have been carried out (Wen, Li et al. 2003, Wen, Yan et al. 2008), ivermectin is not yet available for human use and can be only used as veterinary medicine (Steinmann, Zhou et al. 2008).

1.5.7 Control

Strongyloidiasis has drawn some attentions in recent years from both researchers and public health authorities. In 2011, the strongyloidiasis Information Sharing Platform (https://ezcollab.who.int/ntd/strongyloidiasis) was established by the Department of Control of Neglected Tropical Disease of WHO (Bisoffi, Buonfrate et al. 2013). Later in 2016, an

international research network on strongyloidiasis has been launched (StrongNet) (Albonico, Becker et al. 2016). Both of the two networks aim to connect scientists, encourage them to share and discuss information, and consequently provide knowledge for strongyloidiasis diagnosis, treatment and public health control strategies.

With these efforts, strongyloidiasis has been recognized as one of the major public health problems. In 2017 ivermectin was added to the WHO essential medicine list for strongyloidiasis treatment. However, there is still no global public health strategy for controlling strongyloidiasis. Preventive chemotherapy with ivermectin has shown positive effects against strongyloidiasis, but it is not yet recommended by WHO (Albonico, Bisoffi et al. 2018). Therefore, efforts are still needed, to develop a strongyloidiasis control and prevention strategy for public health, and to integrate it into the WHO's STH control programs.

1.6 Hookworm infection in humans

1.6.1 Hookworm

Hookworm is one of the main soil-transmitted helminths beside roundworm (*Ascaris lumbricoides*) and whipworm (*Trichuris trichiura*), infecting 576-740 million people in the world. The two main hookworm species infecting humans are *Ancylostoma duodenale* and *Necator americanus*, which cause ancylostomiasis and necatoriasis, respectively (Bethony, Brooker et al. 2006). Similar as *S. stercoralis* infection, most hookworm infections are asymptomatic or associated with only mild gastrointestinal symptoms. The main clinical manifestations of hookworm infection are iron-deficiency anemia and protein malnutrition caused by the chronic intestinal hemorrhage. Chronic infection can lead to the intellectual and cognitive impairments of children (Hotez, Bethony et al. 2005).

Similar as *S. stercoralis*, hookworms are mostly prevalent in tropical and sub-tropical areas in Asia, sub-Saharan Africa, middle and South America (figure 8). *N. americanus* is the most common hookworm in humans, whereas *A. duodenale* has a more scattered focal distribution in particular environments (Hotez, Bethony et al. 2005).

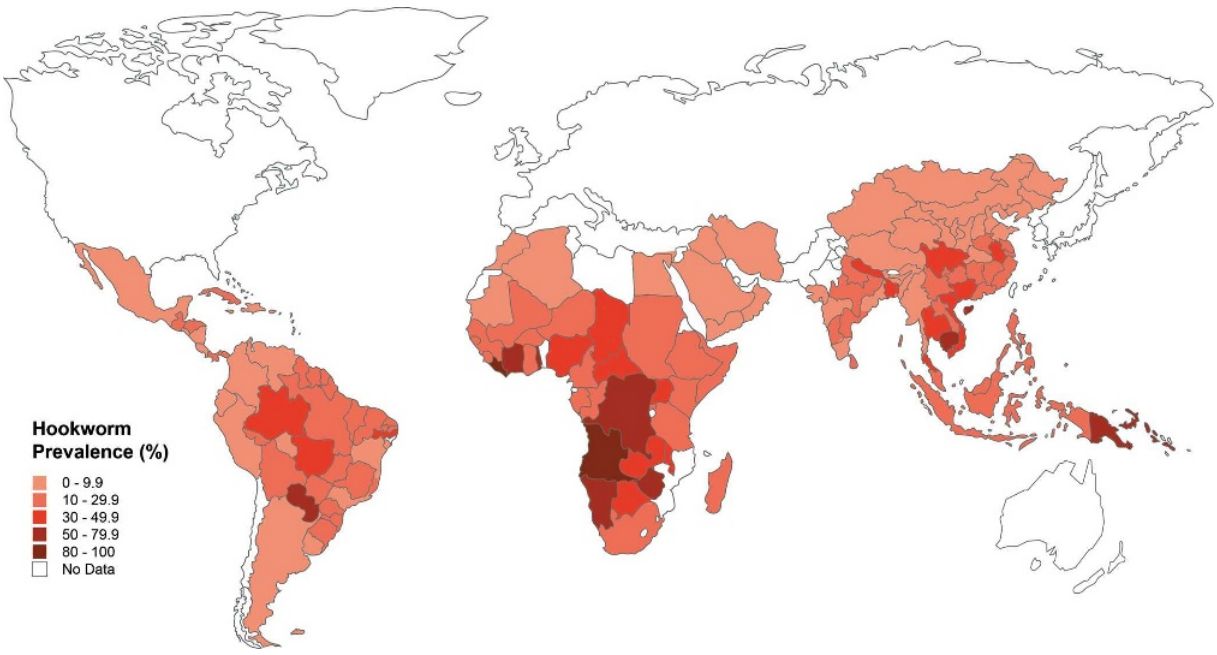

Figure 8. Worldwide prevalence of hookworm infection (Hotez, Bethony et al. 2005)

The transmission route of hookworms is also similar to *S. stercoralis*. Hookworm eggs are brought to environment by defecation, and hatch to rhabditoform L1 under favorable conditions. After two molts, they develop to filariform L3, which are capable of infection. People are infected by direct contacting contaminated soil such as walking barefoot. The hookworm L3 can penetrate human skin and migrate through the vascular system to the lungs, and then coughed up and swallowed by the host. After that they pass the esophagus, arrive the intestine, molt twice and develop into adult parasitic female and males. The parasitic adults mate in the host, and produce large amounts of eggs (9,000 - 10,000 eggs/day for N. americanus and 25,000 - 30,000 eggs/day for A. duodenale), which are released to the environment through defecation (figure 9) (Hotez, Brooker et al. 2004, Hotez, Bethony et al. 2005, Bethony, Brooker et al. 2006).

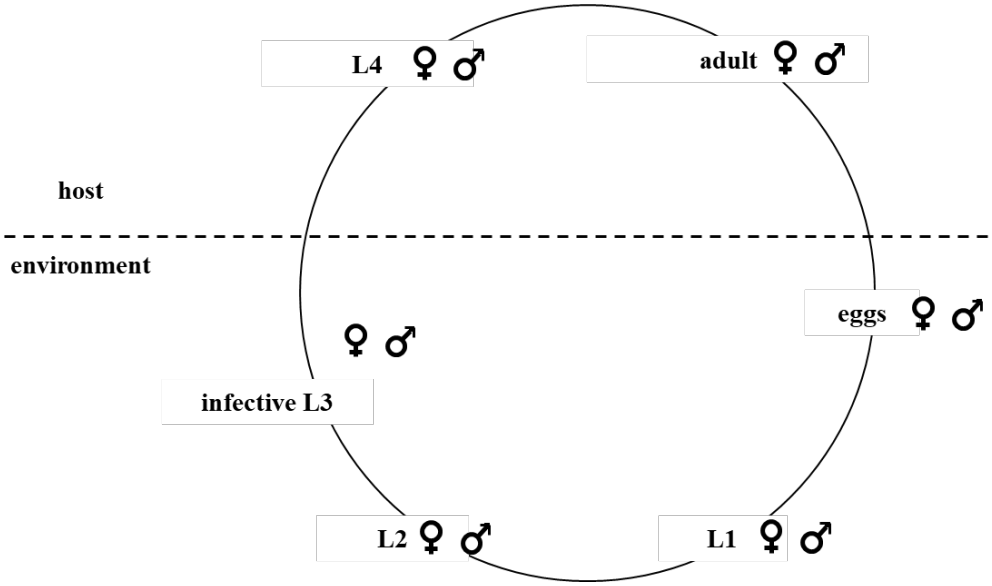

Figure 9. Life-cycle of hookworms (Zhou, Fu et al. 2019).

Diagnosis of hookworm infection mainly depends on the identification of eggs by microscopic examination of stool samples, but the eggs of *N. americanus* and *A. duodenale* are morphologically indistinguishable. To identify the two species, either the hookworms need to be cultured until the L3 stage, which are then morphological differentiable, or molecular diagnostic methods may be used (Hotez, Brooker et al. 2004). cAMP dependent protein kinase, ITS (internal transcribed spacer) of rDNA, and *cox1* gene have been used as molecular markers for hookworm identification (Gasser, Stewart et al. 1996, Hawdon 1996, Hasegawa, Modry et al. 2014).

1.6.2 Hookworm co-infection with *S. stercoralis*

Hookworm co-infections with *S. stercoralis* are often found because the distribution and the transmission routes of these two parasites are comparable. Co-infection has been reported in many countries including Argentina (Echazu, Juarez et al. 2017), Brazil (Ines Ede, Souza et al. 2011), Cambodia (Forrer, Khieu et al. 2018), Tanzania (Knopp, Salim et al. 2014), Cote d'Ivoire (Becker, Sieto et al. 2011), Ghana (Yelifari, Bloch et al. 2005) and Thailand (Jongwutiwes, Charoenkorn et al. 1999).

Normally, hookworms and *S. stercoralis* can be differentiated by their morphology at different stages of life cycle. Hookworms require a few days in the environment for the eggs to hatch and develop to larvae, whereas *S. stercoralis* larvae hatch inside hosts and can be found in fresh stools. In addition, free-living adults and iL3s of *S. stercoralis* can be easily

distinguished from hookworm larvae by body size and morphology. However, at times when young larvae of both worms are present in the stool sample, it is difficult, but for experts possible, to distinguish them based on morphology. Such situations can occur at less than one day of culture, when the hookworm larvae hatch under suitable conditions and the *S. stercoralis* larvae have not developed into iL3s or adult yet, or if the stool samples are left for several days, such that *S. stercoralis* larvae reproduced by free-living adults are already present (Jongwutiwes, Charoenkorn et al. 1999). In these circumstances, PCR-based molecular methods are useful, because they are highly specific and can simultaneously detect multiple helminths.

1.7 Aim of this thesis

Strongyloides is a genus of parasitic nematodes with several fascinating features such as switching between sexual free-living and asexual parasitic generations. It is also of medical and veterinary concern. Many basic biological and parasitological questions of *Strongyloides* spp. remain open. In this thesis I mainly pursued the three projects described below including field work and laboratory experiments:

1.7.1 Zoonotic potential, genetic variation and reproduction of *S. stercoralis*

S. stercoralis is a human parasite but natural infection in dogs has been reported. It was a long-lasting debate whether *Strongyloides* spp. found in dogs are human pathogenic *S. stercoralis* and whether strongyloidiasis is a zoonotic disease. Earlier study from our group has shown that *S. stercoralis* with different 18S rDNA haplotypes co-occurred in the same host in Cambodia, implying the worms had the chance to mate in the recent past. However, no hybrids between different 18S rDNA haplotypes were found, suggesting these different haplotypes representing reproductively isolated populations. Besides, it was unclear whether the free-living adults of *S. stercoralis* reproduce sexually, like other *Strongyloides* spp. do, or they reproduce by sperm-dependent parthenogenesis (pseudogamy), as suggested by old studies based on cytology. The aim of this part of thesis was to isolate *S. stercoralis* from humans and dogs living in the same households in northern Cambodia, genetically and genomically characterize individual *S. stercoralis*, evaluate the taxonomic status of the different 18S rDNA haplotypes, therefore determine if dogs are reservoirs for human infective *S. stercoralis,* and also elucidate if the free-living generation reproduces sexually or clonally. This project was conducted in collaboration with the Cambodian Ministry of Health and the Swiss Tropical and Public Health Institute.

1.7.2 Identification of a non-sexual population of *S. stercoralis*

In the project described above (1.7.1), the existence of different 18S rDNA HVR-I haplotypes in *S. stercoralis* in humans and the absence of hybrids between them has been confirmed. However, the nuclear and mitochondrial phylogenies did not support the separation of different 18S rDNA HVR-I haplotypes into different taxonomic groups. In order to test if the different haplotypes also exist in other geographic locations and to further evaluate their taxonomic status, I organized a sampling excursion to collect and analyze *S. stercoralis* from humans in the rural area of Guangxi, southwestern China. In this project, I also attempted to

sample and characterize the hookworms present in the same area for comparison, because they share important aspects of their biology and infection routes with *S. stercoralis*, although phylogenetically rather distant. This project was done in collaboration with Guangxi Medical University.

1.7.3 Genetic recombination in *S. papillosus* and the improvement of its genome

It had been shown that the large chromosome of *S. papillosus* was formed by the fusion of the X chromosome and one autosome. Interestingly, preliminary study in our lab had suggested that meiotic recombination does not happen in the X-derived region (diminished region) in *S. papillosus*, even in females. If true, this would imply the sex chromatin in *S. papillosus* reproduces essentially asexually, which would be expected to have profound genomic consequences. The aim of this project was to test the hypothesis that meiotic recombination is absent in the diminished region of *S. papillosus*. To do so, I took two complementary approaches. First, in order to detect recombination events directly, I crossed single females and males, analyzed the inheritance of molecular markers in their progeny and calculated the recombination frequencies between different markers. Second, in order to assess recombination at the population level, I analyzed the linkage disequilibrium (LD) by sequencing the genomes of individual *S. papillosus* isolated from a wild population.

However, the rather poor quality of the published *S. papillosus* genome was limiting for some of the analyses. For example, the physical distances between the markers used were unknown. Therefore, another aim of this project was to improve the genome assembly of *S. papillosus*. To this end, I made use of an iso-female isolate, which has a less complex genetic background than the outbred isolate the published sequence was based on and I combined several sequencing technologies including Illumina short read sequencing and PacBio long read sequencing.

2. Result and Discussion

2.1 Different but overlapping populations of *Strongyloides stercoralis* in dogs and humans - Dogs as a possible source for zoonotic strongyloidiasis

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2.1.1 Synopsis

It is well known that *Strongyloides stercoralis* is the major causative agent of human strongyloidiasis, but some fundamental questions about its epidemiology and biology remain open. For instance, there was a long debate whether *Strongyloides* spp. found in dogs in the wild are human pathogenic *S. stercoralis* and whether dogs are a source of zoonotic transmission of *S. stercoralis*. Also, it was not clear whether the free-living generations of *S. stercoralis* undergo sexual reproduction, or, despite the presence of males, they reproduce asexually. It had been speculated that the sperm is only required for triggering parthenogenetic reproduction but do not contribute genetically (pseudogamy).

To address these questions, we isolated *S. stercoralis* from humans and dogs at the same time points and locations in northern Cambodia. Firstly, we compared the hyper variable region I and IV (HVR-I and HVR-IV) sequences of the 18S rDNA (*SSU*) of individual *S. stercoralis* worms isolated from two different villages. HVRs have been widely used for taxonomy of nematodes in general and *Strongyloides* spp. in particular. We found multiple sequence variants of the HVRs and we defined five HVR-I haplotypes (I, II, III, IV, and V) and two HVR-IV haplotypes (A and B). But strikingly, we never detected any hybrids between different HVR haplotypes. While the haplotype A was found in human and dog derived *S. stercoralis*, the haplotype B, which had never been described before, was only found in the *S. stercoralis* isolated from dogs, indicating dogs carry two different populations, possibly sub-species or species of *S. stercoralis.* Secondly, for subsets of *S. stercoralis*, we sequenced the mitochondrial *cox1* gene and the whole genome. Phylogenies based on these data support the

separation of the two HVR-IV populations. Taking together, this illustrates the potential of dogs as a reservoir for zoonotic transmission of human pathogenic *S. stercoralis*, suggesting that dogs should be treated along with their owners in order to reduce the exposure of people to *S. stercoralis*.

Meanwhile, the genetic isolation of different HVR-I haplotypes was not supported by either the *cox1* or the whole-genome-based phylogeny, suggesting they do not represent different species or sub-species, although we detected no hybrids between different HVR-I haplotypes. These findings brought up the hypothesis that asexual reproduction, which would not allow the genetic exchange between different HVR genotypes, could be the normal reproduction mode of *S. stercoralis* in our study area. Reproduction might be asexual for two possible reasons: (1) The *S. stercoralis* in our study area use only the inherently clonal direct/homogonic and auto-infective life cycles. (2) Reproduction in the free-living generation is pseudogamic as explained above. The first appeared rather unlikely given that we observed large numbers of free-living animals of the indirect life cycle both sexes and they readily produced progeny. Therefore, we took two approaches to investigate the mode of reproduction of free-living *S. stercoralis*. We first genotyped mothers and their progeny isolated in the field at two molecular markers. The result showed that the progeny were not the products of clonal reproduction, indicating that a father must have passed genetic material to them. Second, with a *S. stercoralis* laboratory strain, we crossed individual females and males and genotyped the parents and the progeny. The result showed Mendelian inheritance with equal contribution by both parents. With these approaches, we clearly demonstrated that sexual reproduction does occur and it is likely the dominant reproduction model in the free-living generation of *S. stercoralis*, in both, the laboratory isolate and the wild population in our study area.

2.1.2 Own contribution

Tegegn G. Jaleta, Fabian Schär and I collected the samples from dogs and humans in Cambodia. Tegegn G. Jaleta and I genotyped individual worms at rDNA and mitochondrial gene, analyzed the sequences and I calculated phylogeny. I prepared genomic libraries and sequenced the whole genome of individual worms. Felix M. Bemm calculated the phylogeny based on the whole genome sequences. Tegegn G. Jaleta and I performed the crossing experiment, genotyped the individual worms from crossing and analyzed the sequences. Tegegn G. Jaleta, Adrian Streit and I wrote the manuscript.

2.2 Characterization of a non-sexual population of *Strongyloides stercoralis* with hybrid 18S rDNA haplotypes in Guangxi, Southern China

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2.2.1 Synopsis

In a previous study (described in 2.1), we had identified several HVRs haplotypes of *S. stercoralis* in the rural region of north Cambodia. Both mitochondrial and nuclear phylogenies suggested that the different HVR-I haplotypes do not represent separate species/sub-species, despite the fact that we detected no hybrids between different HVR-I haplotypes. In this follow up study we wanted to test, if the same HVRs haplotypes are also present in other regions/countries in the world and if they can interbreed. In the Guangxi Province, Southern China *S. stercoralis* was known to be present due to the occurrence of clinical cases of strongyloidiasis but no HVRs haplotypes had ever been determined in this area.

In this study, we isolated *S. stercoralis* from people living in a village with a high incidence of parasitic helminths and from patients from local clinics in Guangxi. We determined nuclear (*SSU* and for a subset whole genome) and mitochondrial (*cox1*) DNA sequences of individual *S. stercoralis* and compared them with the published *S. stercoralis* datasets from Cambodia, Myanmar and Japan. For comparison, we also analyzed the hookworms isolated from the same host population, as hookworms share the infection route with *S. stercoralis*. We found that the *S. stercoralis* individuals isolated in our study area were much more closely related with each other than were the *S. stercoralis* in Cambodia, whilst the hookworms isolated from the same location in Guangxi showed rather high genetic diversity, indicating a recent common infection source for *S. stercoralis* but not for hookworms.

Interestingly, we found almost all *S. stercoralis* isolated in this study to be hybrids of different 18S rDNA HVR-I and HVR-IV haplotypes, which is completely contrary to what had been described in earlier studies where no or rare 18S hybrids had been found. Meanwhile, we also noticed that except 3 males, all *S. stercoralis* isolated were infective larvae, indicating the *S.*

stercoralis in our study area predominantly, if not exclusively reproduce clonally through the direct/homogonic and/or auto-infective life cycles. To test this hypothesis, we looked for genomic hints of asexuality. Firstly, we compared the heterozygosity of individual *S. stercoralis* isolated from different countries, and we found that the Chinese samples are the most heterozygous population. Secondly, we calculated the ratio of non-synonymous and synonymous mutations (dN/dS), which is expected to be elevated among mutations accumulated under reduced selection pressure due to asexual reproduction. Overall, in the Chinese samples the dN/dS was comparable to the corresponding ratios of sexually reproducing nematode species *C. elegans* and *P. pacificus*. However, we found that putatively new mutations (alleles not shared with the Cambodian samples) were slightly, but significantly, more frequently non-synonymous compared with old mutations (alleles shared with the population in Cambodia). Thirdly, we found that the apparent phylogenetic relationship between individual *S. stercoralis* from China changed depending on the genomic region considered, indicating that recombination occurred in the relatively recent past. Our findings suggest that most new alleles (mutations) in Chinese samples were acquired in sexual populations and the high heterozygosity was caused by recent hybridization events that rendered the population predominantly asexual.

2.2.2 Own contribution

I was responsible for the general organization of the field study. Together with Adrian Streit, Xiaoyin Fu and Dengyu Liu I designed the study and collected the samples from humans in China. With help from Adrian Streit and Pei Pei I isolated individual worms in China. I genotyped individual worms at the rDNA and the mitochondrial gene *cox1*, analyzed the sequences and calculated the phylogeny. I cloned the 18S rDNA sequences of individual worms and analyzed the sequences. I prepared genomic libraries and sequenced the whole genome of individual worms. Christian Rödelsperger and I analyzed the whole genome data. Adrian Streit and I wrote the manuscript with input from Christian Rödelsperger and the other co-authors.

2.3 Sexual and non-sexual reproduction in the same genome - absence of meiotic recombination in the sex chromosome of *Strongyloides papillosus* (un-published portion of the thesis)

2.3.1 Introduction

Meiotic recombination occurs naturally during the meiosis in eukaryotes. It allows the exchange of genetic material between homologous chromosomes, generating novel combinations of alleles which can be passed to the offspring. Such genetic recombination is believed to be beneficial because it can combine the beneficial mutations arisen independently on different homologous chromosomes, and separate the deleterious and beneficial mutations on the same chromosome. Therefore, it can facilitate the fixation of beneficial mutations and the purging the deleterious mutations (Webster and Hurst 2012). Inhibition, or loss of meiotic recombination has been described in the sex chromosomes such as Y or W chromosomes, where the suppression of recombination leads to the maintenance of the male specific region, and this is associated with a major loss of genes as well as a size reduction of the sex chromosome (Graves 2006, Bergero and Charlesworth 2009). In asexual (mitotic parthenogenetic) producing organisms, meiotic recombination is absent in all chromosomes, resulting in the accumulation of mutations and rapid divergence of the homologous chromosomes (Birky 1996, Archetti 2004).

The nematode genus *Strongyloides* contains more than 40 described species, which can parasitize various vertebrates, including humans. It has a unique and rather complex life cycle consisting of parasitic and free-living generations (Viney and Lok 2015). In brief: The infective third stage larvae (iL3), which are all females, infect the host by skin penetration. After migration through the blood circulation system to the lungs, the larvae are coughed up and swallowed by the host, and therefore enter the digestion system and eventually arrive the small intestine where they mature and become parasitic adults. The parasitic adults reproduce parthenogenetically and their eggs or young larvae, depending on the species, are brought to the environment by defecation of the hosts. The progeny of parasitic females have 3 developmental options: (1) They become females, develop into iL3s and search for a new host (direct/homogonic cycle); (2) They become females or (3) males and develop into free-living, non-infective adults (indirect/heterogonic cycle). The free-living females and males reproduce in the environment. In most *Strongyloides* species, their progeny consist of only females, which develop into iL3s and need to find the next host to continue their life cycle (figure 3).

Some *Strongyloides* species, such as the rat parasite *S. ratti* and the human parasite *S. stercoralis*, have 2 pairs of autosomes and employ the XX/XO sex determination, where females have two and males have only one X chromosomes (Nemetschke, Eberhardt et al. 2010, Hunt, Tsai et al. 2016). Other *Strongyloides* species, such as the sheep parasite *S. papillosus*, has a different karyotype: The X chromosome is fused with one of the autosomes, resulting in a pair of large chromosomes (L) and a pair of medium chromosomes (M) in females. Where in males, an internal portion of one L chromosome is removed and this eliminated region corresponds evolutionarily to the X chromosome of *S. ratti* (figure 5). This has been described as chromatin diminution and functionally restores an XX/XO sex-determining system. The breakpoints between eliminated and the flanking non-eliminated regions have been tentatively identified by unpublished previous studies in our lab but await confirmation. It has also been shown that only the intact L chromosome is present in mature sperm, which explains why the progeny of the free-living generation are all females (Albertson, Nwaorgu et al. 1979, Nemetschke, Eberhardt et al. 2010, Kulkami 2015). Old literatures based on cytology had suggested the free-living generation of *Strongyloides* spp. reproduce through sperm dependent parthenogenesis (pseudogamy). However, more recent studies based on molecular evidences showed that the reproduction is sexual at least in *S. ratti* (Viney 1994), *S. papillosus* (Eberhardt, Mayer et al. 2007), *S. vituli* (Kulkarni, Dyka et al. 2013) and *S. stercoralis* (Jaleta, Zhou et al. 2017) (part of this thesis).

In *S. ratti*, which has a free X chromosome, a genetic linkage map has been constructed by analyzing 74 molecular markers (SNPs), and meiotic recombination was observed on all three chromosomes. While recombination is obviously not possible on the X chromosome in males, it does occur in both sexes on the autosomes. In females, the recombination frequency on the X chromosome may be reduced, compared with the autosomes, because the X chromosome appears genetically shorter than the autosomes on the genetic map (Nemetschke, Eberhardt et al. 2010). In *S. papillosus*, meiotic recombination does occur in both sexes on the M chromosome and on the autosomal parts of the L chromosome (Nemetschke, Eberhardt et al. 2010). Interestingly, in the earlier experiments all four diminished markers appeared perfectly linked also in females, which suggested that recombination is absent in the diminished region also in females (Nemetschke, Eberhardt et al. 2010). If true, this would be expected to have profound consequences on this genetic region and it would provide us with an opportunity to study recombining and non-recombining genomes within a single species. However, due to the relatively low quality of genome assembly of *S. papillosus*, the physical distance between the analyzed markers is unknown. Thus, it was not possible to determine if this finding was

true or rather an artifact due to an unfortunate distribution of the rather few genetic markers analyzed in this region.

In order to address this question, we first improved the *S. papillosus* genome assembly using a combination of short read (Illumina) and long read (PacBio) sequencing and an iso-female isolate, which is genetically less complex than the outbred isolate the published (Hunt, Tsai et al. 2016) sequence was derived from. Secondly, we demonstrated with genetic crosses of individual females and males using multiple molecular genetic markers covering most of the diminished region, that recombination occurs only very rarely, if at all, in the X-derived region within the laboratory strain. Thirdly, we show that in a wild population there is very strong linkage disequilibrium over the entire diminished region, arguing that also in the wild population recombination is rare or absent in this part of the genome.

2.3.2 Materials and Methods

2.3.2.1 *S. papillosus* isolates

The Isolates LIN (in this thesis referred to as LIN1 for clarity) and LIN2 were isolated from naturally infected sheep at the agricultural experimental field station “Oberer Lindenhof” (E9°18'14" N48°28'28") of the University of Hohenheim, Stuttgart, Germany and maintained in rabbits and as frozen stocks as described (Eberhardt, Mayer et al. 2007). LIN1 was isolated in May 2005 (Eberhardt, Mayer et al. 2007), LIN2 was isolated in September 2015 as described in 2.3.2.2. QA362 is an iso-female isolate derived from LIN1 by infection of a lamb with a single iL3 (Nemetschke, Eberhardt et al. 2010) and was also maintained in rabbits and as frozen stock.

2.3.2.2 Sampling *S. papillosus* from the wild

Fecal samples were taken directly from the rectum of 110 lambs that had been born during the spring 2015 lambing season and had been dewormed in June 2015 and 18 lambs that were born late and had never been dewormed. Each sample was immediately mixed with an equal volume of sterile sawdust, moisturized and distributed into 9 cm petri dishes and incubated at 25°C for 2 days. Then the worms were isolated using standard Baermann funnels for 2-3 hours at room temperature (Lok, 2007). The resulting worms were visually inspected and searched for *S. papillous* iL3s and free-living adults.

The majority of *S. papillosus* found were iL3s. About 150 iL3s were used to infect a rabbit to start a culture resulting in the isolate LIN2 described above. As for the free-livings, we managed to isolated 78 adult females and 46 adult males from 44 different sheep. These worms were transferred individually into PCR tubes with 10 µl water and frozen.

2.3.2.3 Crossing the free-living *S. papillosus*

Free-living young adults of LIN1 and LIN2 were collected from 1-day cultures of infected rabbit feces by the Baermann funnel technique, as described above. One virgin female and one male (from the same isolate) were transferred into one well of the 96-well-plate with 40 µl tap water and 10 µl of water from a Baermann funnel. The plate was incubated at 25°C overnight in a water-saturated atmosphere to allow the couple to mate. On the next day, the plates were visually inspected. If a female contained developing embryos in the uterus, indicating that the mating was successful, the corresponding male was transferred into a PCR tube containing 10 µl of water and frozen for later use. The female was washed with PBS supplemented with antibiotic (0.05 mg/ml streptomycin and 0.048 mg/ml penicillin) to minimize the bacteria from feces and transferred to fresh NGM plates spotted with *E. coli* OP50 as the food source (Stiernagle 1999). The female was inspected daily until it ceased laying eggs, which usually took three days. Then the female was processed like the male. After the eggs hatched, the larvae, which were at the L1 or L2 stage, were transferred individually into PCR tubes with 10 µl water and frozen.

2.3.2.4 Single worm genotyping

DNA of individual worms from crossing was prepared by single worm lysis as follows: Individual worms in 10 µl water were frozen and thawed 3 times with liquid nitrogen. Then 10 µl 2X lysis buffer (20 mM Tris-HCl pH 8. 3, 100 mM KCl, 5 mM $MgCl_2$, 0.9% NP-40, 0.9% Tween 20, 240 µg/ml Proteinase K) were added. The samples were incubated at 65°C for 2 hours followed by for 15 minutes at 95°C in order to inactivate the proteinase K. The worm lysates were stored at -20°C until use.

2 µl of single worm lysate were used as template for PCR amplification with *Taq* DNA polymerase (M0267, New England BioLabs) and the primers listed in table 2 according to the manufacturer's instructions. Cycling protocol: An initial denaturation step at 95°C for 30 sec was followed by 40 cycles of denaturation at 95°C for 20 sec, annealing at 55°C for 15 sec, extension at 68°C for 90 sec and a final extension step of 5 minutes at 68°C.

1-2 µl of the PCR product were used as template for sequencing. The sequencing reactions were done using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following to the manufacturer's instructions. The reactions were submitted to the in-house sequencing facility at the Max Planck Institute for Developmental Biology at Tübingen for electrophoresis and base calling. The sequences were analyzed using the Lasergene package version 12 (DNAStar, Inc., Madison, WI USA). In order to detect homozygous and heterozygous worms the chromatograms were visually inspected for ambiguous signals at the known polymorphic sites.

Table 2. Primers used for genotyping *ytP* markers

Markers	Forward primer	Reverse primer	Sequencing primer
*ytP*129[a]	LN3162 ATCTCGGCATCCAGTGATTC	LN3163 TCCTAGTCTTCGCGCTACATC	LN3162 ATCTCGGCATCCAGTGATTC
*ytP*130[a]	LN3509 CCAGACCCAAAAATCAGAATC	LN3510 ATTCCAACACGGGCTACAAG	LN3509 CCAGACCCAAAAATCAGAATC
*ytP*295[b]	ZS7087 TCCTGAGATGCACCTTTTATTCA	ZS7088 TCACAATTCGAAGTTCCAGAA	ZS7088 TCACAATTCGAAGTTCCAGAA
*ytP*51[a]	LN2758 CTGGAGAGGTAAAAGCTGCTG	LN2759 ATCTTGGCAACCTCCAAAAC	LN2759 ATCTTGGCAACCTCCAAAAC
*ytP*134[a]	LN3361 TGAAACAGAGGCTATTATGTTTTGA	LN3360 TGAAGATATCCGAGAATCGTTG	LN3361 TGAAACAGAGGCTATTATGTTTTGA
*ytP*135[a]	LN3530 CGTTCATTTTGTTGTGATGGA	LN3531 GCACGTGCTCGTGGTATTAG	LN3530 CGTTCATTTTGTTGTGATGGA
*ytP*214[b]	AK5016 CGATATGGCAATTGTTTATGGA	AK5017 TGACGCAATCTGTACTGAGGA	AK5017 TGACGCAATCTGTACTGAGGA
*ytP*244[b]	AK5336 CTGAATTCACGGGGAAAGAT	AK5337 GGATTTTAGCATGTTGGAGCA	AK5336 CTGAATTCACGGGGAAAGAT
*ytP*291[b]	ZS7073 TTGAATGTGCCCTCCCAGTT	ZS7074 ATCATCGTCGTTCCAATGCC	ZS7073 TTGAATGTGCCCTCCCAGTT

[a] marker described in (Nemetschke, Eberhardt et al. 2010).

[b] unpublished markers. The marker sequences and polymorphic positions can be found in the Appendix 4.1.

2.3.2.5 Whole genome sequencing of *S. papillosus* individuals and linkage analysis

24 free-living *S. papillosus* adults isolated from sheep as described in 2.3.2.2 were used for whole genome sequencing and linkage analysis. DNA of individual worms was prepared using the MasterPure DNA Purification Kit (Epicenter MC85201) according to the manufacturer's instructions, and the DNA was stored frozen in 10 µl of TE buffer. Genomic libraries of individual worms were prepared with Clontech Low Input Library Prep Kits

(Takara Bio, USA) following the manufacturer's protocol. Libraries were submitted to the in-house sequencing facility for sequencing on an Illumina HiSeq 3000 instrument (150 bp paired-end).

Raw reads were mapped to the *S. papillosus* reference genome (version PRJEB525.WBPS11) (Hunt, Tsai et al. 2016) using bwa mem with default settings (Li and Durbin 2010). For comparison, we in parallel also analyzed the published whole genome sequences of 23 *S. stercoralis* individuals from Cambodia (Jaleta, Zhou et al. 2017)part of this thesis) using the *S. stercoralis* genome (version PRJEB528.WBPS11) (Hunt, Tsai et al. 2016) as reference. Aligning of the sequence reads, SNP calling and the analysis of linkage disequilibrium (LD) pattern were done by Christian Rödelsperger from our department. The LD was calculated as average r^2 measuring physical separation between 100 kb windows across the scaffolds of the published assembly genome (Hunt, Tsai et al. 2016).

2.3.2.6 Whole genome sequencing of *S. papillosus* for *de novo* genome assembly

Preparing *S. papillosus* for sequencing

Feces from rabbits infected with the iso-female isolate QA362 were cultured at 25°C for 1 day and 7 days for collecting free-living adults and iL3s, respectively. The worms were collected with Baermann funnels and washed two times with water. iL3s were transferred to 1.5 ml EP tubes and approximately 4 ml worm pellet were collected. Free-living adults were transferred to a watch glass and females and males were separated by hand picking. Approximately 10000-20000 females and males were collected. The worms were allowed to sediment and as much liquid as possible was removed. The worm pellets were stored at -20°C until use.

DNA preparation, library construction and PacBio sequencing

Approximately 2 ml iL3 worm pellet was homogenized into a fine powder with a pestle and grinder, while keeping the materials frozen by regular addition of liquid nitrogen. Then the liquid nitrogen was allowed to evaporate and the high molecular weight (HMW) genomic DNA was isolated immediately, by using the Qiagen Genomic-tip kit (500/G) following the manufacturer's instructions. The size of DNA was examined by pulsed field gel electrophoresis. About 60 µg of HMW DNA were submitted to the in-house sequencing facility for library preparation and sequencing on a PacBio Sequel platform.

DNA preparation, PCR-free library construction and Illumina sequencing

2 µg genomic DNA of iL3s, males and females (isolated from 10000-20000 worms each) were prepared using MasterPure DNA Purification Kit (Epicenter MC85201) following the manufacturer's instructions. The genomic libraries were prepared using Illumina TrueSeq DNA PCR-Free kit following the manufacturer's instructions. Libraries were submitted to in-house sequencing facility on an Illumina MiSeq instrument (250 bp paired-end).

De novo genome assembly

The *de novo* assembly was done by Felix M. Bemm from department VI of the Max Planck Institute for Developmental Biology at Tübingen. Since I did not contribute to this part, it is not described in detail in this thesis.

2.3.2.7 Determination of the diminished and non-diminished regions

The short reads derived from free-living females and males were mapped to the new assembly and used to assign the diminished and non-diminished regions. The read coverage of each scaffolds was compared between females and males using deepTools bamCompare with the default setting (Ramirez, Ryan et al. 2016). A contig was assigned to "sex chromosome" (diminished region) if its mean coverage in males was 0.5 - 0.75 times that of in females, whereas it was assigned to "autosome" (non-diminished region) if the mean coverage in males was 0.9 - 1.3 times that of in females. Contigs which did not fall in any of these classes were assigned as "undetermined".

2.3.2.8 Accession numbers

The whole genome sequences of individuals for the linkage analysis (described in 2.3.2.5) are available from GenBank under the accession number PRJNA531114. The whole genome sequences (Illumina and PacBio) for genome assembly (described in 2.3.2.6) are available from GenBank under the accession number PRJNA531238. The new genome assembly of *S. papillosus* (version draft_pacbio_201804) (described in 2.3.2.7) is available from GenBank under accession number SRSI00000000.

2.3.2.9 Ethics Statements

All animal experiments were in accordance with the German Animal Protection Law (Tierschutzgesetz), the German Animal Protection Laboratory Animal Ordinance (Tierschutz-Versuchstierverordnung) and EU Directive 2010/63/EU on the protection of animals used for

scientific purposes. The procedures for animal maintenance and experiments performed at the Max Planck Institute for Developmental Biology were ethically and administratively approved by the local govermental authorities in charge (Regierungspräsidium Tübingen, Anzeige vom 15. 07. 2015, AZ: 35./9185.82-5). The sample collection from sheep was approved by the animal welfare officer of the University Hohenheim (AZ: S 422/15 THY). For the analysis of the linkage disequilibrium of *S. stercoralis* pre-existing sequence data (Jaleta, Zhou et al. 2017) were re-analyzed and no new sampling of human subjects or dogs was conducted.

2.3.3 Results and Discussion

2.3.3.1 Improvement of the genome assembly of *S. papillosus*

12 Gb of long reads (N50 = 16,189 bp, longest read length = 94,723 bp, coverage = 198x) were generated on 2 SMRT cells on the PacBio Sequel platform. In addition, 25 Gb of 250 bp short reads were generated on the Illumina Miseq platform. The reads were assembled into 79 contigs (N50 = 1,445,102 bp) and the assembled genome size is 58.2 Mb. This represents a significant improvement compared to the published genome which has 4,353 contigs (N50 = 86 kb) (Hunt, Tsai et al. 2016). The details and the comparison with the *S. ratti* and *S. stercoralis* assemblies are shown in table 3.

Table 3. Genome assemblies of *S. papillosus*, *S. ratti* and *S. stercoralis*.

	Assembly size (Mb)	Number of scaffolds	Maximum scaffold length (Mb)	N50 of scaffolds (kb)	N50 number
S. papillosus (old)[a]	60.2	4 353	1.7	68	129
S. papillosus (new)[b]	58.2	79	4.9	1 445	12
S. ratti[a]	43.1	115	16.8	11 700	2
S. stercoralis[a]	42.6	675	5.0	431	16

[a]: assemblies from (Hunt, Tsai et al. 2016).

[b]: assembly from this thesis. Notice in this assembly numbers in the table are referring to contigs instead of scaffolds.

N50 of scaffolds: 50% of the total assembled genome are in scaffolds of this size or larger.

N50 number: the minimal number of scaffolds that contain 50% of the total genome.

The completeness of the assembled genome was evaluated using the Benchmark of Universal Single-Copy Orthologs (BUSCO) with the nematode dataset containing 982 genes which are present in more than 90% nematode species as single-copy orthologs (Simao, Waterhouse et al. 2015). BUSCO quantitatively evaluates the completeness of a genome assembly by examining the presence/absence of these near-universal single-copy orthologs. For comparison, I also included the published genome assemblies of *S. papillosus*, *S. ratti* and *S. stercoralis* (Hunt, Tsai et al. 2016). The new assembly of *S. papillosus* has slightly less single-copy genes compare to the old version (71.0% vs. 73.4%), while the duplicated and fragmented genes numbers are comparable (table 4). The upcoming BioNano data will hopefully increase the continuity as well as the completeness of the *S. papillosus* assembly.

Table 4. BUSCO assessment of the new and old assemblies of *S. papillosus* in comparison with *S. ratti* and *S. stercoralis*.

	Single-copy (S)	Duplicated (D)	Fragmented (F)	Missing (M)
S. papillosus (old)[a]	721 (73.4%)	35 (3.6%)	70 (7.1%)	156 (15.9%)
S. papillosus (new)[b]	697 (71.0%)	35 (3.6%)	69 (7.0%)	181 (18.4%)
S. ratti[a]	744 (75.8%)	23 (2.3%)	70 (7.1%)	145 (14.8%)
S. stercoralis[a]	740 (75.4%)	30 (3.1%)	63 (6.4%)	149 (15.2%)

[a]: assemblies from (Hunt, Tsai et al. 2016).

[b]: assembly from this thesis.

2.3.3.2 Determination of the diminished and non-diminished region

Contigs were assigned to diminished or non-diminished regions as described in materials and methods (2.3.2.7). 9 contigs (14,038,911 bp) were assigned to the diminished region, 67 contigs (43,972,767 bp) were assigned to the non-diminished region and only 3 contigs (220,770 bp) were assigned as undetermined. For details see the Appendix 4.2 (xlsx file).

The coverage comparisons between the female and male samples are shown in figure 10. 7 out of the 9 contigs assigned to the diminished regions are larger than 1 Mb (C1-C7). The contig C4 contains a large diminished region and a small non-diminished region, the junction of which might indicate a break point. Noticeably, for the 3 diminished contigs (C1, C3, C7), an internal part shows non-diminished assignment which is flanked by two diminished parts. These are presumably assembling errors caused by for example repetitive sequences

bordering the internal and flanking parts. Therefore, this genome assembly is waiting to be corrected and improved with the upcoming BioNano sequences.

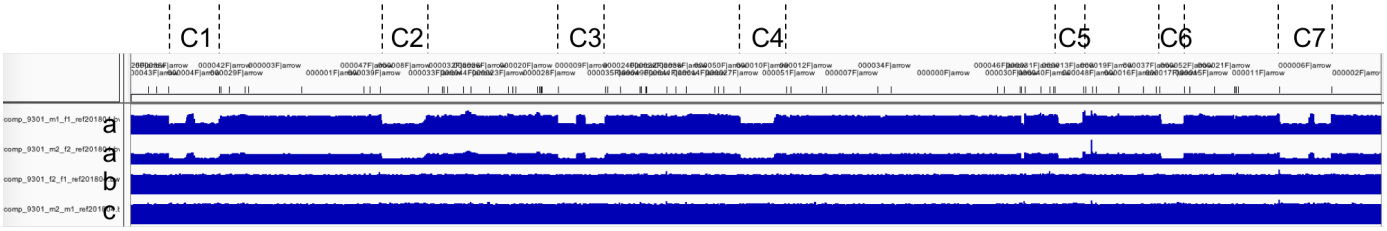


Figure 10. The read coverage comparisons between samples calculated as ratios per 50 bp window. Each bar indicates one contig and all 79 contigs representing the whole genome are shown.

a: between male and female, two replicates; b: between the two female replicates; c: between the two male replicates.

C1-7: the seven diminished contigs > 1 Mb. Dash lines indicate the contig boundary.

2.3.3.3 Crosses indicating the absence of recombination in the diminished region

Position of markers

Six diminished molecular markers (*ytP*51, *ytP*134, *ytP*135, *ytP*214, *ytP*244 and *ytP*291), each of which is located on a different contig and three non-diminished markers (*ytP*129, *ytP*130 and *ytP*295) were selected for the genetic analysis of crosses. It had been known that *ytP*129 and *ytP*130 locate on the L chromosome on opposite sides of the diminished region (Nemetschke, Eberhardt et al. 2010). Since the heterozygosity at *ytP*130 is rather low in our laboratory isolates, I used *ytP*295, which is on the same contig as *ytP*130 as an alternative option, in order to increase the number of informative crosses. The exact positions of the 9 markers on contigs are shown in table 5 and the presumed positions on the chromosome are shown in figure 11.

Table 5. The location of genetic markers on the new assembly

Marker	Chromatin region	Contig	Contig length (bp)	Position on contig (bp)
*ytP*129[a]	Non-diminished	000002F|arrow	2 538 622	2 269 450-2 269 875
*ytP*130[a]	Non-diminished	000025F|arrow	820 894	442 238-441 925
*ytP*295[b]	Non-diminished	000025F|arrow	820 894	666 016-666 408

*ytP*51[a]	Diminished	000008F|arrow	2 218 631	571 331-571 839
*ytP*134[a]	Diminished	000006F|arrow	2 423 176	733 561-732 890
*ytP*135[a]	Diminished	000017F|arrow	1 159 132	986 342-985 960
*ytP*214[b]	Diminished	000004F|arrow	2 370 071	2 366 879-2 366 476
*ytP*244[b]	Diminished	000009F|arrow	2 211 228	1 517 846-1 517 371
*ytP*291[b]	Diminished	000010F|arrow	2 145 945	997 355-997 612

[a] markers described in (Nemetschke, Eberhardt et al. 2010).

[b] unpublished markers. The marker sequences and polymorphic positions can be found in Appendix 4.1.

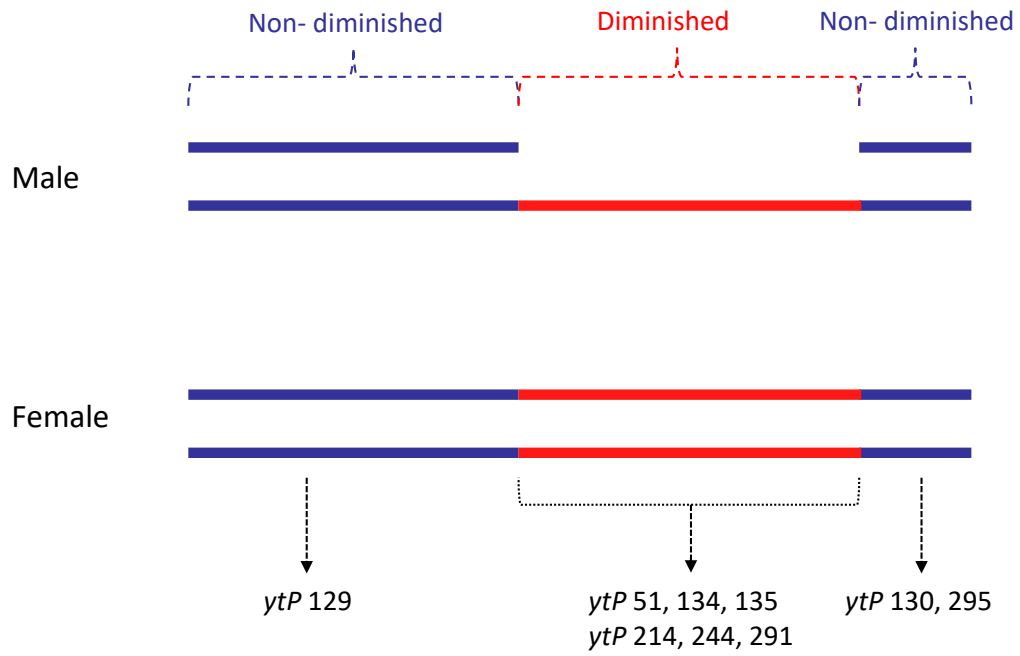


Figure 11. Presumed positions of markers on the L chromosome.

Recombination frequencies between markers

Crosses between single free-living females and males were conducted with the outbred isolates LIN1 and LIN2 as described in materials and methods (2.3.2.3). The crosses were set up "blind" and only the post mortem genotyping of the parents revealed if a particular cross was informative to detect recombination between two markers. The parents were first genotyped at all markers. Only progeny from informative crosses were genotyped at the particular informative markers. The genotypes of parents and their progeny at informative markers can be found in Appendix 4.3 (xlsx file).

The recombination frequencies between *ytP*129 and diminished markers, between *ytP*130 (or *ytP*295) and diminished markers, and among the diminished markers were calculated for each cross. If both parents were heterozygous (only possible in non-diminished markers), two possible recombination events (during spermatogenesis and oogenesis) could be detected in one progeny therefore each progeny was scored twice; if only one of the parents was heterozygous (appear in both non-diminished and diminished markers), each progeny was scored only once.

The recombination frequencies are shown in figure 12. In the non-diminished regions, numerous recombination events were detected at frequencies of 5.5% (42/762) between *ytP*129 and diminished markers and 8.6% (47/544) between *ytP*130 (or *ytP*295) and diminished markers. We also detected one recombination event between *ytP*130 and *ytP*295. On the two non-diminished regions, recombination events were observed in the gametes of both males and females (Appendix 4.3). Within the diminished region, 5 recombination events were detected. Noticeably, 3 of them appeared to have occurred in the context of double cross-over events. This is rather unlikely and a possible alternative explanation would be that these were gene conversions at one marker instead of double cross-overs.

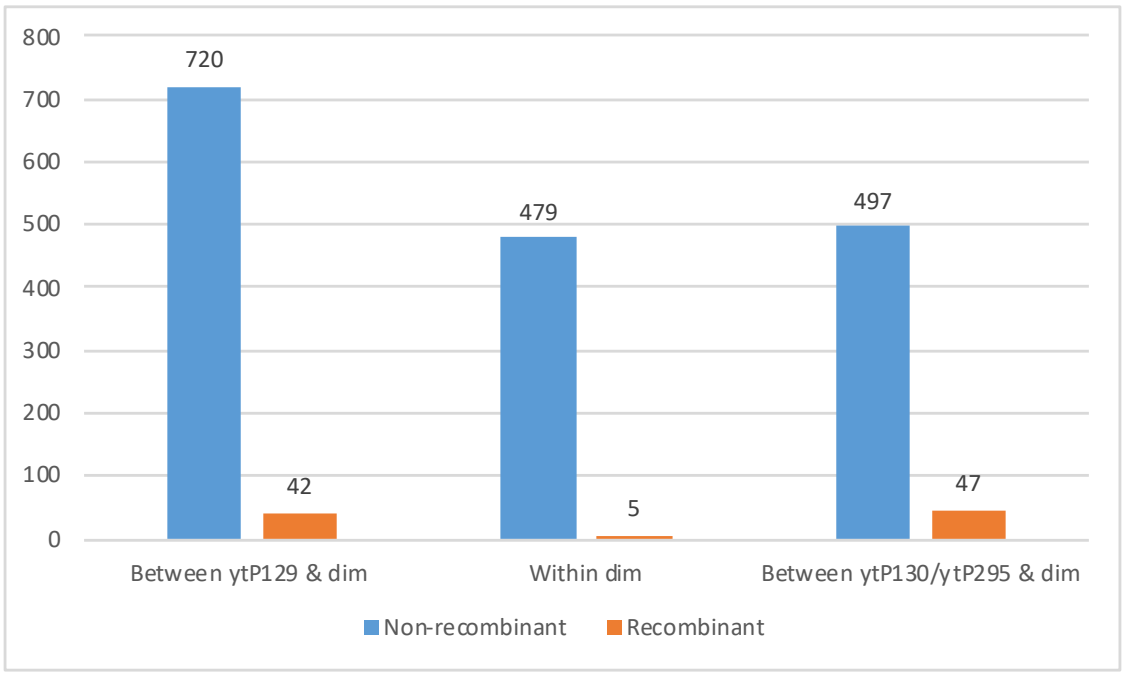


Figure 12. Recombination in diminished and non-diminished chromosomal regions. Notice that for a few crosses, not all the diminished markers were informative. For details see the Appendix 4.3 (xlsx file).

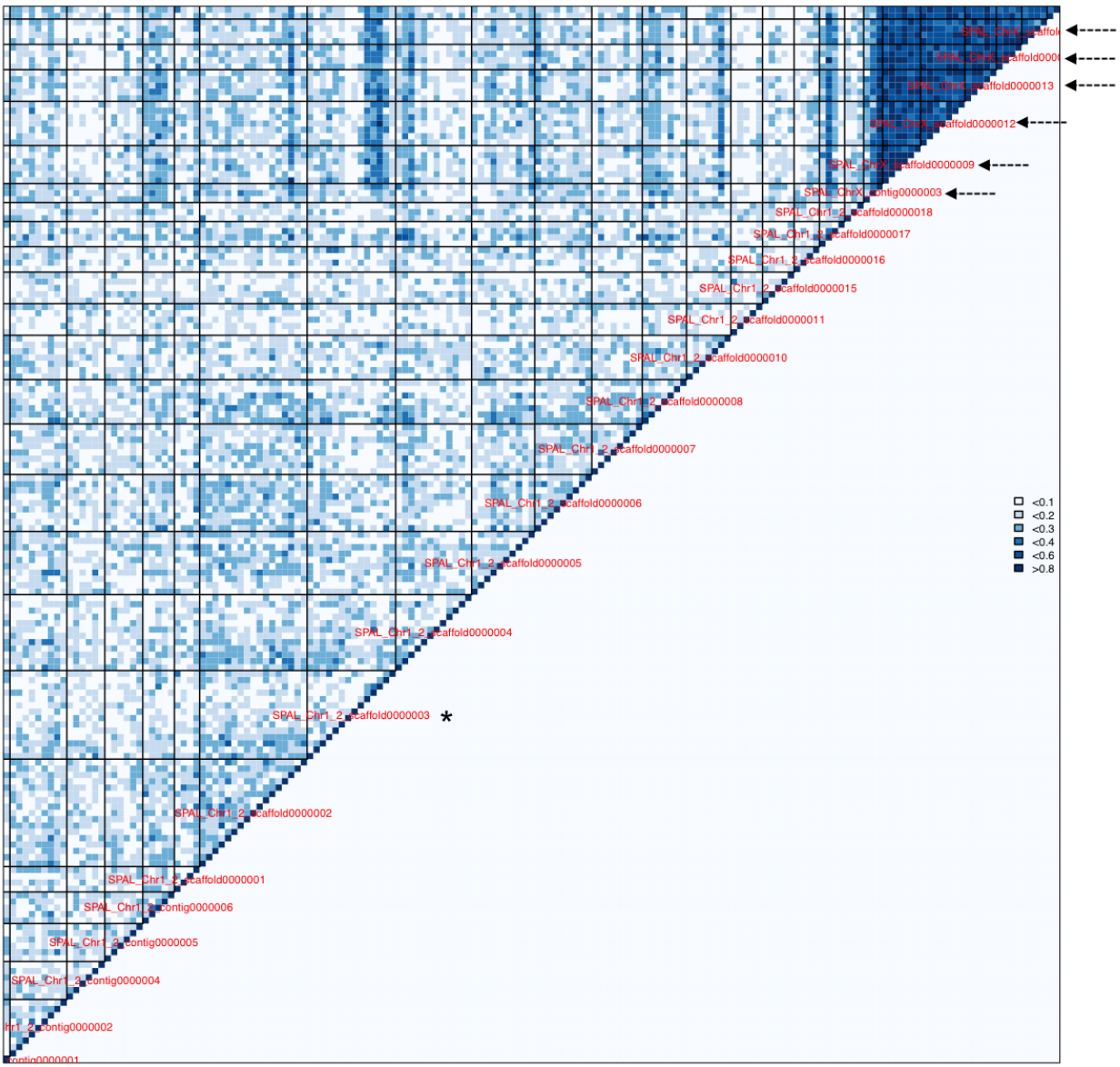
It must be noted that, although it was well established that *ytP*129 and *ytP*130 are on opposite sides of the diminished region, their assignment to the longer and the shorter arm was preliminary. Also, the order of the 6 diminished markers is unknown although preliminary findings in our lab had suggested that *ytP*244 is physically linked with non-diminished markers (Kulkami 2015). Nevertheless, the 6 diminished markers investigated in this study locate on 6 different scaffolds representing 12.5 Mb of the 14.0 Mb (89.2 %) of the whole diminished region and only 2 recombination events (0.41%) were detected. Thus, the result of the genetic crossing indicates that recombination rarely occurs in the diminished region of the *S. papillosus* L chromosome, if at all.

2.3.3.4 A high linkage disequilibrium implying little or no genetic recombination in the diminished region in a wild population

We sequenced the whole genomes of 24 individuals and assayed them for linkage disequilibrium as described in materials and methods (2.3.2.5). The LD was analyzed using the published genome rather than the new assembly. We found a higher LD over the entire diminished region than between any other parts of the genome (figure 13A). The relatively high LD between a portion of Chr1_2 scaffold0000003 and the diminished loci, might suggest this scaffold is physically close to diminished chromosomal region. The result clearly demonstrates a strong genetic linkage over the entire diminished region in the *S. papillosus* population under study. Therefore, the LD pattern agrees with the crossing experiment, that the diminished region does not or only rarely exchange genetic information between the two homologous chromosomes.

In order to test if lack of recombination is a general feature of *Strongyloides* spp. sex chromatin and is also observed in species with a free X chromosome, we also analyzed the LD pattern of *S. stercoralis*, based on the 23 whole genome sequences of individuals collected in Cambodia described in (Jaleta, Zhou et al. 2017) (part of this thesis). The LD pattern of *S. stercoralis* is shown in figure 13B. Low LD is found across the whole genome, indicating recombination does occur in both autosomes and X chromosome in *S. stercoralis*. Crossing overs were also observed on the X-chromosome in *S. ratti* females in laboratory crosses (Nemetschke, Eberhardt et al. 2010).

A


SPAL_ChrX_scaffold0000013
SPAL_ChrX_scaffold0000012
SPAL_ChrX_scaffold0000009
SPAL_ChrX_contig0000003
SPAL_Chr1_2_scaffold0000018
SPAL_Chr1_2_scaffold0000017
SPAL_Chr1_2_scaffold0000016
SPAL_Chr1_2_scaffold0000015
SPAL_Chr1_2_scaffold0000011
SPAL_Chr1_2_scaffold0000010
SPAL_Chr1_2_scaffold0000008
SPAL_Chr1_2_scaffold0000007
SPAL_Chr1_2_scaffold0000006
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SPAL_Chr1_2_scaffold0000002
SPAL_Chr1_2_scaffold0000001
SPAL_Chr1_2_contig0000006
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SPAL_Chr1_2_contig0000004
<0.1
<0.2
<0.3
<0.4
<0.6
>0.8

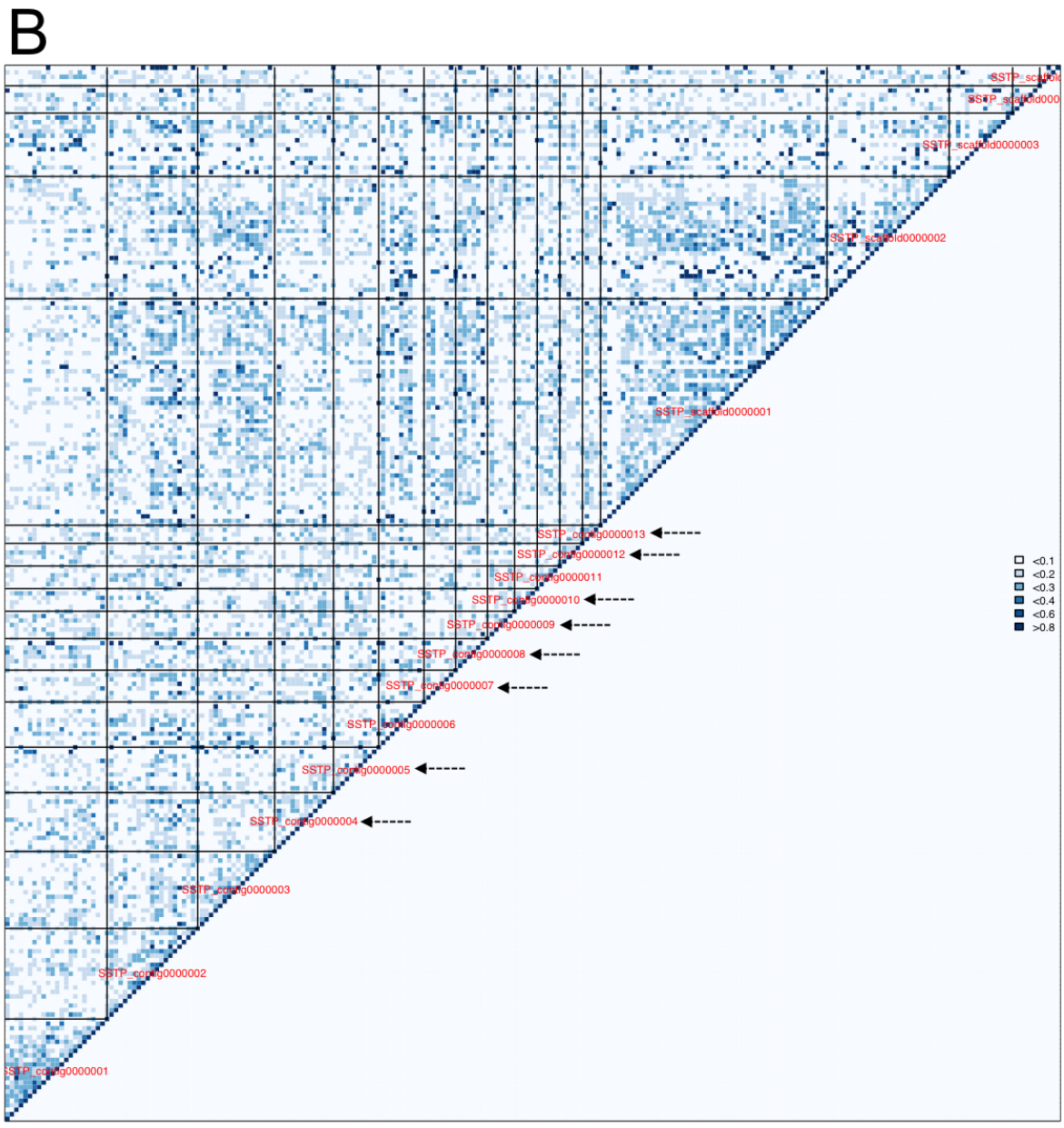

Figure 13. (A) Linkage disequilibrium of *S. papillosus* and (B) *S. stercoralis*
The plots show average LD measured as r^2 between pairs of 100 kb windows. The names of contigs or scaffolds are labelled in red. Dark blue indicates high LD level. Arrows indicate the scaffolds/contigs located on the diminished region (*S. papillosus*) or X chromosome (*S. stercoralis*). Asterisk indicates Chr1_2 scaffold0000003 of *S. papillosus*.

Taken together, my results show that genetic recombination rarely happens in the sex chromatin (diminished region) of *S. papillosus*, if at all, meaning that around 1/3 of the genome reproduces essentially asexually and the other 2/3 sexually. The same is not true for the sex chromosomes of its closely related species, *S. stercoralis* and *S. ratti*. It can be expected that the BioNano sequencing, which is still in process, will strongly improve the

scaffolding of the current *de novo* assembly of *S. papillosus*. With the added information about the order of SNPs and contigs, we will also get a higher resolution of the LD pattern and be able to estimate the physical distances between the markers used.

2.3.4 Contribution

Adrian Streit and I collected *S. papillosus* from field station with the help of other people from our research group. I preformed the crossing experiment, genotyping and analyzed the recombination frequency. I collected materials and prepared libraries for population genetic study. Christian Rödelsperger analyzed the LD pattern. I collected the materials for *de novo* assembly and prepared the libraries for Illumina sequencing. Christa Lanz prepared the libraries for PacBio sequencing. Felix M. Bemm assembled the genome with the discussion of Adrian Streit and I.

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4. Appendix

4.1 Single copy molecular genetic markers

>*ytP*291

TTGAATGTGCCCTCCCAGTTGAGGAGGAAGATGACGGTATAATTGAAGTTATTGG
TACACTAGCTGATGATGTACAAGTACTACCTTTACAGTAACAAACTGCTGGTCCA
TTTGGATCATAACCATTTTTTAATAATAATTCAACAATTTCTTTTTGCTTATGCGCC
ACTGCCATATGAAGAGTGTTATATAATGCACATGTTGGTTTGCCACTGGAGTGAG
TTGATAATGTTGAGGAGGCATTGGAACGACGATGAT

>*ytP*295

TCCTGAGATGCACCTTTTATTCATGAAGAAAATGTCAAATTATATAAGTGTTTGTA
GGAGTTCAGTATATCACAAGGACATATAATTGTAAATCATAATTTTTTAATATCTT
TTAATAATAAGTTAATATTCAATCACATAAACTATATTCTCTTTTGTTAGAAAAAA
TTGATCTTTAAATTTTGAATAAAATTAAAGTCAAGTTCTAGAATTACAATATTTTC
AATTAAAAAAAGATCTTTATTATAATTATACTACTTTTGTCGGCATTACATAAAGCA
TTAGTCAATATTAGACCTGCACACAATCTTATTTATAAATTTCTGTAATATAAAAA
GAAAAGACTTCCTTGAAGTTGTCAAAGTGTTAGAAATTCTGGAACTTCGAATTGT
GA

> *ytP*214

CGATATGGCAATTGTTTATGGAATAAGTGTTGGTACAGCACTAATTGCGTCTACA
TTATTATCATACATTGTTGTTATTGGAGTTTTTGTTGCAAAATTTTTAAATTTTGAT
CCTGATAATATTGTTGGACCAATTGCTGCTTGTCTAGGTGATGTTATAACAGTTAT
AATTATGGTCGGTTTTATTATCTTAATATATGTTAGTAATCACTATTTTTTAATAAT
TTCAGATGTATTATGGTCAACTAATAGATAGTTATTATCAAAACTATTTATGGGTA
CCAATAGTTTGTTTAATAATTATTTATTTATGGTCAATTATAATGATTCCAATTGC
TTGGCAATACCCGAAAACCAAGTTGGTCCTTAAGTATGGATGGTTCTCTACAATA
ATAGCAATGGTGCGACGAGACGAATGGAGACAAGAC

> *ytP*244

CTGAATTCACGGGGAAAGATAGTTTCTTTTCTAAAGAAAATAATAATGATTTTGG
AGAAGATTCTAAAGGTTTATCCCGTGATAAAAGGTACATTATTTCTAATGAATCA
ACAATACCAGGTTGTAATGATGATGCTGTCATCCTTGGATGGCTTCTAAAAGACT
ACAACTATCTACAAATTCCAGGTAATGGTCATGTTAAAGTAGAAGTTGAGATATG
GATTCAGGAAGTTTCAAAGATAATTGAAATTACATCTGAATTTGAGGTTGATTTA
TATGTCACAGAAATGTGGAATGATCCTTCATTAGTCTTTTCTCATTTATCACCATG
TAAATCAAATATGTCTGTTGATGGCCCAAAAGTAATAGAACAGATTTGGAAGCCA
CAAGGATGTTTTATTAATTCAAAGGACGCAAAAATTCACTCAAGTCCTGTTAAAA
ATATCTTCCTACAAATCTATGATAATGGAAATGTACTGCTGTATCAAGCCATG

Polymorphic positions are under laid in red.

4.2 The *de novo* genome assembly and the assignments of diminished and non-diminished regions

The name and length of contigs, the mean read coverage per contig comparisons between samples, and the contig assignments into diminished or non-diminished regions are indicated.

Attached as an excel file.

4.3 Recombination frequencies between molecular genetic markers

For each cross, the genotypes of parents and their progeny at different *ytP* markers are indicated. Recombinants and non-recombinants were calculated (1) between *ytP* 129 and diminished markers, (2) within diminished markers, and (3) between *ytP*130/295 and diminished markers.

Attached as an excel file.

5. Publications

Different but overlapping populations of Strongyloides stercoralis in dogs and humans - Dogs as a possible source for zoonotic strongyloidiasis

Siyu Zhou*, Tegegn G. Jaleta*, Felix M. Bemm, Fabian Schär, Virak Khieu, Sinuon Muth, Peter Odermatt, James B. Lok, Adrian Streit.

PLOS neglected tropical diseases.

Published: August 9, 2017.

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*Shared first authorship, published as Jaleta, Zhou et al.

Characterization of a non-sexual population of Strongyloides stercoralis with hybrid 18S rDNA haplotypes in Guangxi, Southern China

Siyu Zhou, Xiaoyin Fu, Pei Pei, Marek Kucka, Jing Liu, Lili Tang, Tingzheng Zhan, Shanshan He, Yingguang Frank Chan, Christian Rödelsperger, Dengyu Liu and Adrian Streit.

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Different but overlapping populations of *Strongyloides stercoralis* in dogs and humans—Dogs as a possible source for zoonotic strongyloidiasis

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Data Availability Statement: All relevant data are either within the paper and its Supporting Information files or deposited in publicly accessible databases. Cox1 and new *SSU* sequences were submitted to genbank (accession numbers KU724124-KU724129 and KX226367-KX226384 and KY548505). Whole genome data are available at ftp://ftp.tuebingen.mpg.de/pub/PLOS_NTD_Jaleta_2017_whole_genome_data and were submitted to the European Nucleotide Archive (accession number PRJEB20999)

Abstract

Strongyloidiasis is a much-neglected soil born helminthiasis caused by the nematode *Strongyloides stercoralis*. Human derived *S. stercoralis* can be maintained in dogs in the laboratory and this parasite has been reported to also occur in dogs in the wild. Some authors have considered strongyloidiasis a zoonotic disease while others have argued that the two hosts carry host specialized populations of *S. stercoralis* and that dogs play a minor role, if any, as a reservoir for zoonotic *S. stercoralis* infections of humans. We isolated *S. stercoralis* from humans and their dogs in rural villages in northern Cambodia, a region with a high incidence of strongyloidiasis, and compared the worms derived from these two host species using nuclear and mitochondrial DNA sequence polymorphisms. We found that in dogs there exist two populations of *S. stercoralis*, which are clearly separated from each other genetically based on the nuclear 18S rDNA, the mitochondrial *cox*1 locus and whole genome sequence. One population, to which the majority of the worms belong, appears to be restricted to dogs. The other population is indistinguishable from the population of *S. stercoralis* isolated from humans. Consistent with earlier studies, we found multiple sequence variants of the hypervariable region I of the 18 S rDNA in *S. stercoralis* from humans. However, comparison of mitochondrial sequences and whole genome analysis suggest that these different 18S variants do not represent multiple genetically isolated subpopulations among the worms isolated from humans. We also investigated the mode of reproduction of the free-living generations of laboratory and wild isolates of *S. stercoralis*. Contrary to earlier literature on *S. stercoralis* but similar to other species of *Strongyloides*, we found clear evidence of sexual reproduction. Overall, our results show that dogs carry two populations, possibly different species of *Strongyloides*. One population appears to be dog specific but the other one is shared with humans. This argues for the strong potential of dogs as

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reservoirs for zoonotic transmission of *S. stercoralis* to humans and suggests that in order to reduce the exposure of humans to infective *S. stercoralis* larvae, dogs should be treated for the infection along with their owners.

Author summary

Infections of humans with the nematode *Strongyloides stercoralis* can persist for a very long time, due to the capacity of this pathogen to undergo an autoinfective life cycle and re-infect the same host over and over again. Clinical manifestation, known as human stongyloidiasis, may be fatal and can arise many years and generations of worms after the initial infection occurred. Although *Strongyloides stercoralis* has been known as the causative agent of strongyloidiasis for a very long time, some key questions about its biology and epidemiology remain open. Here we address two of them. Firstly, it has long been known that dogs can serve as experimental hosts for *S. stercoralis* but it is a matter of debate whether *Strongyloides* spp. found in dogs in the wild are human pathogenic *S. stercoralis*, and whether dogs therefore are a source of zoonotic transmission of this parasite. Here we show that dogs carry two genetically different populations of *Strongyloides* spp. one of which is shared with humans. This demonstrates that dogs represent a possible reservoir for zoonotic strongyloidiasis. Secondly, the all female, parthenogenetic parasitic generations may alternate with single facultative free-living generations, which consist of both females and males. In spite of the presence of both sexes, it had been postulated that males do not contribute genetic material to the progeny and that sperm are merely required to trigger parthenogenetic embryonic development. Here we show that the free-living adults of *S. stercoralis* reproduce sexually.

Introduction

Soil-transmitted helminthiasis (STH) affects up to one in four individuals in the world, disproportionately impacting impoverished populations with less access to clean water, sanitation, and opportunities for socioeconomic development [1]. Strongyloidiasis is one of the most neglected tropical diseases [2,3]. Estimates of the number of people infected with the causative agent *Strongyloides stercoralis* vary and go up to 370 million worldwide [2,4,5]. The local prevalence can reach more than 40% in some tropical and subtropical countries [3,6]. Factors such as high temperature, high moisture, poor sanitation and sharing premises with domestic animals may contribute to high prevalence of *S. stercoralis* [3,7,8]. *S. stercoralis* is the major causative agent of human strongyloidiasis [9] but there are also reports of people infected with *Strongyloides fuelleborni* and *Strongyloides fuelleborni kellyi*, in Africa and in Papua New Guinea [9]. Based on molecular data, *S. fuelleborni kellyi* should probably be considered an independent species rather than a subspecies of *S. fuelleborni*, [10]. Although *S. stercoralis* infection frequently remains asymptomatic, immuno-compromised patients can develop a systemic infection, which may lead to fatal forms of strongyloidiasis. The medical relevance of this parasite has probably been grossly underestimated due to difficulty of diagnosis [4,5,11]. Also, it should be noted that *Strongyloides* is not limited to tropical and underdeveloped areas, and the presence of *S. stercoralis* and fatal cases caused by it have also been reported from well-developed regions with temperate climates such as the European Union and North America [12–18]. *S. stercoralis* has a complex, rather unique life cycle (Fig 1) consisting of parasitic and free-living generations [19–21]. In brief: infective third stage larvae (L3i), which are all females,

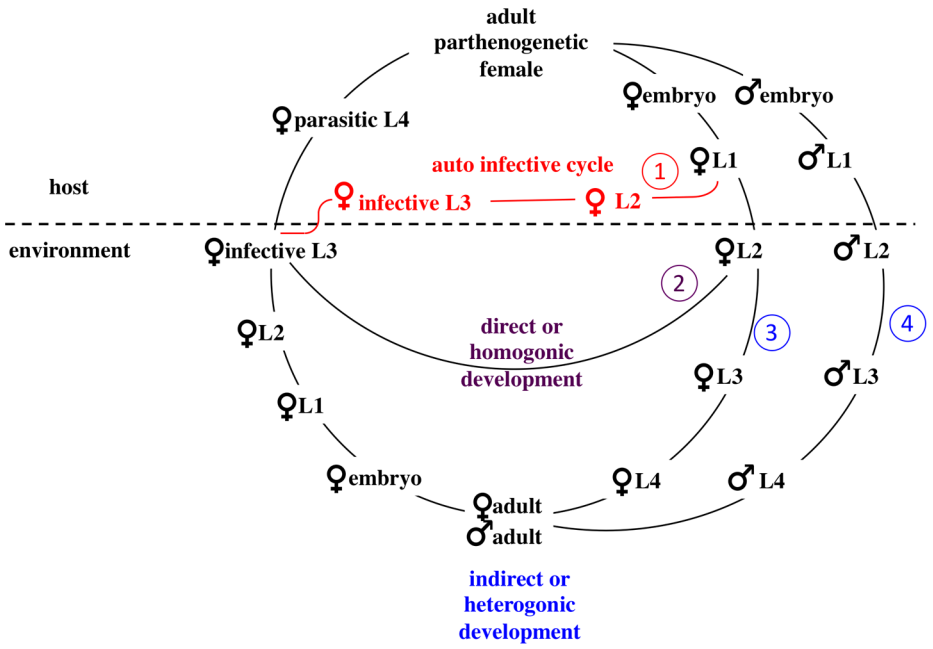

Fig 1. The life cycle of *S. stercoralis*. The life cycle of *Strongyloides stercoralis*. The numbers refer to the numbers of the developmental options in the description of the life cycle in the text.

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invade a new host by skin penetration and, after migrating through the blood and the lungs, are coughed up and swallowed and eventually establish in the small intestine of the host. The parasitic adult females reproduce by parthenogenesis. The progeny of the parasitic females have four developmental options: 1) Firstly, they may become female, and develop into infective third stage larvae (iL3) within the host and re-infect the same host individual (autoinfective cycle); 2) Secondly, they may become female, but this time leave the host as first-stage larvae, develop into iL3 and search for a new host (direct/homogonic development); 3) Thirdly, they may become female and leave the host, but this time develop into free-living, non-infective third stage larvae and subsequently into adult females (indirect/heterogonic development); 4) Or fourthly, they become male and leave the host and develop into free-living adult males (indirect/heterogonic cycle). The free-living adults mate and reproduce in the environment and all their progeny are females and develop to iL3s. No male iL3s have been reported in any *Strongyloides* species. For two species of *Strongyloides* (*Strongyloides ratti* and *Strongyloides papillosus*), it has been shown that the reproduction in the free-living generation is sexual, in spite of some earlier literature that had described it as pseudogamic (by sperm dependent parthenogenesis) [22–24]. For *S. stercoralis* prior to this report no genetic analysis of the mode of reproduction had been conducted and non-sexual (pseudogamic) reproduction as proposed based on cytological observations remained an option [25,26]. Whilst all species of *Strongyloides* may undergo homogonic or heterogonic development, the autoinfective cycle (option 1) appears to be specific for *S. stercoralis* and maybe a few other less well-investigated species [19]. This autoinfective cycle allows the parasite to persist in a particular host individual for many years, much longer than the life expectancy of an individual worm. Usually, healthy individuals tolerate such long lasting infections well and control them at very low levels [5]. These people have no clinical symptoms and the infection is unlikely to be detected. However, if such a chronically infected person becomes immunodeficient due to disease or immunosuppressive treatment (i.e. cancer chemotherapy or organ transplantation), this may lead to failure to control the infection and consequentially to

self-enhancing progression of strongyloidiasis (hyperinfection syndrome and disseminated strongyloidiasis), which is lethal if not treated [5].

Parts of the 18S rDNA (small Subunit, *SSU*) sequence, in particular the hypervariable regions (HVR) I and IV, are widely used as nuclear markers for molecular taxonomy of nematodes in general (e.g. [27–33]) and *Strongyloides* spp. in particular [8,34–36]. Whilst some sequence variation in HVR I within *S. stercoralis* was reported [35,37], HVR IV appears virtually invariable within this species. To our knowledge, there is only one report to date of a single nucleotide difference within this region [38](accession number M84229).

Whilst humans are their natural hosts, dogs, cats, and non-human primates have also been proposed to be suitable hosts for *S. stercoralis* [39–42]. To what extent strongyloidiasis is a zoonotic disease has been the subject of controversy in the literature for several decades. Originally Brumpt (1922) [43], later supported by Augustine (1940) [44] split the *Strongyloides* of dogs from *S. stercoralis* and described it as a separate species, called *Strongyloides canis*. Recent comparative analyses of the mitochondrial locus cytochrome c oxidase subunit 1 (*cox*1) [6,36,38,45] and the whole genome sequence of 33 individual *S. stercoralis* from Japan and Myanmar [46] indicated that there is substantial genetic diversity among *S. stercoralis* isolated from human hosts and [38] suggested that there might exist human- and dog-specialized subpopulations. On the other hand, dogs have long been known to be suitable experimental hosts for human derived *S. stercoralis* [39,41], and many authors consider *Strongyloides* in dogs and humans to belong to the same species, i.e. *S. stercoralis*. While the more recent literature appears to favor separation, it remains unclear whether *S. stercoralis* naturally infecting dogs and humans belong to the same populations or not, and correspondingly, what the potential is for dogs to serve as a source for human *S. stercoralis* infections (recently reviewed by Thamsborg and colleagues [42]). In order to address this question, we compared individual *Strongyloides* isolated at the same time and location from humans and dogs, which, to our knowledge, had never been done. In our study area, rural communities in Northern Cambodia, people share their premises closely with their dogs, and the prevalence of strongyloidiasis is high [3]. We compared the sequences of the nuclear *SSU* HVR I and HVR IV and, for a selected subset of worms, the mitochondrial *cox*1 gene and whole genome sequences. Further, we characterized reproduction in the free-living generations of wild and laboratory isolates of *S. stercoralis*. Our results show that dogs carry *S. stercoralis* genetically indistinguishable from the ones in humans in addition to a dog specific population. Further, we demonstrate that reproduction in the free-living generation of both wild and laboratory isolates of *S. stercoralis* is sexual and not pseudogamic. Overall, our observations strongly support the hypothesis that dogs are a potential source for human *S. stercoralis* infection and suggest that in order to reduce the exposure of people to infective *Strongyloides* larvae, dogs should be treated along with their owners in settings where people are exposed to dog excrement.

Materials and methods

Study area

Fecal samples were collected from humans and dogs of the same households in the villages Anlong Svay (AS) and Chom Long (CL) in May 2013 and in Damnak Chin (DC) and Kampot (KP) in June 2016. All villages are in the Rovieng District (13˚21′N 105˚07′E) in Preah Vihear province in Northern Cambodia.

Stool sample collection and *S. stercoralis* isolation

Human stool samples were collected and *S. stercoralis* larvae were isolated according to established methods [47]. In brief, stool samples were collected for two consecutive days from each

member of the household who agreed to participate in this study. In May 2013 all the fecal samples collected from humans were analyzed within 3 hours after collection using Baermann and Kato-Katz methods. The sediments of positive Baermann funnels were preserved and transported to our laboratory in Tübingen in 70% ethanol at ambient temperature. In June 2016 the fecal samples were mixed with an approximately equal volume of sawdust, moisturized and cultured at ambient temperature for 24–48 hours and analyzed using the Baermann method. From positive Baermann funnels a portion of the worms were transferred individually into 10 μl of water or, for those intended for whole genome sequencing, 10 μl of Tissue and Cell Lysis Solution (component of the MasterPure DNA Purification Kit, Epicenter MC85201) and the remaining worms were preserved as batches in 70% ethanol. While the work was ongoing the samples were stored in the hotel freezer. For transport to our laboratory the samples were refrigerated using wet ice but not frozen. In the majority of cases, worms from the 2016 sample come from those that had been picked individually whilst alive into water or Tissue and Cell Lysis Solution. If any ethanol preserved specimen from 2016 was used, this is explicitly stated.

Fecal samples were also collected from dogs found in the proximity of *S. stercoralis* positive households. The samples were taken directly from the rectum of the animals with the help of the owners and the field assistants. The samples were further processed like the human samples except that for some samples, 3 g of feces were placed on NGM agar plates [48] and incubated for 24–48 hours at ambient temperature and emerging *S. stercoralis* were picked directly from the plates instead of setting up saw dust cultures followed by baermanization.

Single worm DNA preparation for PCR and whole genome sequencing

For ethanol fixed samples, single worms were picked and washed twice with Phosphate-buffered saline (PBS) and then incubated in 20 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 2h, followed by incubation at 95˚C for 15 min. 2 μl (for *SSU*) or 4 μl (or single copy loci) of this lysate were used as template for PCR amplification.

For worms stored in 10 μl water, 10 μl 2x lysis buffer were added, after which the samples were treated as described above. For samples preserved in Tissue and Cell Lysis Solution, single worm DNA was prepared using the MasterPure DNA Purification Kit (Epicenter MC85201) according to the manufacturer's protocol, and the DNA was stored frozen in 10 μl of TE buffer. 1 μl was used for *SSU* amplification, and the reminder for sequencing library construction.

PCR for *SSU*, *cox*1 and single copy locus genotyping

PCR reactions were done in a total volume of 25 μl (up to 25 μl nuclease-free water, 2.5 μl 10X ThermoPol Reaction buffer (New England BioLabs), 0.5 μl dNTP's (2mM each), 0.5 μl 10 mM forward primer (Table 1, Fig 2), 0.5 μl 10 mM reverse primer (Table 1, Fig 2), 0.3 μl Taq DNA polymerase (New England BioLabs), 1 μl to 4 μl template as specified above). Thermocycling program: 94 ˚C for 2 min, followed by 35 cycles of denaturing (94 ˚C for 30 sec), annealing (temperature given in Table 1 for 15 sec), extension (72 ˚C for time given in Table 1), and a post amplification final extension (72˚C for 10 min) and cooling to 4 ˚C.

Sequencing of PCR products and sequence analysis

1 μl of the PCR reaction and either one of the PCR primers or, in the case of *SSU* HVR I, a designated sequencing primer were used in sequencing reactions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems,) according to the manufacture's protocol. The reactions were submitted to the in-house sequencing facility at the Max Planck Institute for Developmental Biology at Tübingen for electrophoresis and base calling. Sequences were

Table 1. Primers and PCR conditions.

	Primer		Sequence	Ann	Ext	Prod
SSU HVR I	Fw	SSU18A[a]	5'-AAAGATTAAGCCATGCATG-3'	52˚C	90"	863 bp
	Rev	SSU26R[a]	5'-CATTCTTGGCAAATGCTTTCG-3'			
	Seq	SSU9R	5'-AGCTGGAATTACCGCGG-3'			
SSU HVR IV	Fw	18SP4F[b]	5'-GCGAAAGCATTTGCCAA-3'	57˚C	90"	712 bp
	Rev	18SPCR[b]	5'-ACGGGCGGTGTGTRC-3'			
*cox*1	Fw	TJ5207	5'-TTTGATTGTTACCTGCTTCTATTTT-3'	50˚C	90"	650 bp
	Rev	TJ5208	5'-TTTTACACCAGTAGGAACAGCAA-3'			
ytP274	Fw	TJ6026	5'-CAGGACCACCTGGACAAGTT-3'	54˚C	90"	543 bp
	Rev	TJ6027	5'-CTTTCCATCCTGATGCCACT-3'			
ytP289	Fw	ZS6420	5'-TGAAACAGGAAAACACATCTACTGA-3'	49˚C	90"	765 bp
	Rev	ZS6421	5'-AGTGTTCAAGATATTCACGCAG-3'			
ytP289 nested	Fw	ZS6472	5'-AAATGGTTCAAGTTTGGGAC-3'	49˚C	60"	431 bp
	Rev	ZS6473	5'-TGACATACCATTAGCTTCACCA-3'			
ytP290	Fw	ZS6490	5'-TGCTGCCTCAACAATGTACA-3'	49˚C	60"	431 bp
	Rev	ZS6491	5'-TTATAGGCATCTAAAAGGCTTT-3'			
ytP290 nested	Fw	ZS6448	5'-GCTGTACAGGATGCTTTGGA-3'	49˚C	60"	203 bp
	Rev	ZS6449	5'-TGTGCGATACATAATTTTCTGATGAA-3'			

[a]Taken from [10];

[b] taken from [35].

Rev reverse; Seq sequencing; Fw forward; Ann annealing temperature for PCR; Ext extension time for PCR; Prod PCR product length.

https://doi.org/10.1371/journal.pntd.0005752.t001

analyzed with SeqMan Pro version 12 (Lasergene package; DNAStar, Inc., Madison, WI USA). Chromatograms were visually inspected to detect ambiguous signals indicating mixed sequences (heterozygous worms). For comparison with published sequences, we used BLAST against the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For the *SSU*, we used the GenBank entry AF279916 as reference sequence. All position numbers refer to this entry. For *S. stercoralis cox*1, we used the sequence LC050212 as reference. 552 base pairs, of which 63 were polymorphic, were considered (for the full sequences of the different haplotypes see S1 Text). For *ytPxxx* markers, position 1 is the first base of the (non-nested) forward primer (for the sequences of the markers see S1 Text).

Phylogenetic analysis of the *cox*1 sequences was done using MEGA7 [49] with default settings. As an outgroup species we used *Necator americanus* (AJ417719). The Maximum Likelihood tree is shown in Fig 3. For comparison we also reconstructed Maximum Parsimony and Neighbor Joining trees, which resulted in the same tree topology as far as well-supported nodes are concerned.

Whole genome sequencing and analysis

DNA of 23 single free-living males *S. stercoralis* from different hosts (11 males from 9 different humans and 12 males from 10 different dogs) was prepared as described above. Whole-genome sequencing libraries were prepared with Clontech Low Input Library Prep Kits (Takara Bio, USA) following the manufacturer's protocol. Samples were submitted to in-house sequencing on an Illumina HiSeq 3000 instrument (150 bp paired-end). An approach similar to a previous *S. stercoralis* population study [46] was used to analyze the whole genome data. In brief, raw reads were trimmed with skewer [50] (version 0.1.123; -q 30 -Q30 -l 60). Trimmed reads were mapped to the *S. stercoralis* reference genome (GCA_000947215.1) and the small subunit ribosomal RNA (*SSU* rRNA; AF279916) using bwa mem [51] (version 0.7.12; MEM algorithm with

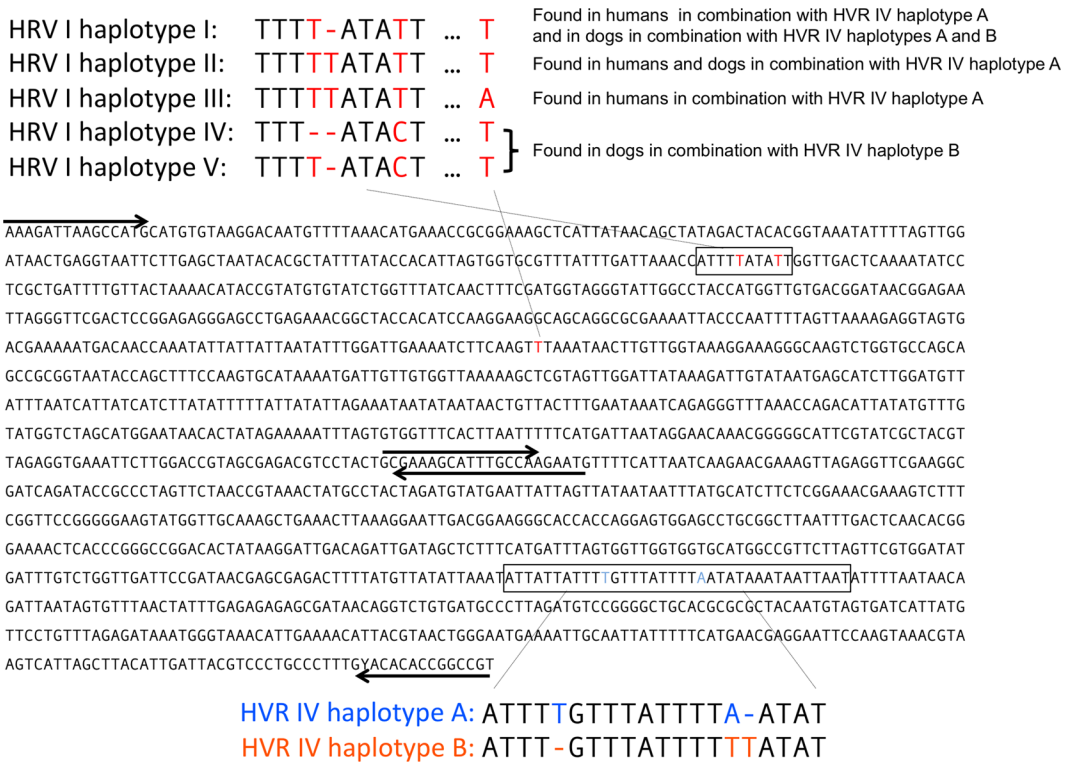

Fig 2. The different *SSU* HVR variants found. The sequence of the portion of the *SSU* amplified for genotyping of the two HVRs. Arrows indicate the positions of the primers used for amplification, from top to bottom SSU18A, 18SP4F, SSU26R, 18SPCR (c.f. Table 1). Note that forward primers are above the sequence while reverse primers are below the sequence. HVR I and HVR IV as defined by Hasegawa and colleagues [35] are boxed. For sequencing results for both *SSU* HVR and *cox*1 for each individual worm see S1 Table.

https://doi.org/10.1371/journal.pntd.0005752.g002

defaults). Duplicate reads were marked with Picard tools (http://broadinstitute.github.io/picard) (version 2.2.1; MarkDuplicates with defaults). Variants were called with GATK [52] (build 2016-09-27-g026f7e8; HaplotypeCaller and GenotypeGVCFs both with defaults) after Indel realignment (GATK IndelRealigner with defaults) and freebayes [53] (version 1.0.2-6-g3ce827d, defaults). The intersection of both variant calls was imported into GNU R using SNPRelate [54] (version 1.8.0; method = "biallelic.only"). Single-nucleotide polymorphism (SNPs) were recursively removed using a sliding window approach and a Linkage Disequilibrium (LD) threshold of 0.05 and or a minimum allele frequency (MAF) of 0.05. The resulting SNP set was used to calculate the fraction of identity by state (IBS) for each pair of samples. Results were stored in a genomic identity-by-state relationship matrix and used to estimate a phylogeny with the BIONJ algorithm implemented in ape [55] (version 4.1; defaults). A second phylogeny was estimated with a reference-free approach. Trimmed short reads were used to generate a k-mer count graph with khmer (version 0.2.0; load-into-counting.py with a k-mer size of 19 otherwise defaults). k-mer counts were used to calculate pairwise distances between samples using kWIP [56]. The resulting distance matrix was imported into GNU R to estimate a phylogeny; again using the BIONJ algorithm.

Analysis of free-living females and their progeny

Free-living females were isolated after two days of culture as described above and placed individually onto NGM plates seeded with *E. coli* OP50 [48]. The plates were inspected daily.

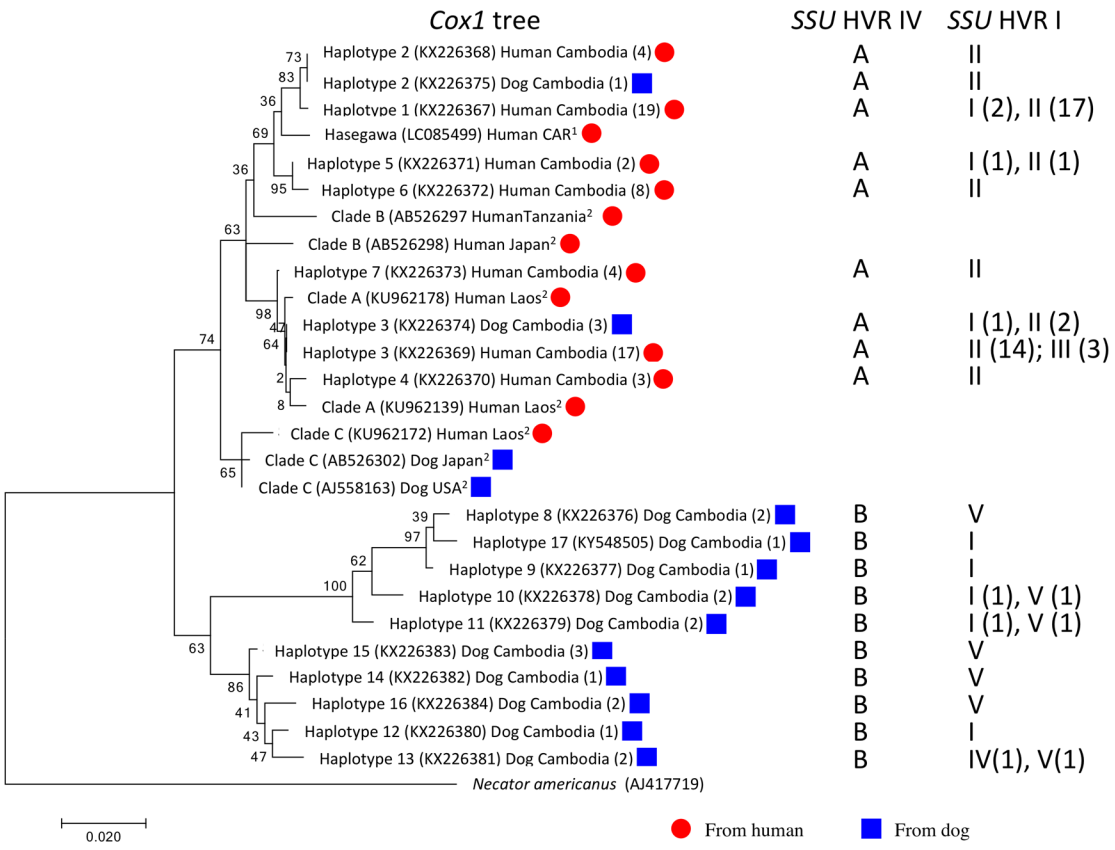

Fig 3. Gene tree of the mitochondrial gene *cox*1. Maximum likelihood tree of the 17 different *cox*1 sequences we found and representative previously published sequences. The numbers are bootstrap values based on 1000 bootstraps. For haplotypes isolated in this study the labels have the following format: Haplotype number (accession number) host country (number of individuals this haplotype was found in). [1]For sequences previously published by Hasegawa and colleagues [36] the label starts with Hasegawa. [2]For sequences previously published by Laymanifong and colleagues [6] the label starts with the *cox*1 clade this reference assigned the particular sequence to. This is followed by: (accession number) host country (CAR = Central African Republic). The hosts are also highlighted by red circles (human) and blue squares (dog). Entries to the right of the tree indicate for each *cox*1 haplotype the *SSU* haplotypes it was found together in the same individual. If a given *cox*1 haplotype existed in the context of multiple *SSU* haplotypes, the number of worms with this particular combination is given in parentheses. Note: the *cox*1 haplotypes 2 and 3 were found in both hosts and are included twice in this tree. For the sequencing results for both *SSU* HVR and *cox*1 for each individual worm see S1 Table.

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Females that had produced progeny but presumably had ceased reproduction because they no longer contained embryos in their uterus were picked and prepared for genotyping as described above. One day later, larvae from these females were also isolated and processed. For 17 of these families the mothers and the progeny were picked individually and alive into 10 μl of water and processed as described above. Five more families (Females 7, 8, 14, 15, 16) were preserved in Ethanol (one tube per family) and the individuals were only separated when they were genotyped. The mothers and the progeny were genotyped at *ytP289* and *ytP290* as described above. *ytP289* contains single nucleotide polymorphisms at positions 308 (A/G), 359 (A/G), 416 (C/T) and 566 (C/T). Three different combinations (alleles) existed in our samples. Allele 1 has the combination G+A+C+C, allele 2 A+G+T+T, and allele 3 A+A+C+C. *ytP290* contains single nucleotide polymorphisms at positions 291 (A/C) and 310 (A/G). Two different combinations (alleles) existed in our samples. Allele 1 has the combination A+A and allele 2 C+G. For the full sequences see S1 Text.

Crossing free-living stages of *S. stercoralis* (laboratory strain)

The UPD strain and PV001 line of *S. stercoralis* were maintained in immuno-suppressed dogs and cultured in fecal cultures with charcoal as previously described [41]. Free-living L4 larvae and young adults (males and virgin females) were isolated from 1-day fecal cultures at 22 ˚C using Baermann funnels as described [41].

Single virgin females and males were handpicked and transferred in male-female pairs onto NGM plates spotted with 30 μl of OP50 [48] and 20 μl of water from a Baermann funnel and incubated at 22 ºC for 24 hrs. On the next day, from pairs where the female contained developing embryos in the uterus, the males were transferred into PCR tubes containing 10 μl of lysis buffer (see above) and frozen for later use. Once the females contained no embryos any longer (after three days) they were processed like the males. After all the eggs had hatched, all the L1/L2 were transferred individually into PCR tubes as described for the parents. Single worm lysis was performed as described above for ethanol fixed specimens without the PBS washing step. The parents and eight progeny per cross were genotyped at the marker *ytP274* as described above. *ytP274* has a single SNP (T/C) at position 236. For the full sequence of *ytP274* see S1 Text.

Ethics statements

The sampling of material in Cambodia was approved by the National Ethics Committee for Health Research (NECHR), Ministry of Health, Cambodia and the ethics committee of the cantons of Basel-Stadt and Basel-Land (EKBB), Switzerland. All participants were informed of the study procedures and provided written informed consent prior to enrolment. All data handled were strictly confidential. All individuals infected with *S. stercoralis* were treated with Ivermectin (single oral dose of 200 μg/kg BW). Co-infections with other intestinal helminths were treated according the Cambodian treatment guidelines. The experiments requiring culture of *S. stercoralis* in host animals were all done at the University of Pennsylvania with the approval of the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). The *S. stercoralis* UPD strain and PV001 isolate were maintained in prednisolone-treated dogs according to IACUC protocols 702342, 801905, and 802593. All IACUC protocols, as well as routine husbandry care of the animals, were conducted in strict accordance with the *Guide for the Care and Use of Laboratory Animals of the National Institutes of Health*. No human subjects were used in this part of the study.

Accession numbers

The sequences obtained from this study are available from GenBank (accession numbers KU724124-KU724129 and KX226367-KX226384 and KY548505).

The whole genome data are available permanently from the FTP server of the Max Planck Institute for Developmental Biology (ftp://ftp.tuebingen.mpg.de/pub/PLOS_NTD_Jaleta_2017_whole_genome_data). They have also been submitted to the European Nucleotide Archive (accession number PRJEB20999).

Results

Prevalence of *S. stercoralis* in humans and dogs

In May 2013, we collected stool samples from a total of 537 persons from 128 households. Of these, 177 individuals (32.96%) living in 95 different households were positive for *S. stercoralis*. From positive households we obtained rectal fecal samples from a total of 88 dogs. Of these, 78 (88.63%) originating from 44 different households were positive. In June 2016, 20 of 169

(11.8%) people from 17 out of 62 households tested positive for *S. stercoralis*. Of the 29 dogs from 14 households tested, 22 (75.9%) living in 12 different households were positive. It should be noted that the prevalence in the two hosts cannot be compared directly because of the biased sampling of dogs.

Multiple *SSU* HVR I haplotypes among *S. stercoralis*

Firstly, we sequenced the region around the HVR I of the *SSU* from individual *S. stercoralis* isolated from humans and dogs [10,34–36,27–33]. Overall, we found the five different haplotypes described in Fig 2. The two most dissimilar haplotypes (III and IV) show four differences (2 indels and 2 SNPs). Earlier studies [27–30,35,36] found this fragment to be largely invariable within species and two or more differences to be fairly reliable indicators of different species. Therefore, a within species variability as we observed was rather unexpected but not entirely implausible. In order to obtain additional information, we next sequenced the *SSU* HVR IV [35,36].

Dogs carry two different populations of *S. stercoralis* identifiable by the *SSU* HVR IV sequence

Among all *Strongyloides* individuals from dogs, we found two sequence variants in the *SSU HVR IV* as defined in [35]. The two haplotypes differed at three positions (two indels, one base substitution, Fig 2). One of these variants, from now on referred to as HVR IV haplotype A, is the one previously described as the HVR IV sequence of *S. stercoralis* [35], and was found (Table 2) in 11 (11.5%) out of 96 dog derived worms in the 2013 sample and 39 (31%) out of 126 dog derived worms in the 2016 sample (in total 50 (22.5%) out of 222). This variant was also found in all human derived worms of both samples (in total 521 worms from a total of 85 host individuals, S1 Table). The other variant, referred to hereafter as HVR IV haplotype B, has, to our knowledge, not been described previously and was present in 85/96 (88.5%) dog derived worms in the 2013 sample, and in 87/126 (69%) dog derived worms in the 2016 sample. Notably, this haplotype was not found in any of the 521 human derived worms. If the two haplotypes indeed indicate two reproductively isolated groups, which have been separated for some time in evolution, this should also be reflected in their mitochondrial and nuclear genomes. Therefore, from the 2013 sample we sequenced a portion of the mitochondrial *cox*1 gene of 21 dog-derived worms (17 with *SSU* HVR IV haplotype B and 4 with *SSU* HVR IV haplotype A) and 57 human derived worms (all with *SSU* HVR IV haplotype A) [6,36,38]. In total we identified 17 different *cox*1 haplotypes. Seven were associated with *SSU* HVR IV haplotype A and 10 with *SSU* HVR IV haplotype B (Fig 3, S1 Table). No haplotypes were shared between worms with the two *SSU* HVR IV haplotypes. In a phylogenetic analysis, the *cox*1 haplotypes associated with the two HVR IV haplotypes were well separated from each other. From the 2016 sample we selected 23 worms for whole genome sequencing and compared them over their entire genome using two different approaches (Fig 4). Both methods robustly separated the worms with *SSU* HVR IV haplotype B form all worms with *SSU* HVR IV haplotype A. The

Table 2. *SSU* HVR IV haplotype distribution in *S. stercoralis* from humans and dogs.

	From dogs			From humans		
HVR IV haplotype	**A**	**B**	**% A**	**A**	**B**	**% A**
2013 sample	11	85	11.5%	340	0	100%
2016 sample	39	87	31.0%	181	0	100%
Total	50	172	22.5%	521	0	100%

https://doi.org/10.1371/journal.pntd.0005752.t002

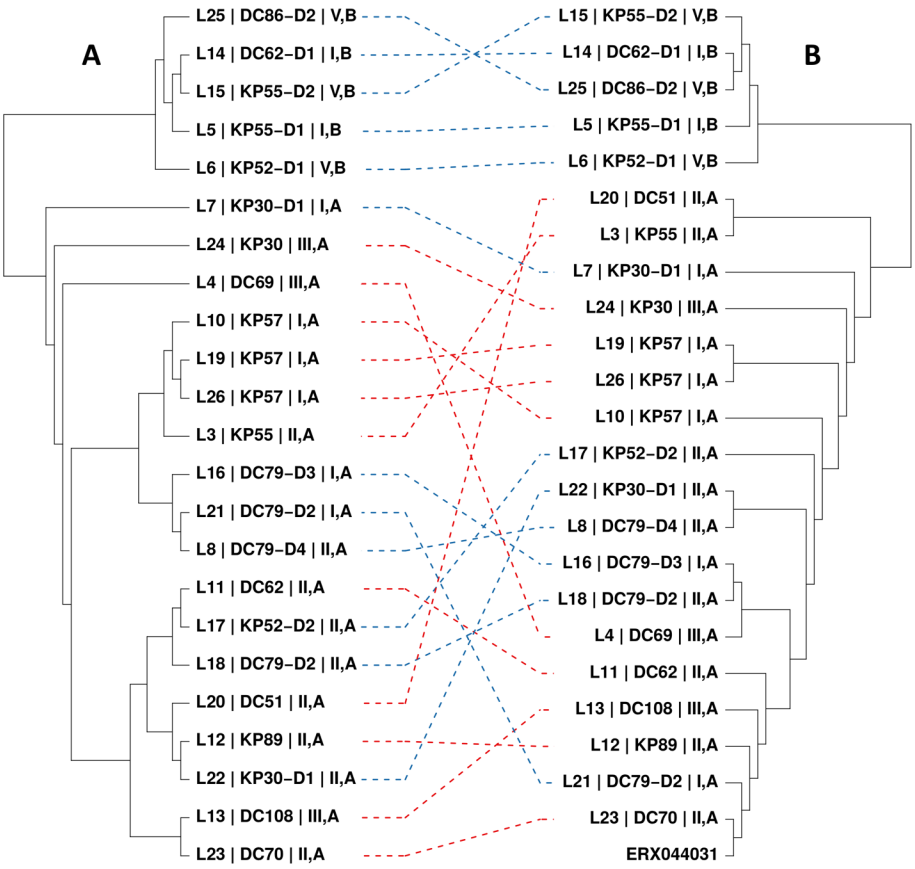

Fig 4. Sample relatedness analysis on whole genome data. A) Neighbor joining tree based on a genomic identity-by-state relationship matrix in cooperating 1326 SNPs (thresholds: LD = 0.05, MAF = 0.05). B) Neighbor joining tree based on pairwise similarities of the 23 individual genomes estimated using kWIP. The same samples in the two trees are connected with dotted lines colored in red for human derived worms and in blue for dog derived worms. ERX044031 indicates the reference genome short read data set [57], which has HVR I haplotype I and HVR IV haplotype A. The labels contain: [identifier of the worm] | [identifier of the host individual] | [HVR I haplotype, HVR IV haplotype], with each attribute separated by vertical lines.

https://doi.org/10.1371/journal.pntd.0005752.g004

two methods yielded different results on the exact topology of the tree within the two *SSU* HVR IV defined groups but provided no indication that the dog derived worms with *SSU* HVR IV haplotype A form a group different from the human derived worms. From this we conclude that dogs in our study area carry two populations of *S. stercoralis*, which are distinguishable by their *SSU* HVR IV haplotype. One population, to which the majority of the *Strongyloides* belong, was not found humans. The other population, however, is shared with humans, strongly indicating that *S. stercoralis* with *SSU* HVR IV haplotype A can shuttle between the two vertebrate host species.

Different HVR I haplotypes appear not to reflect genetically separated populations

When we analyzed the region around HVR I of *S. stercoralis* with HVR IV haplotype A, we found the same three variants (haplotypes I, II, III c.f. Fig 2) as described by Schär, Guo and colleagues [37] at various frequencies (Table 3). In combination with HVR IV haplotype B, which occurs only in dogs, we also found three variants of HVR I (haplotypes I, IV, V c.f. Fig 2). It is noteworthy that only HVR I haplotype I occurred in combination with both HVR IV

Table 3. *SSU* HVR I haplotypes of *S. stercoralis* with HVR IV haplotype A isolated from humans and dogs, 2013 and 2016.

	2013		2016	
HVR-I haplotype	From humans	From dogs	From humans	From dogs
I	28	5	5	8
II	298	6	153	31
III	14	0	23	0
Total	340	11	181	39

https://doi.org/10.1371/journal.pntd.0005752.t003

haplotypes (c.f. Fig 2) [37]. If the different HVR I haplotypes reflect at least partially separated populations this should be reflected in the whole nuclear genome and the separation should also be visible in the mitochondrial genome. However, the mitochondrial *cox*1 phylogeny is not correlated with the HVR I differences (Fig 3), indicating that there is mixing of the nuclear genome between the different mitochondrial matrilinages. In addition, data from whole genome sequencing (Fig 4) are not consistent with the null hypothesis that worms with the same HVR I haplotype are more closely related to each other than to individuals with a different HVR I haplotype. The two methods lead to different tree topologies, none of which correlates with the HVR I haplotypes. Therefore this analysis provides no evidence for the existence of genetically isolated subpopulations within the worms with HVR IV haplotype A. The same is true for the worms with HVR IV haplotype B and different HVR I haplotypes. These results show that among the worms analyzed, other than *SSU* HVR IV haplotypes, not every *SSU* HVR I haplotype appears to represent its own separate genetically isolated population.

S. stercoralis free-living adults reproduce sexually

As a possible explanation for the absence of hybrids, Schär, Guo and colleagues [37] proposed that *S. stercoralis* might not reproduce sexually, either because the free-living generation undergoes pseudogamy (sperm dependent parthenogenesis) as proposed earlier [25], or because in the study area the population propagates only through the non-sexual homogonic cycle. We showed above that the nuclear genome and the mitochondrial genomes, which are normally maternally inherited, do not evolve in parallel. This argues strongly for at least occasional sexual reproduction. Large numbers of free-living *S. stercoralis* of both sexes were present in our field and laboratory isolates and males were clearly required for reproduction in the free-living generation. Of a total of 480 females (96 from field isolates and 384 from a laboratory isolate) placed individually on plates before they had a chance to mate, none produced progeny. This result indicates that female autonomous reproduction by parthenogenesis or self-fertilization occurs rarely or not at all. However, the result is not an argument against pseudogamy, because, although they do not contribute genetic material to the progeny, males and their sperm are required to activate embryogenesis in this mode of reproduction. Further, although mitochondria are normally inherited only from the mother, in other nematodes male derived mitochondria occasionally fail to be degraded and are incorporated in zygotes [58,59]. This could also happen upon pseudogamic interaction between oocyte and sperm and lead to a recombination of nuclear and mitochondrial genomes. Therefore, we sought to demonstrate sexual reproduction more directly.

To this end we genotyped 22 individual mothers (all derived from humans) and several of their progeny at two single-copy loci (Table 4). These results demonstrate clearly that the progeny were not the product of clonal reproduction because we found larvae with genetic material absent from the mother but presumably derived from the male and/or larvae that did not have alleles present in the mother, indicating that the mother passed on only half of its genetic material as expected for sexual but not for clonal reproduction.

Table 4. Genotypes of free-living mothers and their progeny.

Female number	Host individual[a]	Informative marker and female Genotype[2]	Progeny genotypes
F1	DC51	*ytP289* 1/2	2x 1/1, 1x 2/2
F2	DC51	*ytP289* 1/2	2x 1/1, 2x 2/2
F3	DC51	*ytP289* 1/2	3x 1/1, 3x 2/2
F4	DC51	*ytP289* 1/2	1x 2/2
F5	DC51	*ytP289* 1/1	3x 1/1, 1x 1/2
F6	DC51	*ytP289* 1/2	3x 1/2, 2x 2/2
F7	DC51	*ytP289* 1/2	1x 1/1, 3x 1/2, 4x 2/2
F8	DC51	*ytP289* 1/2	2x 1/1, 3x 1/2, 2x 2/2
F9	KP57	*ytP289* 1/1	1x 1/1, 2x 1/3
F10	KP57	*ytP289* 2/3	1x 1/2, 5x 2/3, 4x 3/3
F11	KP57	*ytP289* 1/3	6x 1/2, 2x 1/3
F12	KP57	*ytP289* 2/3	1x 2/2, 1x 2/3
F13	KP57	*ytP289* 2/3	4x 1/2, 1x 1/3, 1x 2/2
F14	DC108	*ytP289* 1/2	1x 1/1, 2x 1/2
F15	DC108	*ytP289* 1/2	1x 1/1
F16	DC108	*ytP289* 1/2	2x 1/2, 3x 2/2
F17*	DC69	*ytP289* 1/2	1x 1/2, 1x 1/1
F17*	DC69	*ytP290* 1/2	3x 1/2, 2x 1/1, 2x 2/2
F18	KP31	*ytP290* 1/2	3x 1/1, 2x 2/2
F19	KP31	*ytP290* 1/2	2x 1/1, 8x 1/2, 5x 2/2
F20	KP31	*ytP290* 1/2	1x 1/1, 6x 1/2, 4x2/2
F21	KP31	*ytP290* 1/2	2x 1/1, 1x 1/2
F22	KP31	*ytP290* 1/2	3x 1/2, 1x 1/1, 2x 2/2

[a]The host individual is defined by the two-letter code for the village followed by the host individual number.

[2]*ytP289* contains single nucleotide polymorphisms at positions 308 (A/G), 359 (A/G), 416 (C/T) and 566 (C/T).

Allele 1 has the combination G+A+C+C, allele 2 A+G+T+T, and allele 3 A+A+C+C. *ytP290* contains single nucleotide polymorphisms at positions 291 (A/C) and 310 (A/G). Allele 1 has the combination A+A, allele 2 C+G.

*F17 was the only case where both markers were informative.

https://doi.org/10.1371/journal.pntd.0005752.t004

We sought to further solidify this in a fully controlled experiment with known males and a larger number of progeny. To this end, we set up crosses with single females and males using a laboratory isolate of *S. stercoralis*. The results (Table 5) were fully consistent with Mendelian inheritance with equal genetic contribution by males and females but not with clonal reproduction.

Are there hybrids between *SSU* haplotypes?

Given the results above, one would expect to observe animals that are hybrids between the different *SSU* HVR I haplotypes. However, like in an earlier study [37], we failed to find any hybrids among the 436 larvae from 97 different host individuals (68 humans 29 dogs) isolated in 2013. The *SSU* locus is on the X chromosome in *S. stercoralis* (information extracted from a previously published dataset [57]). Therefore, hybrid males could not be detected because they only contain one X chromosome. However, for [37] and in our 2013 sampling, young larvae that were the progeny of parasitic worms were analyzed. The sex of these larvae was unknown but given that the field isolates produced both sexes, it is very likely that a substantial number of females were among them. In 2016 we tested adult worms and used predominantly males because, unlike females, they may not contain genetic material from other individuals (sperm,

Table 5. Genotypes of free-living parents and their progeny at *ytP274*.

Cross	Female genotype[a]	Male genotype[a]	Progeny genotypes[a]
C1	C/T	C/T	4x C/T, 1x C/C, 3x T/T
C2	C/T	C/T	4x C/T, 2x T/T, 2x C/C
C3	C/T	C/C	4x C/C, 4x C/T
C4	T/T	C/T	4x C/T, 4x T/T
C5	T/T	C/T	4x C/T, 4x T/T
C6	C/C	C/T	3x C/C, 5x C/T
C7	C/T	C/C	4x C/C, 4x C/T
C8	C/T	T/T	3x C/T, 5xT/T
C9	T/T	C/T	4xC/T, 4x T/T
C10	C/C	C/T	4x C/C, 4xC/T
C11	C/T	T/T	4x C/T, 4x T/T
C12	C/T	C/T	4x C/C, 3x C/T, 1x T/T
C13	C/T	C/T	5x C/T, 1x C/C, 2x T/T
C14	C/T	C/T	4x C/T, 1x C/C, 3x T/T
C15	C/T	C/C	5x C/C, 3x C/T
C16	C/T	C/T	3x C/T, 2x C/C, 3x T/T
C17	C/C	C/T	3x C/T, 5x C/C
C18	T/T	C/T	6x C/T, 2x T/T

[a]The marker *ytP274* has a single nucleotide polymorphism (T/C) at position 236.

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embryos). Only 30 of the 307 worms from 2016 listed in Table 2 were pre-reproductive females. None of them was a hybrid.

However, of the gravid females described in Table 4 one (Female 17) and one of its progeny showed a mixture of HVR I haplotypes II and III. While the mixed signal in the female might have been caused by sperm and embryos derived from a male with a different haplotype than the mother, the larva was most likely a true hybrid. This prompted us to test fully mature females that had been isolated from cultures, after they had a chance to mate, preserved in ethanol and separated by host individuals. We analyzed 9 to 23 worms from each of six host individuals (four humans, two dogs), from which we had already isolated *S. stercoralis* of different HVR I haplotypes. In five of the cases we found worms with mixed haplotypes, along with individuals with only one *SSU* haplotype (Table 6). Two observations are noteworthy. Firstly, both dogs had worms with HVR IV haplotypes A and B. In total, 11 out of 21 worms had mixed HVR I haplotypes but only a single one showed a mixture between the two HVR IV

Table 6. *SSU* haplotypes found in individual gravid females.

Host (species)	Non-hybrid genotypes HVR I + HVR IV (number of individuals)	Mixed genotypes HVR I + HVR IV (number of individuals)	Mixed/Total
DC44 (Human)	II+A (12)	-	0/12
DC69 (Human)	II+A (6), III+A (3)	II/III+A (14)	14/23
KP30 (Human)	II+A (3)	II/III+A (8)	8/11
KP57 (Human)[a]	II+A (5)	I/II+A (9)	9/14
DC79D2 (Dog)	II+A (4), V+B (1)	I/II+A (1), V/IV+B (3)	4/9
KP52D2 (Dog)	II+A (4), IV+B (1)	V/IV+B (6), V/II+A/B (1)	7/12

[a]from this host individual we also genotyped 12 pre-reproductive females and 19 males.
All females and 14 males were of haplotype II+A, 5 males were I+A.

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haplotypes, which might indicate that males and females with different HVR IV haplotypes tend to avoid each other. Secondly, from host individual KP57 (human), 9 of 14 worms had mixed haplotypes. From the same host individual we had also genotyped 12 pre-reproductive females, none of which was a hybrid. This finding suggests that the females genotyped in this experiment were not true hybrids but contained sperm and developing embryos derived from males of the other haplotype detected.

Discussion

In rural communities in Cambodia, many people share their premises with domestic animals and the general hygenic, water and sanitation infrastructures are precarious [60,61]. Therefore, the conditions appear very favorable for human to animal and animal to human transmission of STH including *S. stercoralis* [3]. In order to find evidence for or against zoonotic transmission of *S. stercoralis* under such circumstances, we isolated large numbers of *S. stercoralis* from humans and dogs at the same time and in the same households and analyzed individual worms using molecular genetic markers. To our knowledge there had been no such study of *S. stercoralis* of comparable scale undertaken anywhere. It should be noted that our experimental strategy aimed to sample individuals with a large potential for transfer of *Strongyloides* spp. between the two hosts (e.g., only dogs found close to households with positive people were sampled). Therefore our study was not designed to yield accurate estimates of haplotype frequencies in the entire population. We also point out that we did not directly demonstrate transmission from dogs to humans and therefore cannot exclude that the transmission is mostly or exclusively from human to dog. Nevertheless, our results strongly suggest that there is a considerable risk for dog to human transmission. This would not be in agreement with conclusions by Takano and colleagues [62] who found that humans in households with *Strongyloides*-infected dogs were not more likely to be parasitized by *S. stercoralis* than those with parasite free dogs and concluded that natural transmission does not occur between humans and dogs. However, this study was conducted in Japan, in areas with presumably much better sanitary conditions than in the Cambodian villages where the present study was conducted. Consequently, only five *Strongyloides* positive dogs were found and none of their owners was infected. Likewise, a study conducted in Southern China, in a setting probably more comparable to our study area [47], did not identify the presence of animals as a statistically significant risk factor for human strongyloidiasis. However, this conclusion was based on only 21 infected individuals (11.7% of the tested), and no details about the exposure to dogs are given. In rural settings dogs are usually semi-domesticated and roam freely such that the risk of exposure to contamination by canine feces among people who do not own a dog themselves might be approximately equal to the risk among people who do. Therefore, the lack of statistical significance cannot be taken as evidence against zoonotic transmission. Interestingly, a later study in a similar setting [63] revealed that anthelmintic treatment of people alone was not sufficient to significantly reduce the prevalence of *S. stercoralis*.

Overall, the present findings strongly suggest that dogs must be seriously considered as sources for human strongyloidiasis. Whether dogs are the only non-human carrier of concern or if other animals also have the same potential remains to be determined. However, our results also show that at least in our study area, the majority of the *Strongyloides* present in dogs is of a genotype that we never found humans. Therefore, the number of *Strongyloides* spp. detected in dogs by coproscopic diagnosis might be an inaccurate index of the risk of exposure to *S. stercoralis* that dogs pose to humans.

The 18S rDNA HVR IV appears to be diagnostic for the two separate *Strongyloides* populations in dogs. This agrees with the findings of Hasegawa and colleagues [35] who found this region to be invariable within *S. stercoralis*. Both, the mitochondrial *cox*1 and whole genome

sequence analyses confirmed that the two HVR IV variants represent separate phylogenetic groups. Therefore, our results support the proposal by Brumpt and Augustine [43,44] of a separate species, *Strongyloides canis*.

Consistent with [37] we found three different 18S rDNA HVR-I genotypes in human derived *S. stercoralis*. Worms of different haplotypes sometimes co-existed in the same host individual. In comparison with other nematodes [27–33] it is unusual that such differences in this region of the *SSU* occur within one species. However, species status can never be inferred from sequence information alone. Nevertheless, in those examples in nematodes where the same fragment around the HVR I was used and more rigorous criteria for species separation (e.g. mating experiments) could be applied, a sequence difference of more than one position was a safe indicator of a distinct species [28–33]**.** Most of the time, this region appears completely invariable within a species and there are several examples where even separate species do not differ in their HVR I. However, our comparative analyses of the mitochondrial *cox*1 locus and of whole genome data suggest that in *S. stercoralis*, different HVR I genotypes do not indicate separate species, but rather that in *S. stercoralis* the HVR I is more variable than in other nematodes. It should be noted that all the well-studied cases mentioned above involve obligate sexual nematode species, some of which are capable of self-fertilization. Asexual reproduction through the homogonic cycle, which may be a frequent mode of reproduction in *S. stercoralis*, might contribute to a higher variability within the species. Nevertheless, although we show that the different HVR I genotypes are not diagnostic for different species, our results do not exclude the existence of cryptic species among *S. stercoralis*. It is striking that, like Schar et al. [37], we failed to detect hybrids between different HVR I haplotypes among all worms that were the progeny of parasitic mothers and that were definitely unmated. However, in mature free-living females and in their progeny, we frequently found mixed signals. While, for reasons described above, we doubt that these females were true hybrids, their offspring presumably were. We think that this indicates that adults of different HVR I haplotype do mate, at least in laboratory fecal cultures, and that at least some of the progeny develop to larval stages. We can only speculate about why such hybrids are not found in the progeny of the parasitic generation. It might be that the hybrids, or even the progeny of the free-living generation in general, are sub-viable and only rarely develop into successful fertile parasitic females. In this case genetic mixing between subpopulations would occur only rarely. Over long periods of time, even rare exchange might be significant and cause enough mixing of the genomes that we were not able to detect genetic differentiation between subpopulations. Alternatively, in the *S. stercoralis* populations in our study area there might be very high inbreeding (brother sister mating) under natural conditions. This would lead to a very high degree of homozygosity in the population. Both scenarios described above would lead to a very low number of *SSU* haplotype heterozygotes in the population, and we may have simply missed the rare hybrids. In order to address this, controlled crosses and experimental infections are required. In the context of this study we had no opportunity to do such experiments because, for logistic and for legal reasons, we were not able to bring live worms into our laboratory.

Our results demonstrate, however, that such crossing experiments are possible. We demonstrate that in contrast to earlier claims [25], sexual reproduction in the free-living generation of *S. stercoralis* does occur and is likely the predominant, if not the only the mode of reproduction in this generation. With this, the number of species of *Strongyloides* for which genetic analyses demonstrated that the reproduction in the free-living generation is sexual rises to four out of four tested (*S. ratti* [23,24], *S. papillosus* [22], *S. vituli* [64], *S. stecoralis*, this study). Sexual reproduction by *S. stercoralis* has also been confirmed recently by experimental crossing of free-living male and female worms harboring discrete reporter transgenes[65]. Although these findings do not exclude that asexual species or strains might exist, even within what is currently referred to

as *S. stercoralis*, they do suggest strongly that sexual reproduction rather than pseudogamy is the prevailing mode of reproduction in free-living *Strongyloides* spp.

Conclusions and outlook

Our results provide a compelling solution for the long-standing controversy about whether the *Strongyloides* sp. of dogs is identical to the *S. stercoralis* of humans or not. In fact, both scenarios appear to be true. Dogs, at least in our study area, host two different populations. These either represent separate species or well-separated sub-species of *Strongyloides* spp, and only one of them is shared with humans. It remains to be determined if the different types of *Strongyloides* we observed in humans and dogs also occur in other regions of the world. Among *S. stercoralis* in humans there is variability in the rDNA sequence. While we did not find further genomic evidence supporting multiple genetically separate populations in humans the absence of hybrids between the different *SSU* HVR I haplotypes is striking. It will be most interesting to ascertain whether different *SSU* HVR I types indeed interbreed and, even more importantly, if they might be associated with different clinical outcomes. Therefore, we suggest using molecular diagnostics for *Strongyloides* spp. wherever possible. In order to generate comparable data, we propose following the lead of Hasegawa and colleagues [35,38], and using the *SSU* HVRs I and IV and the mitochondrial *cox*1 locus as primary markers as was done in this study. With respect to strongyloidiasis control and prevention, this study suggests that dogs should be seriously considered as a source for human *S. stercoralis* infection at least in settings similar to our study area. Prevention of human contact with dog feces and of dog contact with human excrement as well as anthelmintic treatment of dogs are likely to reduce the exposure of humans to infective *S. stercoralis* larvae.

Supporting information

S1 Text. Sequences of the *cox1* haplotypes and the molecular markers. The sequences of the mitochondrial *cox1* haplotypes and the sequences of the nuclear molecular markers are given. GenBank accession numbers are in (KX226367-KX226384). For the nuclear markers the polymorphic positions are indicated by red boxes.
(DOCX)

S1 Table. *SSU* HVR I and IV and *cox 1* haplotypes of all individuals analyzed. Sheet 1: worms collected 2016 from humans. Sheet 2: worms collected 2016 from dogs. Sheet 3: worms collected 2013 from humans. Sheet 4: worms collected 2013 from dogs.
(XLSX)

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RESEARCH ARTICLE

Characterization of a non-sexual population of *Strongyloides stercoralis* with hybrid 18S rDNA haplotypes in Guangxi, Southern China

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Abstract

Strongyloidiasis is a much-neglected but sometimes fatal soil born helminthiasis. The causing agent, the small intestinal parasitic nematode *Strongyloides stercoralis* can reproduce sexually through the indirect/heterogonic life cycle, or asexually through the auto-infective or the direct/homogonic life cycles. Usually, among the progeny of the parasitic females both, parthenogenetic parasitic (females only) and sexual free-living (females and males) individuals, are present simultaneously. We isolated *S. stercoralis* from people living in a village with a high incidence of parasitic helminths, in particular liver flukes (*Clonorchis sinensis*) and hookworms, in the southern Chinese province Guangxi. We determined nuclear and mitochondrial DNA sequences of individual *S. stercoralis* isolated from this village and from close by hospitals and we compared these *S. stercoralis* among themselves and with selected published *S. stercoralis* from other geographic locations. For comparison, we also analyzed the hookworms present in the same location. We found that, compared to earlier studies of *S. stercoralis* populations in South East Asia, all *S. stercoralis* sampled in our study area were very closely related, suggesting a recent common source of infection for all patients. In contrast, the hookworms from the same location, while all belonging to the species *Necator americanus*, showed rather extensive genetic diversity even within host individuals. Different from earlier studies conducted in other geographic locations, almost all *S. stercoralis* in this study appeared heterozygous for different sequence variants of the 18S rDNA hypervariable regions (HVR) I and IV. In contrast to earlier investigations, except for three males, all *S. stercoralis* we isolated in this study were infective larvae, suggesting that the sampled population reproduces predominantly, if not exclusively through the clonal life cycles. Consistently, whole genome sequencing of individual worms revealed higher heterozygosity than reported earlier for likely sexual populations of *S. stercoralis*. Elevated heterozygosity is frequently associated with asexual clonal reproduction.

which also provided funds for other project costs through the core budget of the MPI for developmental Biology (http://www.eb.tuebingen.mpg.de) and the FM-Laboratry (http://www.fml.tuebingen.mpg.de) (no grant numbers). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Author summary

The vast majority of multicellular organisms reproduce sexually. Sexual reproduction is believed to be advantageous because meiotic recombination separates beneficial and deleterious mutations and generates new, possibly better allele combinations. However, sexual reproduction comes at a cost. Beneficial allele combinations are broken up and, in gonochoristic species, there is the "two-fold cost of sex" due to the investment in "unproductive" males. *Strongyloides stercoralis*, the causing agent of the grossly neglected but sometimes fatal human strongyloidiasis, appears to get the best of both worlds. Depending on the environmental conditions, *S. stercoralis* switches between asexual parasitic and sexual free-living generations. In the Guangxi province (China) we identified a population of *S. stercoralis* that appears to have recently transitioned to predominantly if not exclusively reproducing asexually. We failed to detect sexual stages and, in the genomes, we found indication of asexuality such as a high heterozygosity, compared with other populations of *S. stercoralis*. Additionally, global within-species phylogenetic analysis showed that in our study area, all *S. stercoralis* form one group of fairly close relatives, suggesting a rather recent common origin. In contrast, the hookworms (*Necator americanus*), which are phylogenetically distant from but share the infection route with *S. stercoralis*, in a within-species phylogenetic comparison fall into various groups, suggesting much greater diversity.

Introduction

Soil-transmitted helminths (STHs) are parasitic worms that infect hosts by transmitting their eggs or larvae through contaminated soil. Among them are parasitic nematodes like giant roundworms (*Ascaris lumbricoides*), hookworms (*Necator americanus* and *Ancylostoma* spp.), whipworms (*Trichuris*) and threadworms (*Strongyloides*). STHs infect up to one quarter of the world's population and cause helminthiases, which are considered to be neglected tropical diseases (NTDs). Impoverished populations with limited access to clean water, sanitation, and opportunities for socioeconomic development are disproportionately affected [1]. *Strongyloides stercoralis* is the prime causative agent of human strongyloidiasis [2]. Estimates of worldwide human infection with *S. stercoralis* vary but go up to 370 million [3–5]. *S. stercoralis* is generally more prevalent in tropical and subtropical countries, and local prevalences of over 40% in particular regions have been reported [6, 7]. However, the presence of *S. stercoralis* and strongyloidiasis, including fatal cases, have also been reported from well-developed regions with temperate climates such as the European Union and North America [8–15].

S. stercoralis has a rather unique life cycle (Fig 1A) with the possibility of forming free-living adults in between parasitic generations [16–18]. The parasitic adults are all females and reproduce by mitotic parthenogenesis. Nevertheless, they can produce progeny of both sexes. Their female progeny have three developmental options. They can develop into infective third stage larvae (iL3) within the host (1 in Fig 1A) and infect the same host without ever leaving the host (auto infective cycle, notice that auto infective iL3s are not normally present in naturally deposited feces and are therefore not detectable with the methods employed in this study). Alternatively, the female larvae can leave the host with the feces as early (first stage) larvae and continue their development in the environment. There, in approximately one to two days, they either become iL3s (2 in Fig 1A), which search for another host (direct/homogonic cycle) or develop into free-living adults (3 in Fig 1A), as do all the males (4 in Fig 1A) (indirect/heterogonic cycle). The free-living adults reproduce sexually. Their progeny are all females and invariably develop into iL3s. The auto infective cycle appears to be specific for *S. stercoralis* and it is a

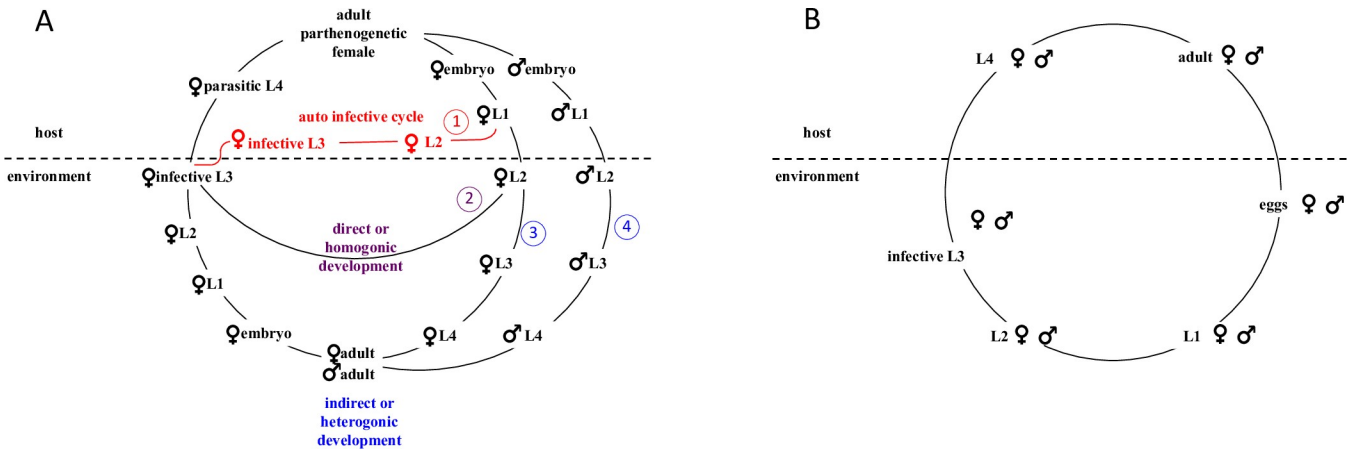

Fig 1. The life cycles of *S. stercoralis* and *N. americanus*. (A) The life cycle of *Strongyloides stercoralis*. The numbers refer to the numbers of the developmental options in the description of the life cycle in the text. This figure was reproduced from [50] under the creative commons license. Notice that auto infective iL3s are not normally observed in naturally deposited feces and were therefore not detectable with the methods used in this study. (B) The life cycle of *N. americnaus*.

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prerequisite for the severe pathogenicity caused by this species [16]. This explains why strongyloidiasis in humans is a severe disease but *Strongyloides* spp. are of only very minor veterinary concern [4, 19], with the exception of great apes in captivity [20] and dogs [21], which are also hosts for *S. stercoralis*. The auto infective cycle allows the infection to persist in one host for many years, which is much longer than the life expectancy of an individual worm. Most of the time, infection with *S. stercoralis* is asymptomatic in healthy hosts, rendering it rather unlikely to be detected. If the host becomes immuno-deficient, the control of the infection may fail, resulting in a self-enhancing progression of strongyloidiasis, known as hyperinfection syndrome and disseminated strongyloidiasis, which is lethal if not treated [3–5, 22].

All species of *Strongyloides* investigated so far may undergo homogonic or heterogonic development. The switch between the two life cycles is influenced by various environmental factors, such as the immune status of the host, temperature or food availability, but also by genetic pre-disposition [23], such that different isolates may show very different homogonic to heterogonic ratios even under standard laboratory conditions [24].

Hookworms are among the most prevalent parasitic nematodes in humans. The estimation of hookworm human infection is between 576–740 million (estimate of the CDC, https://www.cdc.gov/parasites/hookworm/index.html, assessed January 21st 2019) [25]. *Necator americanus* and *Ancylostoma duodenale* are the most common human hookworm species but an increasing number of presumably zoonotic infections with *Ancylostoma ceylanicum* has been reported from Asia [26]. In China, all these three species are present. Infections with a small number of hookworms are normally asymptomatic, while more severe infections cause medical problems associated with the blood sucking life style of these worms. The life cycle of hookworm is rather simple (Fig 1B). The female and male parasitic adults mate inside the host, producing eggs which are passed by defecation. The larvae hatch and develop into iL3s in the environment and are then ready to infect the next host [27].

Hookworms and *Strongyloides* spp. are phylogenetically rather distant from each other, belonging to different major clades and their parasitic life styles have presumably arisen independently in evolution [28–30]. Nevertheless, their modes of transmission are very similar. The iL3s of hookworms and *Strongyloides* mature in the environment, penetrate the skin of the host and undergo a similar body migration. Therefore, hookworm and *Strongyloides* co-

infection were frequently observed all around the world (Argentina [31], Brazil [32], Cambodia [33], Tanzania [34], Côte d'Ivoire [35], Ghana [36], Thailand [37]).

Parts of the ribosomal *cox1* gene and the nuclear small ribosomal subunit rDNA (18S rDNA, *SSU*) sequence, in particular the hyper variable regions I and IV (HVR-I and HVR-IV), are frequently used as markers for molecular taxonomy in nematodes (e.g. [29, 38–45]) including *Strongyloides* spp. [46–53]. In *S. stercoralis* isolated from humans the HVR-IV appears virtually invariable. Only one instance of a single nucleotide difference to the reference sequence AF279916 has been reported [49] (accession number M84229). It should, however be noted that [50] found a different HVR-IV sequence in a large portion of the *Strongyloides* sp. isolated from dogs, which are generally also considered to be *S. stercoralis*. In HVR-I, several sequence variants were found by multiple authors [47, 50, 51, 53]. In three recent studies, by genotyping individual *S. stercoralis* isolated from humans in Cambodia [50, 53] and Myanmar and Japan [51], two polymorphic positions were identified in the region around the HVR-I of human derived *S. stercoralis*. One is a sequence of four or five consecutive Ts located in the HVR-I proper corresponding to position number 176–179 of the reference sequence AF279916, the other one is an A/T polymorphism at position 458. Interestingly, although all three studies found worms of different haplotypes to occur sympatrically, sometimes even within the same host individual, [53] and [50] found no and [51] only very few hybrids between different haplotypes, sparking the question if multiple, genetically isolated populations of *S. stercoralis* exist in humans. Whole genome analysis of individual worms suggested that substantial genetic diversity exists among *S. stercoralis* isolated from human hosts [51, 54] but did not support the hypothesis of separate populations defined by the different *SSU* HVR-I haplotypes [50].

To differentiate the different hookworm species *Necator* spp. and *Ancylostoma* spp., the commonly used nuclear molecular markers are ribosomal ITS rather than 18S sequences [40, 41, 55–58]. Based on ITS and mitochondrial *cox1* sequences, [40, 41] defined multiple genetically separated groups of what would generally be considered *N. americanus* in humans in Africa and proposed that they should possibly be considered different species, i.e. *N. americanus* and *N. gorillae*.

Here we describe the isolation and genomic characterization of a population of *S. stercoralis* from a village and local clinics in the Guangxi province in Southern China. In contrast to earlier studies, the vast majority of individuals in this population appear heterozygous for different *SSU* haplotypes and they reproduce predominantly, if not exclusively, through the non-sexual auto-infective and homogonic life cycles. Consistent with asexual reproduction, we found elevated heterozygosity in the genomes of individual worms, compared with individuals from other populations of *S. stercoralis*. All *S. stercoralis* isolated in Guangxi were closely related arguing for a rather recent common source of infection. As a comparison, we also analyzed the hookworms isolated from the same village. While all these hookworms belonged to the species *Necator americanus*, they showed a rather high genetic diversity, even within host individuals, in comparison with other *N. americanus* populations.

Materials and methods

Ethics statements

The sampling of human derived material including the procedures to obtain informed consent, was approved by the "Medical Ethics Committee" of the Guangxi Medical University (no project specific number issued). All participants were volunteers and gave informed consent. Because not all people in the study area are literate, a protocol of oral consent was used as follows. The people were informed about the study by representatives of the local Center for Disease Control (CDC). The participants, or in the case of children their parents, showed their

consent in two steps. First, they registered for the study and claimed collection containers. At this step, the participants were added to a list and assigned a number such that the health care providers could later identify, inform and treat the infected people but the sample was anonymized for the scientific analysis. Second, the participants submitted the sample the next day. Also this step was voluntary and a number of people did not return samples in spite of having claimed collection containers. Participants received a small financial compensation according to local habits. All participants found to be infected with pathogens were treated with anthelminthic drugs by the local disease control and prevention center (CDC) according to the related treatment guidelines.

Experiments involving *S. stercoralis* culture in host animals were in accordance with the "Guiding Opinions on the Treatment of Laboratory Animals" (issued by the Ministry of Science and Technology of the people's republic of China) and the Laboratory Animal Guideline for Ethical Review of Animal Welfare (issued by the National Standard GB/T35892-2018) and were reviewed and approved by the "Animal Care and Welfare Committee" of the Guangxi Medical University (S5 File).

Study area

Human fecal samples were collected in Long An (LA) (23˚13'N 107˚25'E) and Qing Xiu (QX) (22˚46'N 108˚39'E) in May and June 2018. Both districts are located in the region around Nanning, Guangxi province, Southern China. These two districts were chosen either because a generally high incidence of helminth parasites had been noticed in an earlier survey (LA) or because an inhabitant had recently been diagnosed with strongyloidiasis in a local hospital (QX).

Sample collection, stool examination and worm isolation

Sample boxes were distributed to the people who agreed to participate in this study and in the following day fecal samples were collected. In both districts (LA and QX) fecal samples were collected for two consecutive days.

To identify the helminth eggs, approximately 1g fresh feces from each sample were examined by the Acid Ether Sedimentation method as described [59–61]. In brief, feces were mixed with 7 ml 19% hydrochloric acid and large debris were removed. Then 3 ml ether was added, mixed thoroughly and centrifuged at 1500 rpm for 5 min. The centrifugation resulted in four layers, which were the ether, lipid debris, hydrochloric acid, and the sediment. After removing the upper three layers, one drop of 2% iodine was added to the sediment to stain the fixed eggs, which were observed microscopically.

To isolate worms, the rest of the fecal samples were processed as described [50]. In brief, feces were mixed with sawdust and moisturized with water, then cultured at ambient temperature for 24–48 hours to allow the larvae to develop to a stage where individuals destined to become iL3s, free-living males and free-living females can unambiguously be distinguished morphologically. Then the worms were isolated with Baermann funnels. Notice that auto infective iL3s are not normally isolated with this methodology. From the positive Baermann funnels, worms were transferred individually into 10 μl water and stored at -80˚C. The samples from local clinics we obtained in the form of isolated worms conserved in 70% ethanol and stored at ambient temperature. These worms were washed twice in water, and transferred individually into 10 μl water and stored at -80˚C.

Fecal samples were also collected from dogs from *S. stercoralis* positive households with the consent and the help of the owners. The samples were taken directly from the rectums of the animals. Feces were placed on NGM agar plates and incubated for 24–48 hours at ambient temperature. Any emerging worms were directly examined and collected.

Single worm DNA preparation

Worms stored in 10 μl water were frozen and thawed 3 times with liquid nitrogen. Then 10 μl 2X lysis buffer (20 mM Tris-HCl pH 8. 3, 100 mM KCl, 5 mM $MgCl_2$, 0.9% NP-40, 0.9% Tween 20, 240 μg/ml Proteinase K) were added and incubated at 65˚C for 2 hours. The worm lysates were stored at -80˚C until they were transported to Tuebingen on wet ice and stored again at -80˚C until analyzed.

PCR and sequencing of the *SSU*, ITS rDNA and the mitochondrial gene *cox*1

2.5 μl of worm lysate were used as template for PCR amplification of *SSU* HVR-I, *SSU* HVR-IV, ITS and *cox*1 by using *Taq* DNA polymerase (M0267, New England BioLabs) according to the manufacture's protocol. Cycling protocol: An initial denaturation step at 95˚C for 30 sec was followed by 35 cycles of denaturation at 95˚C for 20 sec, annealing for 15 sec, extension at 68˚C for 90 sec and a final extension step of 5 minutes at 68˚C. The primers and the respective annealing temperatures are listed in Table 1.

Sequencing reactions were done with 1 μl of the PCR products and the sequencing primers listed in Table 1 using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. The reactions were submitted to the in-house sequencing facility at the Max Planck Institute for Developmental Biology at Tuebingen for electrophoresis and base calling.

Table 1. Primers and annealing temperatures for *SSU*, ITS rDNA and *cox*1 amplifications and sequencing.

	Primer		Sequence (5'-3')	Anneal	Product
SSU HVR-I (SS and HW)	Fw	RH5401	AAAGATTAAGCCATGCATG	52˚C	862 bp (SS) 883 bp (HW)
	Rev	RH5402	CATTCTTGGCAAATGCTTTCG		
	Seq	RH5403 (SS) ZS6492 (SS) RH5401 (HW)	AGCTGGAATTACCGCGGCTG AAACATGAAACCGCGGAAAG AAAGATTAAGCCATGCATG		
SSU HVR-IV (SS and HW)	Fw	18SP4F	GCGAAAGCATTTGCCAA	57˚C	712 bp (SS) 702 bp (HW)
	Rev	18SPCR	ACGGCCGGTGTGTAC		
	Seq	ZS6269 (SS) 18SP4F (HW)	GTGGTGCATGGCCGTTC GCGAAAGCATTTGCCAA		
ITS-1, ITS-2 (HW)	Fw	NC5	GTAGGTGAACCTGCGGAAGGATCATT	50˚C	1095 bp
	Rev	NC2	TTAGTTTCTTTTCCTCCGCT		
	Seq	ZS6991 (ITS-1) NC2 (ITS-2)	TTAGTTTCTTTTCCTCCGCT GCTGCGTTTTTCATCGAT		
*cox*1 (SS)	Fw	ZS6985	GGTGGTTTTGGTAATTGAATG	47˚C	837 bp
	Rev	ZS6986	ACCAGTYAAACCACCAATAGTAA		
	Seq	ZS6990	GGTTGATAAACTATAACAGTACC		
*cox*1 (HW)	Fw	ZS6985	GGTGGTTTTGGTAATTGAATG	47˚C	963 bp
	Rev	ZS6989	TCACCACAAACTAATACCCGT		
	Seq	ZS6989	TCACCACAAACTAATACCCGT		
SSU (SS)	Fw	ZS6492	AAACATGAAACCGCGGAAAG	65˚C	1625 bp
	Rev	ZS6495	CGACTTTTGCCCGGTTCAAA		
	Seq	RH5403 (HVR-I) ZS6269 (HVR-IV) M13F M13R	AGCTGGAATTACCGCGGCTG GTGGTGCATGGCCGTTC GTAAAACGACGGCCAG CAGGAAACAGCTATGAC		

Fw: forward primer; Rev: reverse primer; Seq: sequencing primer; SS: *S. stercoralis*; HW: Hookworm.

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rDNA and *cox1* haplotype determination and phylogenetic analysis

The sequences were analyzed with SeqMan Pro version 12 (Lasergene package; DNAStar, Inc., Madison, WI USA) and inspected manually. For position numbering the following sequences were used as reference: AF279916 for *S. stercoralis SSU* sequences; LC050212 for *S. stercoralis cox1* sequences; AJ920348 and AF217891 for hookworm *SSU* and ITS, respectively; AJ417719 for hookworm *cox1*. For *S. stercoralis cox1* the same 552 bp as in [50] were considered. For hookworm *cox1* the same 670 bp as in [40] were considered.

The *cox1* sequences were aligned and phylogenetic analysis was performed using MEGA7 [62] with the maximum-likelihood (ML) model as described previously [50]. For the *S. stercoralis* tree, *Necator amercanus* (AJ417719) was used as outgroup species. For the *Necator amercanus* tree, *Ancylostoma duodenale* (AJ417718), *Ancylostoma caninum* (FJ483518) and *S. stercoralis* (LC050212) were used as outgroup species. For comparison, selected published *cox1* sequences were also included in the analysis. The corresponding accession numbers and references are listed in Figs 2 and 3.

Cloning of the *SSU* fragments containing HVR-I and HVR-IV of individual *S. stercoralis*

In order to determine which HVR -I and HVR-IV haplotypes occurred in the same allele of the *SSU* heterozygous worms, we amplified 1625 bp out of 1703 bp of the *SSU* (positions 39 to 1663 in the reference AJ417719), including both, HVR-I and HVR-IV of individual *S. stercoralis*. 2.5 μl of worm lysate were used as template for PCR amplification by using Q5 Hot Start high-fidelity DNA polymerase (M0493, New England BioLabs) according to the manufacture's protocol. Cycling protocol: An initial denaturation step at 98˚C for 30 sec was followed by 35 cycles of denaturation at 98˚C for 10 sec, annealing for 15 sec, extension at 72˚C for 90 sec and a final extension step of 2 minutes at 72˚C. The primers and the annealing temperature are listed in Table 1.

The PCR products were purified with Wizard SV Gel and PCR Clean-Up System (A9282, Promega). 3' A-overhangs were added and cloned into pCR 2.1-TOPO vector and transfected into TOP10 competent *E. coli* cells by using the TOPO TA Cloning Kit (45–0641, Invitrogen) following the manufacturers protocol. For each *S. stercoralis*, multiple colonies were selected and cultured overnight at 37˚C and 200 rpm in 2 ml LB medium containing 50 μg/ml ampicillin. Plasmids were isolated using the QIAprep Spin Miniprep Kit (27106, QIAGEN). The presence of an insert was confirmed by *Eco*R I (FD0274, Thermo Fisher Scientific) restriction analysis. Sequencing was done using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems) as described above with 1 μl of plasmid DNA as template and the sequencing primers described in Table 1. The sequences were analyzed as described above.

Infection of *S. stercoralis* in gerbils

4-week-old female gerbils were brought from Zhejiang Medical College and injected with prednisolone acetate (3 mg) 2 days before infection and once per week after infection. Around 300 infective larvae isolated from one human host (QX24) were washed three times in tap water and incubated in PBS with antibiotic (50 mg/L streptomycin and 50 mg/L penicillin) for 1h at ambient temperature. Infective larvae were then washed again in water, and injected subcutaneously at the neck of one gerbil. Feces of the gerbil were collected daily for 1 month starting from 7-day post infection. Feces were moisturized with water and incubate at ambient temperature for 1 day followed by Baermann analysis.

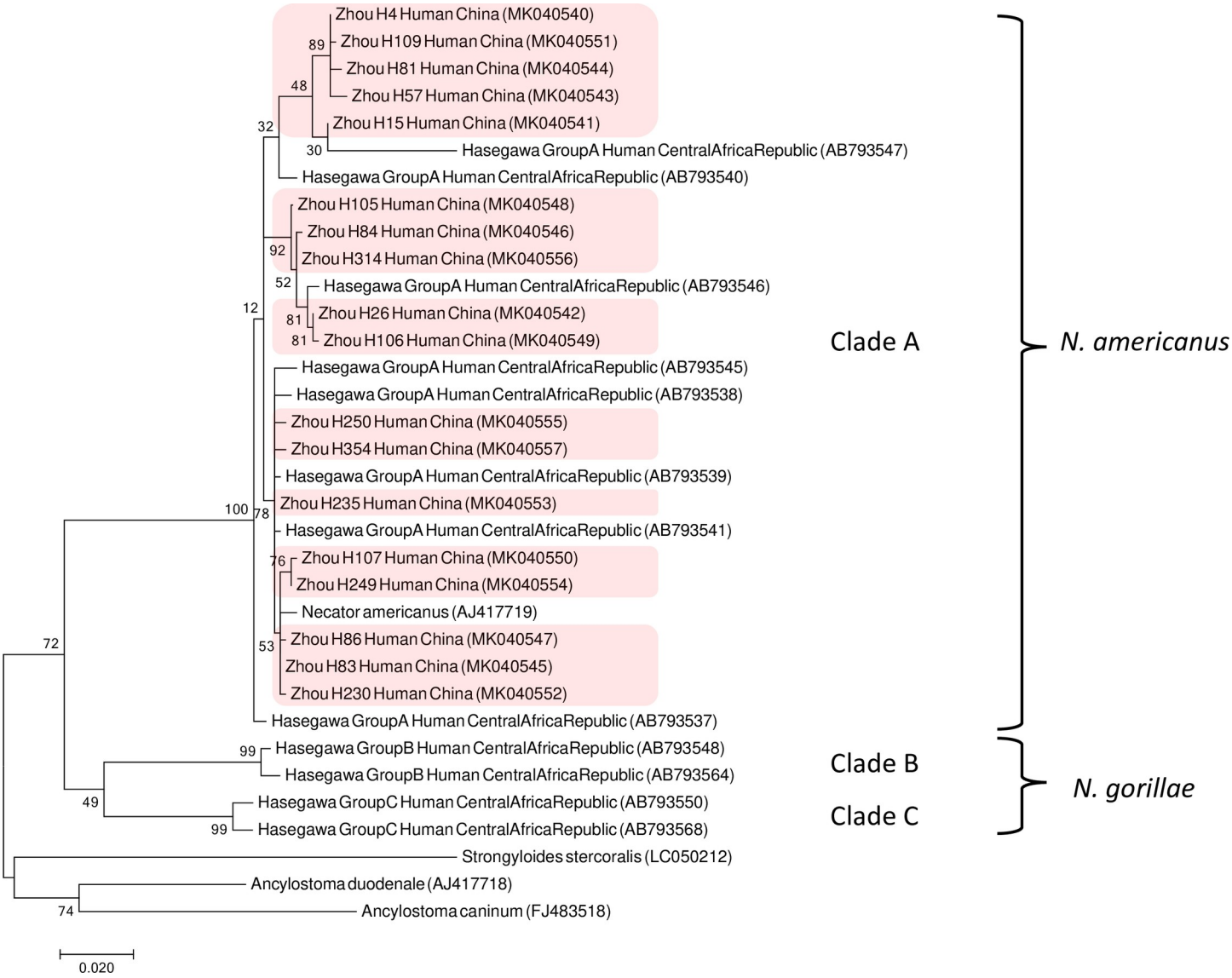

Fig 2. *Cox1* gene tree of different hookworm isolates. Maximum likelihood tree based on 670 bp of the mitochondrial *cox1* gene of the hookworms isolated in this study and selected published sequences. The reference sequence AJ417719 originates from the Zhejiang Province, China [72]. The scale bar represents 0.02 substitutions per site. The boot strap values represent 1000 boot strapping repetitions. The labels are composed as follows: [author of the reference] [haplotypes names according to this reference] [host the isolate was derived from] [country the isolate was isolated from] (accession number). Samples newly isolated in this study are underlaid in red. The clade and species nomenclature on the right is according to [40].

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Whole genome sequencing of individual *S. stercoralis*

Genomic libraries of 29 *S. stercoralis* (26 infective larvae, 3 FL males) isolated from China and 7 *S. stercoralis* (5 FL males, 2 FL females) isolated from Cambodia were prepared as follows: 12 μl worm lysate were mixed with 4 μl paramagnetic bead-immobilized *Tn5* transposomes (*Tn5* expressed and purified according to [63]) and 4 μl 5X TAPS-DMF $MgCl_2$ buffer (50 mM TAPS, 25 mM $MgCl_2$, 50% DMF). The mixture was incubated for 14 min at 55˚C. Following tagmentation, *Tn5* was stripped from DNA using SDS-containing buffer (20 μl, 30 mM Tris pH 8.0, 50 mM NaCl, 0.1% Tween 20, 0.6% SDS) with incubation at 55˚C for 4 min. The beads were then washed twice with 125 μl wash buffer (30 mM Tris pH 8.0, 50 mM NaCl, 0.1%

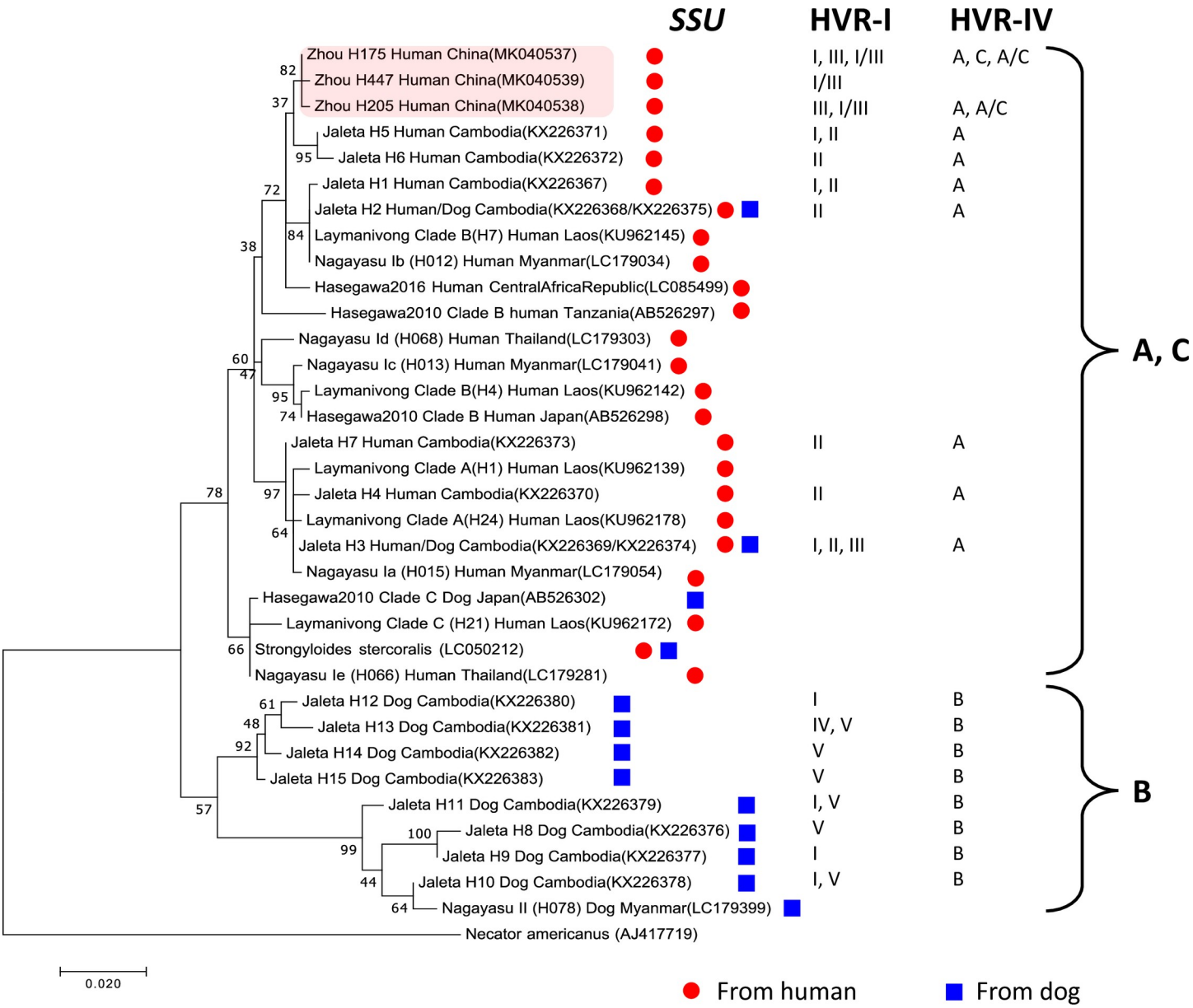

Fig 3. *Cox1* gene tree of different *S. stercoralis* isolates. Maximum likelihood tree based on 552 bp of the mitochondrial *cox1* gene. Shown are the three newly identified (red box) and selected published *S. stercoralis* haplotypes representing the major phylogenetic groups described in recent *S. stercoralis cox1* phylogenies [6, 49, 50, 73]. The scale bar represents 0.02 substitutions per site. The boot strap values represent 1000 boot strapping repetitions. The labels are composed as follows: [author of the reference] [haplotypes names according to this reference] [host the isolate was derived from] [country the isolate was isolated from] (accession number). The two columns on the right indicate the *SSU* HVR-I and HVR-IV haplotypes found among the worm individuals of the respective *cox1* haplotype.

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Tween 20) on a magnetic stand. PCR amplification, adapter extension and barcoding were done by adding 10 μl 5x Q5 reaction buffer, 1 μl 10 mM dNTP, 1.5 μl of 10 μM Nextera i5 primer and i7 primer, 0.5 μl Q5 high-fidelity DNA polymerase (M0491, New England Bio-Labs) and 35.5 μl H_2O followed by the thermocycling program: 72˚C for 5 min, 98˚C for 30 sec, followed by 16 cycles of denaturation (98˚C for 15 sec), annealing (66˚C for 20 sec), extension (72˚C for 90 sec) and cooling to 4˚C. The 250–550 bp fragments of PCR products were selected with HighPrep beads (MagBio Genomics). Libraries were sequenced on an Illumina

HiSeq 3000 instrument (150 bp paired-end) at the Genome Core facility at the MPI for Developmental Biology.

Analysis of the whole genome data

Alignment. Raw reads were mapped to the *S. stercoralis* reference genome (version PRJEB528.WBPS11) using bwa mem with default settings [64]. In addition to the 36 individual *S. stercoralis* sequenced in this study, for comparison, we also included the published whole genome sequences of selected wild isolates from Cambodia [50], Myanmar, Japan [51, 54] and a laboratory reference isolate PV001 derived from USA [65]. For more details see S4 File.

Variants calling. Variants deviating from the *S. stercoralis* reference genome were called as described previously [66]. In brief, raw variants were called using the mpileup, bcftools, and vcfutils.pl programs of the SAMtools suite (version 0.1.18) [67] and filtered for variants with quality values $\geq$20. Heterozygous sites were extracted based on the attributes in the vcf files (AF1$>$0.4 & AF1$<$0.6). Heterozygosity was calculated as the fraction of heterozygous sites on the X chromosome and autosomes. Only samples comprising $>$80% of genomic regions with 15x depth were included for heterozygosity analysis. A Wilcoxon test was performed to evaluate the differences of autosomal heterozygosity between populations. The X chromosome was excluded from the statistical analysis because only females are informative and there were only two females derived from the Cambodian population.

For analysis of population structure, all variant sites were pooled and called in all samples in order to get the full genotypic data including reference alleles.

Population structure. The genome-wide phylogeny was computed by the neighbor-joining method as implemented in the phangorn R package [68] and is based on 1180 variant sites that were called as homozygous in all samples (see [66, 69] for further details). To look for potential evidence of recent or ancient recombination events, a subset of samples (three male samples from China, two reference samples, and four highly covered Cambodian samples) were selected to compute neighbor joining trees for the largest *S. stercoralis* contigs (S1 Fig).

In addition, PCA was performed based on a set of 910 SNPs (distributed across the whole genome, genotyped in all samples, including heterozygous sites, down-sampled to 1 SNP per 5kb) with the help of the smartpca program of the eigensoft package (version 5.0.1) [70].

Nucleotide diversity and analysis of coding sequences. Nucleotide diversity (π) was calculated as the mean fraction of nucleotide differences in pairwise comparisons within and between populations. For these estimates, we ignored heterozygous calls and assumed that 90% of the *S. stercoralis* genome was covered in both samples. Heterozygous non-reference alleles from the Chinese population were extracted and their frequency was quantified in the Chinese and the Cambodian populations. *S. stercoralis* gene annotations (version PRJEB528.WBPS11) were used to assess the effect of substitutions on the coding sequences.

Results

Liver fluke, hookworm and *S. stercoralis* are the gastrointestinal helminths detected in our study area

In the village LA, fecal samples were collected from 108 persons. We detected liver fluke (*Clonorchis sinensis*) (23 = 21.3%) and hookworms (12 = 11.1%) but no *S. stercoralis*. In the village QX, fecal samples were collected from 98 persons. We detected liver fluke (*C. sinensis*) (59 = 60.2%), hookworms (17 = 17.3%) and *S. stercoralis* (7 = 7.1%). For full information see S1 File. Further, we sampled seven of the eight dogs present in the three dog owning households with *S. stercoralis* positive people. No *S. stercoralis* were found in these dogs.

The hookworms in the study area are genetically diverse

Since several species of hookworms are present in China [71] and they are not easily distinguishable by morphology, we determined the *SSU* sequences of 231 hookworms from 19 human hosts (11 from LA, 8 from QX). All of them were identical with the published sequence of *Necator americanus* (AJ920348).

In order to connect our work to [40, 41], which did not report the *SSU* sequences of its isolates, we determined the ITS-1 and ITS-2 sequences of 108 hookworms and 670 bp of the mitochondrial gene *cox1* of 100 hookworms from the 19 host individuals. All of them had the same ITS sequences (MK036418 [ITS-1] and MK036419 [ITS-2]), which correspond to ITS type I as defined by [40].

For *cox1* we identified a total of 18 different haplotypes (GenBank accession numbers MK040540-MK040557). 6 *cox1* haplotypes were present in more than one host individual and multiple *cox1* haplotypes were found in 8 of the 19 host individuals. For more details see S2 File. A phylogenetic comparison with the *cox1* haplotypes identified in earlier studies [40, 41] (Fig 2) showed that all our 18 *cox1* haplotypes fall into clade A as defined by [40]. Taken together, our molecular data indicate that all hookworms isolated in this study are *N. americanus* of ITS type I and *cox1* clade A sensu [40].

The *S. stercoralis* in the study area are very closely related

Partial sequence (552bp) of the mitochondrial gene *cox1* was obtained from 69 *S. stercoralis* representing all seven positive human hosts in the village and one of the patients from a local hospital. All the 53 worms from six hosts shared the same haplotype (H175, GenBank accession number MK040537) while all the 15 worms isolated from the seventh host were of another haplotype (H205, GenBank accession number MK040538), which differed at one position from H175. In the hospital derived sample we identified a third haplotype (H447, GenBank accession number MK040539) that differed from H205 and H175 by 2 and 1 nt, respectively. For more details see S3 File. To examine the phylogenetic relationships, we reconstructed a maximum-likelihood tree with our and selected published *cox1* sequences [6, 48–51] (Fig 3). Our *cox1* haplotypes group clearly with the ones found in Cambodia in humans and dogs but not with the dog specific ones [50]. With moderate bootstrap support, these haplotypes could be assigned to a group described as clade B by [6] or clade Ib by [51]. Our attempt to culture this isolate of *S. stercoralis* in gerbils failed.

Most *S. stercoralis* in the study area are hybrids for different *SSU* haplotypes

A total of 177 *S. stercoralis* from 9 humans were sequenced at the *SSU* HVR-I and/or HVR-IV loci. Only 2 infective larvae and 3 free-living males appeared homozygous or hemizygous (the *SSU* is on the X chromosome) for either one of the HVR-I haplotypes I and III described by [50] (Tables 2 and 3). In HVR-IV the same 5 worms plus another 28 infective larvae appeared homozygous or hemizygous for either haplotype A or C. Haplotype A is the typical haplotype for *S. stercoralis* isolated from humans [50]. Haplotype C is a novel haplotype identified in this study, which has a T deleted at position 1265 (compared with AF279916) (Tables 2 and 3).

Interestingly, unlike in earlier studies [50, 51, 53], in this study the vast majority of *S. stercoralis* individuals appeared to be heterozygous at the *SSU* locus (Table 3). All of them can be explained with the hypothesis that the worms were hybrids of two haplotypes described in Table 2. To confirm this and to determine which combinations of HVR-I and HVR-IV

Table 2. The *SSU* HVR-I and HVR-IV polymorphisms of *S. stercoralis*.

HVR-I	176 bp	458 bp	HVR-IV	1265 bp
Haplotype I	4T ATA T	T	Haplotype A	ATTTT GTTTA TTTT A-A TAT
Haplotype II	5T ATA T	T	Haplotype B	ATTT- GTTTA TTTT TTA TAT
Haplotype III	5T ATA T	A	Haplotype C	ATTT- GTTTA TTTT A-A TAT
Haplotype IV	3T ATA C	T		
Haplotype V	4T ATA C	T		
Haplotype VI	4T ATA T	A		

Haplotype nomenclature: Haplotypes I-V, A,B [50]; haplotype VI [21]; haplotype C this publication. bp indicates the positions in the reference sequence AF279916.

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haplotypes existed we amplified a 1625 bp fragment containing both HVRs from the individual worms described in Table 4, cloned the PCR product and sequenced individual clones.

From host QX32, where all *S. stercoralis* isolated from this host were hybrids in HVR-I but not in HVR-IV, the two dominant *SSU* alleles were haplotypes I and III (HVR-I) in combination with haplotype A (HVR-IV). From the other three hosts (QX24, QX97 and QX105), all *S. stercoralis* were hybrids in both HVR-I and HVR-IV. Here the two dominant *SSU* haplotypes were I (HVR-I) with C (HVR-IV) and III (HVR-I) with A (HVR-IV) (Table 4).

Intra-individual variability of the *SSU* sequence was detected in *S. stercoralis*

In several *S. stercoralis* females analyzed, we identified more (up to 4) than the two *SSU* haplotypes expected for heterozygous animals (Table 4: rows 3–11). For comparison, we repeated the experiment with *S. stercoralis* males from Cambodia [50] (Table 4: rows 12–19). In 2 cases, we found more than one *SSU* haplotype (notice that the *SSU* is on the X chromosome). This suggests that in the population under study and, maybe to a lesser extent, in the population in Cambodia there is appreciable *SSU* sequence variation among the different rDNA copies

Table 3. *SSU* HVR-I and HVR-IV genotypes of individual *S. stercoralis*.

SSU / Host[a]	4T/5T T/A A	- - A	4T/5T T/A A/C	4T/5T T/A -	4T/5T - A/C	- - A/C	5T A A	4T T C	Total
QX105			35	1	6	1	1 (male)		44
QX14				1	1				2
QX24			36	3	4	6		2	51
QX32	26	2							28
QX87						1		1 (male)	2
QX97			19	4	2		1 (male)		26
QX121			1						1
Patient A*					1	14			15
Patient B*				1		7			8

Individual *S. stercoralis* were sequenced at HVR-I and HVR-IV. Rows 1–3 represent polymorphic sequences in HVR-I (176 bp and 458 bp of AF279916) and HVR-IV (1265 bp of AF279916). Rows 4–12 represent one host individual each. Numbers indicate the number of *S. stercoralis* with the HVR-I/IV haplotype combinations specified in rows 1–3.

[a]The host is defined by the letter code for the village followed by the host individual number. Notice that worms from the same host may or may not be clonal siblings.

*Host from local hospital.

-: nucleotide sequence at designated position was not determined.

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Table 4. Combinations of *SSU* HVR-I and HVR-IV haplotypes.

Worm[a]	Country[b]	Sex[c]	I	I	II	II	III	III	VI	I	IV	V	Total
			A	C	A	C	A	C	A	B	B	B	
QX32(1)	Cn	F	8				6						14
QX32(2)	Cn	F	5		1		6		1				13
QX24(1)	Cn	F		3			1						4
QX24(2)	Cn	F		1			10						11
QX97(1)	Cn	F		8		1	4						13
QX97(2)	Cn	F		8		1	3	1					13
QX105(1)	Cn	F		1		1	2	2	1				7
QX105(2)	Cn	F		3			3	2					8
QX105(3)	Cn	F	1	1			16						18
DC44(1)	Kh	M			16								16
DC108(1)	Kh	M					16						16
DC79D4(1)*	Kh	M										18	18
DC86D2(1)*	Kh	M									13		13
DC79D3(1)*	Kh	M	13		5								18
DC79D3(2)*	Kh	M			16								16
KP55D2(1)*	Kh	M								24		2	26
KP55D2(2)*	Kh	M								7			7

A region spanning HVR-I and HVR-IV of individual *S. stercoralis* was PCR amplified and the product cloned. Individual clones were sequenced. Rows 1 and 2 represent the HVR-I and HVR-IV haplotypes. Rows 3–19 represent one worm each. Numbers indicate the number of clones with the HVR-I/IV combination designated in rows 1 and 2.

[a]The worm is defined by the letter code for the village followed by the host individual number, and the worm individual number in brackets. Notice that worms from the same host may or may not be clonal siblings.

[b]Cn: China; Kh: Cambodia.

[c]F: female (iL3); M: male.

*Isolated from dog host.

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within a haploid genome. Intra-individual variability of rDNA sequences, although apparently very rare in animals, has been observed before, for example in American sturgeons [74] or in the plant parasitic nematode *Rotylenchulus reniformis* [75]. So far, the variant 4T+A, which occurred as a minor variant and in combination with haplotype A (HVR-IV), had not been reported in any of the studies from East Asia but has recently been observed in *S. stercoralis* from dogs in Switzerland by [21] and named haplotype VI by these authors.

S. stercoralis populations are geographically clustered

In order to extend our analysis beyond the *cox1* and *SSU* sequences, we sequenced the whole genome of individual *S. stercoralis*. For comparison, the published genome sequences of selected *S. stercoralis* samples from Cambodia [50], Myanmar and Japan [54] were also included in this analysis.

Phylogeny. We reconstructed a phylogenetic tree based on the whole genome sequences. A clear geographical separation was observed. Samples from China, Cambodia/Myanmar, Japan and USA (reference) are grouped into different clades according to their country of origin. The only exception is that the Myanmar and the Cambodian samples show no clear separation. The Cambodian samples show higher within location diversity compared with the Chinese samples (Fig 4A).

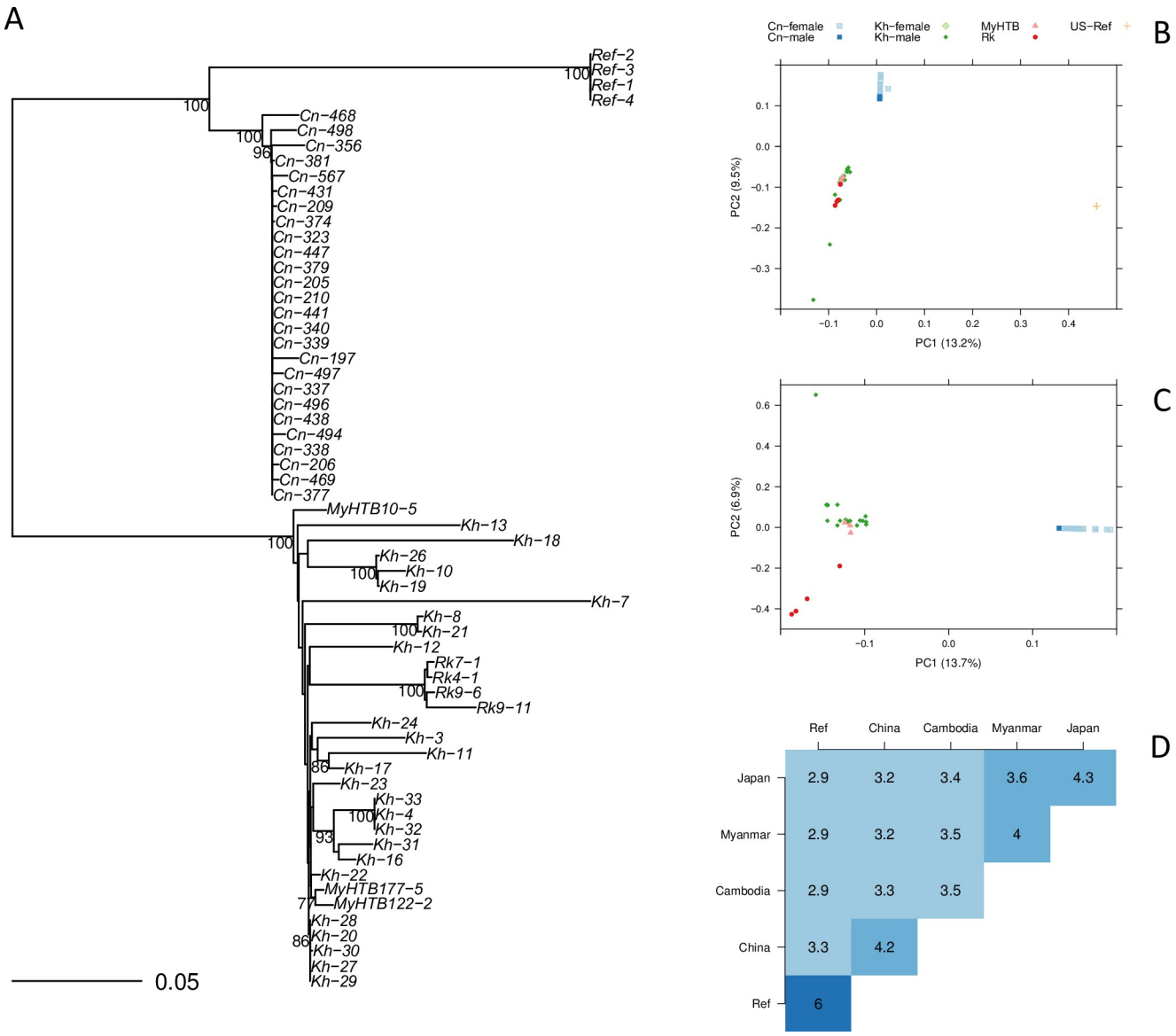

Fig 4. Population structure of *S. stercoralis*. (A) Phylogenetic relationships between the Chinese and selected *S. stercoralis* samples are visualized as a neighbor-joining tree that was calculated from 1180 concatenated variant sites that were genotyped as homozygous in all samples. (B) Principal component analysis was performed on 910 homozygous and heterozygous SNPs that could be genotyped in all samples. (C) Principal component analysis without the reference strain. (D) Nucleotide diversity (π) was calculated from homozygous SNPs as the mean fraction of nucleotide differences in pairwise comparisons within and between populations. The heatmap shows the negative logarithm (base 10) of π. Cn: China; Kh: Cambodia; MyHTB: Myanmar; Rk: Japan; Ref: US-derived reference laboratory strain PV001. For Cn and Kh all but three (very low read coverage) whole genome sequences determined for this study and for [50] were included. For MyHTB and Rk, we selected samples representing one outlier (Rk9-11) and the two clusters in Fig 5 of [54]. Selection within the clusters was random.

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Principal component analysis (PCA). Principal component analysis (PCA) also shows a geographical clustering. All samples collected from different locations are separated from the reference strain [65] by PC1 (13.2%). Samples from China are separated from other populations by PC2 (9.5%) (Fig 4B). Since the US derived reference lab strain PV001 was very different from all the wild samples, we repeated the analysis without the reference. Hereafter, the

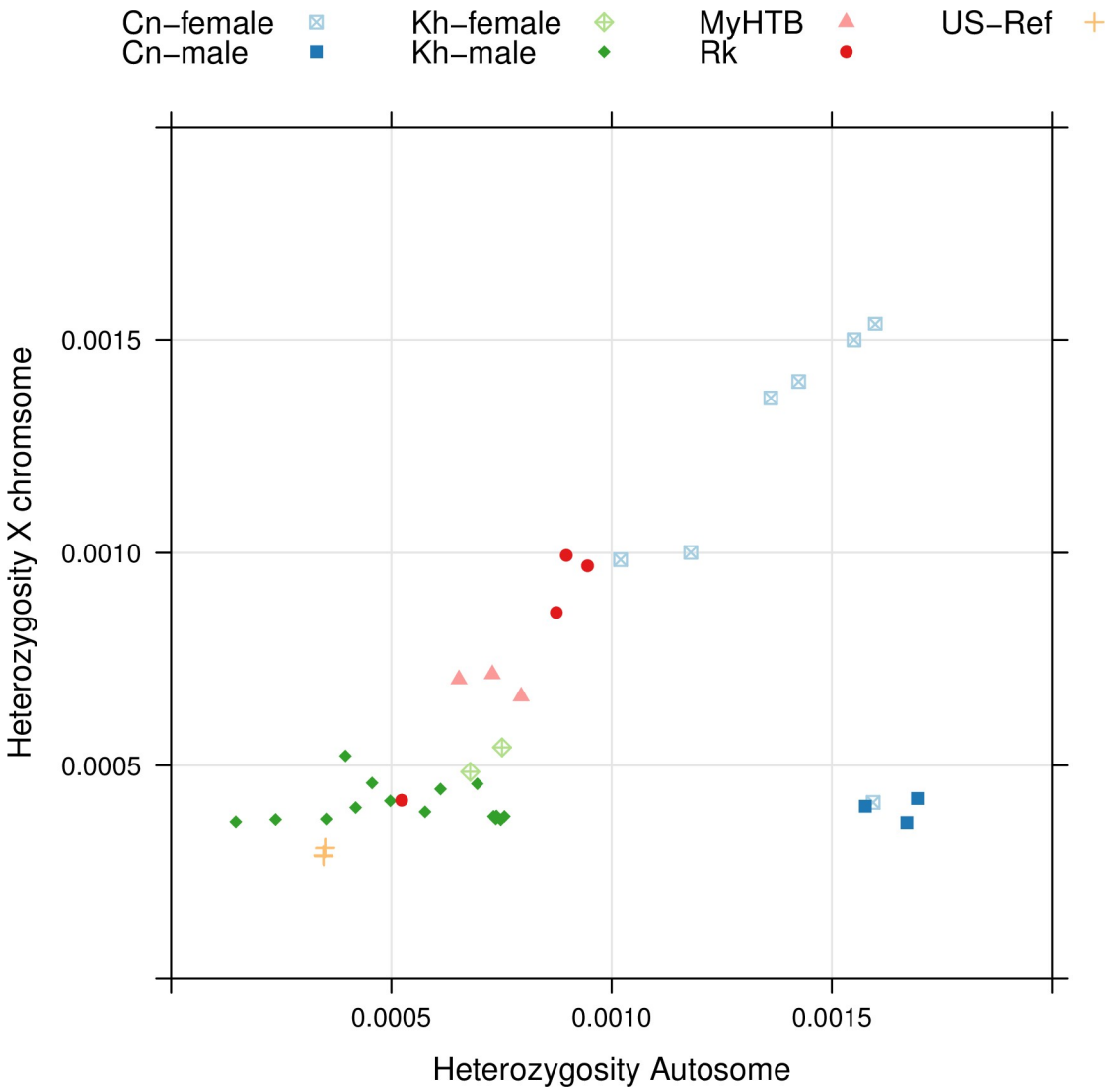

Fig 5. Genomic heterozygosity of individual *S. stercoralis*. The heterozygosity on the autosomes is plotted against the heterozygosity on the X chromosome for *S. stercoralis* individuals from different geographical locations. Cn: China; Kh: Cambodia; MyHTB: Myanmar; Rk: Japan; Ref: US-derived reference laboratory strain PV001. All samples in Fig 4 that fulfilled the read coverage criteria described in Materials and Methods were included in this analysis. The samples from Cn represent four different hosts from the village, two hospital patients and all three *cox1* haplotypes (c.f. S4 file).

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samples from China, Cambodia and Japan are separated by PC1 (13.7%), whereas Myanmar and Cambodian samples remain mixed (Fig 4C).

Nucleotide diversity. Nucleotide diversity (π) within and between geographic populations is shown as a heatmap (Fig 4D). Within the population, the average distance between Cambodian samples is the highest ($10^{-3.5}$) among all the 5 geographic populations, indicating a higher diversity among the Cambodian population. Between locations, the diversity is comparable between Cambodia, China, Myanmar and Japan ($10^{-3.2}$–$10^{-3.6}$). Interestingly, the samples from China appear, to a moderate extent, closer to the US derived reference strain, compared with the other samples.

S. stercoralis in the study area reproduce predominantly, if not exclusively, asexually

Free-living adult *S. stercoralis* were virtually not detected. Noticeably, all (thousands) *S. stercoralis* we observed were infective larvae, except for 3 free-living males (isolated from 3 different human hosts). No free-living female was found. This suggests that *S. stercoralis* in our study area reproduces predominantly, if not exclusively asexually through the homogonic and/or the auto infective cycles. To further test this, we looked for the presence of signs of extended times of asexual reproduction in the genomes.

High heterozygosity across the genome. Due to the absence of meiotic recombination, in asexual organism the divergence between homologous chromosomes is expected to increase in a process known as the Meselson effect [76]. Therefore, if the Chinese population is indeed asexual, one would expect to observe a higher number of heterozygous positions compared with, for example, the samples from Cambodia, where large numbers of sexual free-living individuals were observed and sexual reproduction occurs presumably fairly frequently [50].

To detect such genomic hints for asexuality, we first compared the heterozygosity of individual *S. stercoralis* isolated from the different geographical locations (Fig 5). The heterozygosity on the autosomes and X chromosome were calculated separately. Theoretically, the heterozygosity on the X of males is zero because there is only one copy. The low, but not zero, measured heterozygosity of the X chromosome in males reflects the background of false positives caused for example by sequencing artifacts and errors in the reference genome assembly and variant calling. The heterozygosity in Cambodian samples is generally the lowest among the wild isolates and in the Myanmar samples it is only slightly higher. As described in [54], except for the one outlier, the heterozygosity in the Japanese sample is higher than in Myanmar, a finding these authors attributed to the extended time these worms had reproduced through the clonal auto infective cycle. The Chinese samples are even more heterozygous (Wilcoxon test: $p = 2.371e\text{-}07$ compared with Cambodian samples, $p = 0.001998$ compared with Japanese samples, autosome only).

In general, the females lay very close to the diagonal, indicating there is no difference in heterozygosity between the autosomes and the sex chromosome. The only exception is one Chinese female (Cn-323) with male-like low heterozygosity on the X chromosome (Fig 5). Interestingly, it is the only female from China in this analysis that appeared homozygous at the *SSU*. Read coverage confirmed that the individual is indeed a female with two X chromosomes (see PRJNA517237). A possible explanation is that it was the product of a rare sexual event between close relatives in the process of which the X chromosome but not the autosomes became homozygous.

Most variants arose prior to asexuality. Secondly, since the new mutations never become homozygous in an asexual population, it is expected that purifying selection is reduced and slightly deleterious mutations can accumulate, which is manifested in an increased proportion of nonsynonymous changes [76]. Thus, we asked if there had been an accumulation of nonsynonymous mutations in the Chinese population.

We extracted the heterozygous sites in predicted coding regions present in at least two Chinese samples and calculated the frequency of heterozygous calls in Chinese population (Fig 6A). Then we quantified the frequency of the non-reference alleles in the Cambodian samples. One large fraction of such non-reference alleles was fixed in the Cambodian population. These are old alleles shared between the Cambodian and Chinese populations ("fixed" in Fig 6B). In contrast, in the other large fraction, the reference alleles were fixed in Cambodia. These non-reference variants present in Chinese but not Cambodia population are candidates for new mutations arisen in Chinese population after the transition to asexual reproduction ("not present" in Fig 6B). We

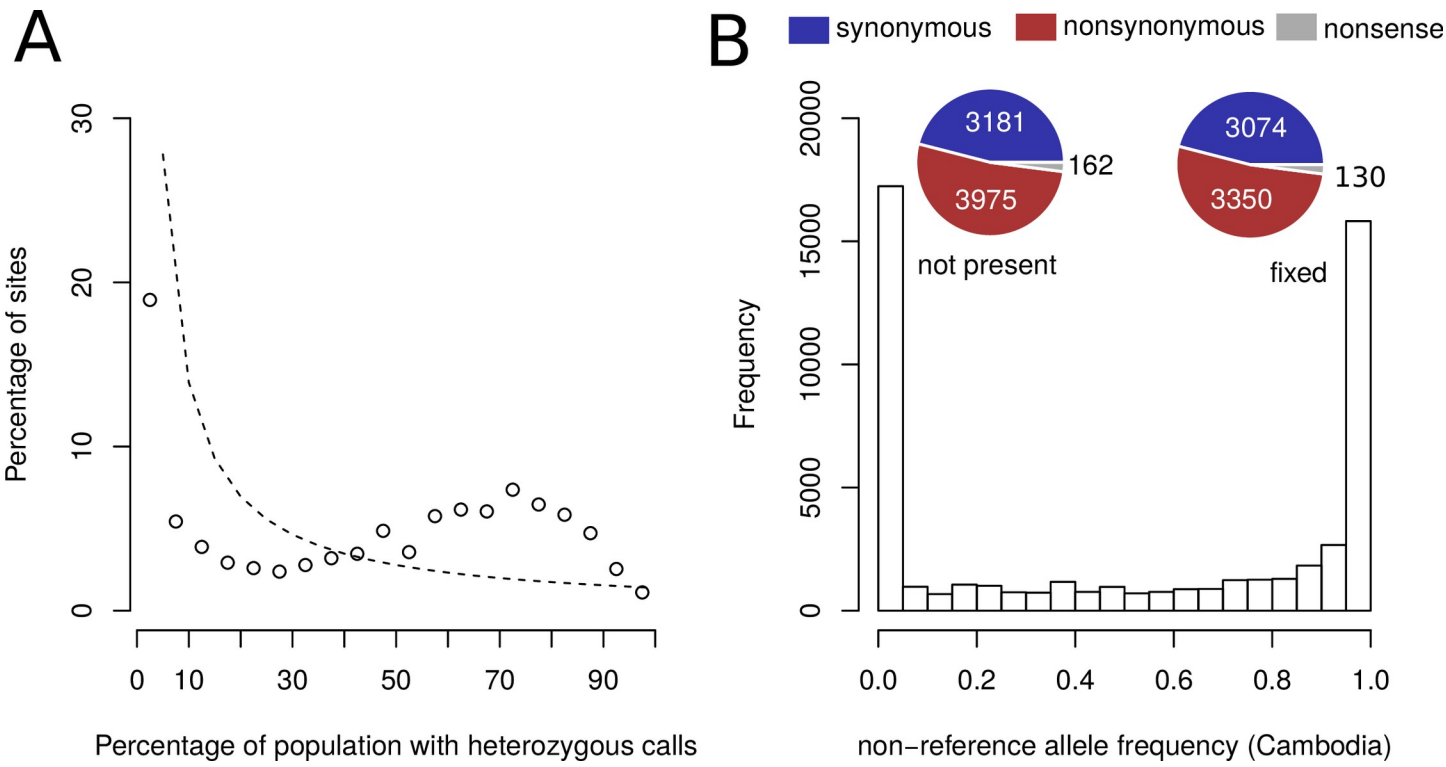

Fig 6. The heterozygosity was derived from ancestral polymorphisms. (A) The frequency of heterozygous calls in the Chinese population was quantified and compared to a neutral expectation of derived allele frequencies under a genetic drift model (1/k where k is the number of samples (dashed line) [66, 77]. (B) The frequency of the non-reference alleles derived from (A) was quantified in the Cambodian population. Large fractions of these alleles are fixed in Cambodian population ("fixed") and the other large fraction of alleles is not found in Cambodian population ("not present"). The numbers of nonsynonymous variants in the "not present" and "fixed" group are shown in pie charts.

https://doi.org/10.1371/journal.pntd.0007396.g006

compared the ratio of nonsynonymous and synonymous mutations between the two groups of variants. The putatively newly derived mutations are slightly but just significantly (54% as opposed to 51%, p = 0.04 Fisher's exact test) more frequently nonsynonymous, which argues against an extended time of mutation accumulation under conditions of relaxed purifying selection.

Recombination happened rather recently. Finally, we asked if the high heterozygosity of Chinese samples is an effect of hybridization events in the relatively recent past. We reconstructed phylogenetic trees of *S. stercoralis* males based on the largest autosomal and X chromosomal contigs (S1 Fig). The apparent phylogenetic relationships between the three Chinese samples changed dependent on the genomic region selected, which is clear evidence for recombination in the rather recent past. One possible interpretation is that rare recombination events did occur after the lineage became mostly asexual. Alternatively, the recombined chromosomes might have been present in the population that gave rise to the asexual lineage.

Taken together, it is unlikely that the high heterozygosity in Chinese population was caused by the accumulation of novel mutations after the transition to asexuality. It appears more likely that the mutations arose in sexual populations and the high heterozygosity was caused by one or a few rather recent hybridization events that also rendered the population largely if not completely asexual.

Discussion

Southern China has a subtropical climate which favors a variety of parasites. The main purpose of this study was the isolation and the genomic/genetic characterization of individual *S.*

stercoralis and not to conduct a prevalence study or a general parasitological survey. Accordingly, the number of people surveyed was rather limited. Nevertheless, there are a few observations worth mentioning. As expected based on our experience as routine diagnostics provider, the predominant helminths detectable by egg floatation were liver fluke and hookworm. Usually, in the routine parasitological diagnosis, the species of the hookworms is not determined. However, in this study we used molecular diagnostic tools and could show that all hookworms found belonged to the species *N*. *americanus*.

Usually, *S*. *stercoralis* is not detectable by egg floatation. Therefore, we used culturing and Baermann funnels to test the presence of this parasite and to isolate live individual worms, which is not normally done in our diagnostic routine.

Very few studies describing *S*. *stercoralis* prevalences in China were recently published in international journals [78–81]. All such studies we are aware of, were conducted by the same research group and in the Yunnan province, which neighbors the Guangxi province. Our study illustrates that *S*. *stercoralis* is also prevalent in Guangxi, a fact that does not come as a surprise if also clinical case reports (usually only available in Chinese language) are taken into account. As a matter of fact, in a review of such cases between 1973 and 2011[82] more human *S*. *stercoralis* cases in Guangxi than in Yunnan were reported.

Hookworm co-infection with *S*. *stercoralis* are common [31]-[37] and they have similar transmission routes. One could therefore expect, that the dynamics and spreading of these two helminths are similar. This study does not support this conclusion. We found only 2 out of the 7 people infected with *S*. *stercoralis* were also positive for *N*. *americanu*s, which corresponds about to the expectation based on the prevalences (17.3% for hookworms and 7.1% for *S*. *stercoralis*). Also, the hookworms were genetically diverse, while the *S*. *stercoralis* were all very closely related. While the auto infection cycle allows *S*. *stercoralis* to maintain an infection for decades, *N*. *americanus*, in absence of new infection, can only persist for the life time of the individual parasites which is in the order of a few years [25]. Taken together, this suggests that in our study population the transmission rate of hookworms is much higher than the one of *S*. *stercoralis*. The *S*. *stercoralis* positive patients have possibly been infected rather long time ago and maintained the infection through the auto infective cycle.

Contrary to earlier studies [50, 51, 53, 54] we found most *S*. *stercoralis* individuals to be heterozygous for different *SSU* haplotypes. While the *SSU* HVR-I haplotypes had all been described in *S*. *stercoralis* before (although almost exclusively in homozygous state), we identified a novel *SSU* HVR-IV haplotype. These findings are of importance for *SSU*-sequence-based diagnostics and taxonomy of *S*. *stercoralis* and closely related species of *Strongyloides*. The occurrence of *S*. *stercoralis* heterozygous for multiple *SSU* haplotypes may, but not necessarily needs to be, related to our second striking observation, namely the virtual absence of free-living adults. The switch between the clonal direct and the sexual indirect cycle in *Strongyloides* spp. is influenced by the environment, in particular the temperature and the immune statues of hosts, and the genetic background [23]. We cannot completely exclude that at different times of the year, when temperatures are different, in our study area, more sexual animals could be found. However, the climatic conditions in Guangxi are comparable with northern Cambodia where we found numerous free-living adults of both sexes at the same time of the year. We can also not fully exclude that the immune status of the hosts in this study very strongly favored females developing into iL3s. However, our genomic analyses suggest that our study population is largely, if not exclusively asexual. We detected a genome wide heterozygosity, which was even higher than the one described as elevated in the Japanese samples by [54]. These authors attributed the observed heterozygosity to the fact that the worms had accumulated mutations during the asexual reproduction through the auto infective cycle since the infection of the particular host individual. We do not think clonal reproduction only within individual patients could explain our observations.

First, in our study the heterozygosity was higher. Second, the worms in the different patients were very closely related. Third, the observed fraction of around 50% nonsynonymous changes is similar to the ones observed in wild populations of the free-living nematode *P. pacificus* and *C. elegans* and considerably lower than in experimental populations of the same two species, where mutations were allowed to accumulate under minimal purifying selection resulting in around 70% - 75% nonsynonymous mutations in coding regions [83, 84]. This indicates that most variants present in our sample did not arise under conditions of relaxed purifying selection. Nevertheless, we did observe a slightly but significantly higher proportion of nonsynonymous changes among the variants not found in the Cambodian population. Given that presumably only a fraction of these variants were recently introduced to our study population. This observation may be a hint for a limited time period of mutation accumulation with reduced purifying selection. Loss of sexual reproduction has been reported for specific isolates of other species of *Strongyloides*. For *S. ratti*, largely asexual populations have been described [24] and in a laboratory strain of *S. venezuelensis* (HH1, originally isolated from Okinawa Japan) no males and only very few free-living females were observed under various conditions [85].

We favor the hypothesis that our study population has rather recently, but prior to the infection of the current host individuals, become predominantly if not exclusively asexual as a consequence of one or several hybridization events between sexual populations of *S. stercoralis*. This is consistent with the observations of high heterozygosity and the origin of most non-reference variants during a period with purifying selection at a level normal for sexual reproduction. It is important to notice that, if this hypothesis is true, the first generation of hybrids must have been capable of sexual reproduction because the results shown in S1 Fig can only be explained if meiotic recombination occurred at least once after the hybridization event. Asexual genotypes then arose in the next generations due to the particular combination of genetic material derived from the parental lines.

Supporting information

S1 File. Fecal sampling and the prevalence of helminths. The table shows the result of two fecal samplings from human hosts in the villages LA and QX, Guangxi, China. Each row represents one person.
(XLSX)

S2 File. Number of hookworms genotyped and the *cox1* haplotypes identified. The table shows the number of hookworms collected, genotyped (*SSU*, ITS, and *cox1*) for each positive host, and number of hookworms of the corresponding *cox1* haplotypes found in each host.
(XLSX)

S3 File. Number of *S. stercoralis* genotyped and the *cox1* haplotypes identified. The table shows the number of *S. stercoralis* collected, genotyped (*SSU* and c*ox1*) for each positive host, and the number of *S. stercoralis* of the corresponding *cox1* haplotypes found in each host.
(XLSX)

S4 File. *S. stercoralis* samples for whole genome analysis. The table shows the sample ID, host ID, country, sex, the mean coverage, the fraction of the genome beyond 15x coverage, the origin of the sequences, and the accession number of individual *S. stercoralis* used for the whole genome analysis.
(XLSX)

S5 File. Copy of the animal experiment approval.
(PDF)

S1 Fig. Phylogenies of the largest *S. stercoralis* contigs. The upper panel shows neighbor-joining trees for the largest sex chromosomal contigs in the three male samples from China, four high quality samples from Cambodia, and two reference samples. Each contig is at least 100kb large and several hundred variant sites were used to reconstruct individual trees. The lower panel shows the equivalent analysis for autosomal contigs.
(TIF)

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