Characterising different wild isolates of human and animal parasitic *Strongyloides* spp. and analysis of poly(UG)-tailed RNAs in *Pristionchus pacificus* and *Strongyloides* spp.

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Veroni Chathunika de Ree
Aus Tangalle, Sri Lanka

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1. Berichterstatter:	Apl. Prof. Dr. Adrian Streit
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Summary

The parasitic nematode *Strongyloides stercoralis* is the main causative agent of human strongyloidiasis, a neglected tropical disease. *S. stercoralis* can undergo three different life cycles. Non-human primates and dogs have been described as possible sources for zoonotic infections of humans. Different isolates of *S. stercoralis* show considerable genetic diversity and different life cycle preferences. Two types of *S. stercoralis* have been identified as 'dog only' and 'human and dog shared'. However, the studies on which these finding are based are concentrated in East Asia, Southeast Asia and Australia. *Strongyloides fuelleborni* can also infect humans. In Asia, *S. fuelleborni* infections were considered to be rare and restricted to zoonotic cases. Given the biological and medical importance and the difficulties of establishing transgenic lines of these obligatory parasites in hosts, genetic tools such as RNAi developed as a straightforward and effective method is highly desirable. In this thesis, I present two projects, one that compares *Strongyloides* wild isolates of different hosts from three countries and the other that explores method development for *Strongyloides* using the free-living model nematode *Pristionchus pacificus* as a stepping stone.

(1) The aim was to enhance the current understanding of the *Strongyloides* spp. responsible for human infections, as well as their potential zoonotic threat by expanding the geographic range for which molecular taxonomic information is available. I approached this by collecting and or analysing human, dog and monkey-derived wild isolates of *Strongyloides* spp. from Iran, Bangladesh and Sri Lanka. Worms collected were subjected to molecular taxonomic and genomic analysis. We found *S. stercoralis* in humans in Iran and Bangladesh and the results further support the existence of multiple populations with different host specificities within *S. stercoralis.* While the samples from Bangladesh did not separate from the Southeast Asian samples, in Iran we found indication for different subpopulations that, however, appear to interbreed at least occasionally. For the first time we observed a genomically 'dog only' type worm in a human and found first evidence of occasional interbreeding between the two *S. stercoralis* types, thus exchange of genetic properties, for example drug resistance, between the two types is conceivable. In Sri Lanka, we did not identify any human *S. stercoralis* cases, suggesting the success of recent STH control measures. Contradicting the literature, we noticed a rather high incidence of human *S. fuelleborni* infections in Bangladesh suggesting *S. fuelleborni* may play a more prominent role in human strongyloidiasis in Asia than previously thought. We also found for the first time, *S. fuelleborni* in dog samples in Sri Lanka, suggesting the possibility of dogs as an additional host for *S. fuelleborni*. Based on the mitochondrial sequences, *S. fuelleborni* from Sri Lanka clustered together with the Asian samples and did not mix with the African samples.

(2) The aim was to explore if a novel RNAi method described for *C. elegans* using poly(UG)-tailed RNA can be used in other nematodes, in particular *Pristionchus pacificus* and *Strongyloides* spp. where long dsRNA-mediated RNAi does not work reliably or not at all. I established this method as a tool in *P. pacificus*. Naturally existing poly(UG)-tailed RNAs throughout the genome were identified in *P. pacificus* using custom 3'-end RNA sequencing. In *S. stercoralis* and in *S. ratti* the detection of poly(UG)-tailed RNAs was not convincing. Although a negative result, we think that this finding indicates that the corresponding biological mechanism is used much less in *S. stercoralis* and in *S. ratti,* compared with *C. elegans* and *P. pacificus*, if it is not absent altogether.

Zusammenfassung

Der parasitäre Fadenwurm *Strongyloides stercoralis* ist der Hauptverursacher der menschlichen Strongyloidiasis, einer vernachlässigten Tropenkrankheit. *S. stercoralis* kann drei verschiedene Lebenszyklen durchlaufen. Nichtmenschliche Primaten und Hunde wurden als mögliche Quellen für zoonotische Fälle bei Menschen beschrieben. Verschiedene Isolate von *S. stercoralis* zeigen eine beträchtliche genetische Vielfalt und unterschiedliche Lebenszykluspräferenzen. Zwei Typen von *S. stercoralis* wurden identifiziert: „nur Hund“ und „Mensch und Hund gemeinsam“. Die Studien, auf denen diese Erkenntnisse basieren, konzentrieren sich jedoch auf Ostasien, Südostasien und Australien. Auch *Strongyloides fuelleborni* kann Menschen infizieren. In Asien galten Infektionen mit *S. fuelleborni* als selten und auf zoonotische Fälle beschränkt. Angesichts der biologischen und medizinischen Bedeutung und der Schwierigkeiten bei der Etablierung transgener Linien dieser obligatorischen Parasiten in Wirten sind genetische Werkzeuge wie RNAi, welches als einfache und effektive Methode entwickelt wurde, äußerst wünschenswert. In dieser Arbeit stelle ich zwei Projekte vor: eines vergleicht *Strongyloides*-Wildisolate verschiedener Wirte aus drei Ländern, das andere betrifft die Methodenentwicklung für *Strongyloides* unter Verwendung des freilebenden Modellfadenwurm *Pristionchus pacificus* als Wegbereiter.

(1) Ziel war es, durch die Ausweitung des geografischen Gebiets für welches molekulartaxonomische Informationen verfügbar ist, das aktuelle Verständnis der für Infektionen beim Menschen verantwortlichen *Strongyloides* spp. sowie deren Potential für zoonotische Infektionen zu verbessern. Hierzu sammelte und analysierte ich Wildisolate von *Strongyloides* spp. von Menschen, Hunden und Affen aus dem Iran, Bangladesch und Sri Lanka. Die gesammelten Würmer wurden molekulartaxonomisch und genomisch analysiert. Wir fanden *S. stercoralis* beim Menschen im Iran und in Bangladesch, und die Ergebnisse untermauern die Existenz mehrerer Populationen mit unterschiedlichen Wirtsspezifitäten innerhalb von *S. stercoralis*. Während sich die Proben aus Bangladesh nicht von den südostasiatischen Proben unterscheiden liessen, fanden wir im Iran Hinweise auf verschiedene Subpopulationen, die sich allerdings zumindest gelegentlich zu kreuzen scheinen. In Bangladesh beobachteten wir erstmals einen Wurm vom genetischen Typ „nur Hund“ bei einem Menschen und fanden erste Hinweise darauf, dass sich beiden *S. stercoralis*-Typendoch gelegentlich kreuzen. Ein Austausch genetischer Eigenschaften, beispielsweise einer Medikamentenresistenz, zwischen den beiden Typen ist daher denkbar. In Sri Lanka konnten wir keine menschlichen *S. stercoralis*-Fälle nachweisen, was darauf hindeutet, dass die jüngsten STH-Kontrollmaßnahmen erfolgreich waren. Entgegen der Literatur stellten wir in Bangladesch eine recht hohe Inzidenz von *S. fuelleborni*-Infektionen beim Menschen fest. Dies lässt darauf schließen, dass *S. fuelleborni* bei der menschlichen Strongyloidiasis in Asien eine wichtigere Rolle spielen könnte als bisher angenommen. Außerdem fanden wir erstmals *S. fuelleborni* in Hundeproben in Sri Lanka, was darauf hindeutet, dass Hunde möglicherweise als zusätzlicher Wirt für *S. fuelleborni* fungieren. Basierend auf den mitochondrialen Sequenzen fiel *S. fuelleborni* aus Sri Lanka mit den asiatischen und nicht mit den afrikanischen Proben zusammen.

(2) Ziel war es zu untersuchen, ob eine für *C. elegans* beschriebene neue RNAi-Methode, die auf RNSn mit Poly(UG) Extensionen basiert, auch bei anderen Nematoden, angewendet werden kann, insbesondere bei *P. pacificus* und *Strongyloides* spp., bei denen lange dsRNS-vermittelte RNAi nicht zuverlässig oder gar nicht funktioniert. Ich habe diese Methode bei *P. pacificus* etabliert und ich habe mittels RNS-3'-End-Sequenzierung gezeigt, dass Poly(UG)-RNSn von mehreren tausend Genen in *P. pacificus* natürlicherweise vorkommen. Bei *S. stercoralis* und *S. ratti* fiel der Nachweis von Poly(UG)-RNSn nicht überzeugend aus. Dies ist ein negatives Resultat, das wir dahingehend interpretieren, dass der entsprechende biologische Mechanismus bei *S. stercoralis* und *S. ratti* weniger verwendet wird als bei *C. elegans* und *P. pacificus*, wenn er nicht gar komplett fehlt.

List of publications

***Strongyloides stercoralis* genotyping in a human population in southwestern Iran**

Molouk Beiromvand, Alireza Ashiri, **Veroni de Ree**, Dorothee Harbecke, Christian Rödelsperger, Adrian Streit, Abdollah Rafiei

Parasites and Vectors. 2024 January 16; 17(21)
doi: 10.1186/s13071-023-06103-6

Genomic analysis *of Strongyloides stercoralis* and *Strongyloides fuelleborni* in Bangladesh

Veroni de Ree, Tilak Chandra Nath, Priyanka Barua, Dorothee Harbecke, Dongmin Lee, Christian Rödelsperger, Adrian Streit

PLOS Neglected Tropical Diseases. 2024 September 03; 18(9)
doi: 10.1371/JOURNAL.PNTD.0012440

Poly(UG)-tailed RNAs are involved in the control of thousands of genes predominantly in the germline in *Pristionchus pacificus*

Veroni de Ree, Dorothee Harbecke, Hanh Witte, Christian Rödelsperger, Catia Igreja, Adrian Streit

bioRxiv. 2025 September 01
doi: https://doi.org/10.1101/2025.09.01.673539

Introduction

Phylum Nematoda

The Phylum Nematoda of the animal kingdom consists of nematodes or roundworms, which can be found in nearly all aquatic and terrestrial habitats (Lee, 2002; Perry and Wharton, 2011). Nematodes can be free-living or parasitic within plants and animals, including livestock and humans, rendering them of significant economic, veterinary and medical importance (Lee, 2002).

Nematoda is putatively the most species-rich phylum, comprising close to 2.7×10^4 described species and many still undescribed (Hugot, Baujard and Morand, 2001). The estimated number of species is at least 1×10^6 (Lambshead, 1993, 2004), and the estimated parasite species of vertebrates alone is about 2.9×10^4 (Carlson *et al.*, 2020).

The name Nematoda is derived from the Greek word "nēma", meaning thread (Liddell *et al.*, 1940), and the suffix "-oda", denoting the form referring to the phylum's characteristic elongated, thread-like body morphology. The name Nematoda was derived from Nematoidea which was proposed by Rudolphi in 1808 (Rudolphi, 1808). The group was first formally classified as a phylum by Cobb in 1919 (Cobb, 1919).

In the earlier days the α-taxonomic classifications were largely based on morphology, morphometrics and geography. Light microscopy, *camera lucida* drawings, scanning electron microscopy (SEM) and later in the sixties, protein-based biochemical techniques have been employed in these classifications (Coomans, 2000). Later, due to different research groups using different criteria (Inglis, 1983) and using morphological characters alone proved challenges in the classification process (Coomans, 2000; De Ley *et al.*, 2005). In 1998, Blaxter used Small SubUnit (SSU) ribosomal DNA sequences for phylogenetic analysis and proposed five major clades (Figure 1) within the phylum Nematoda (Blaxter *et al.*, 1998). Parasitic nematodes were found in all five clades, showing the independent rise of parasitism throughout the phylum (Blaxter *et al.*, 1998; Dorris, De Ley and Blaxter, 1999). Vertebrate parasites were found in four clades: clades I, III, IV and V. This phylogeny was later revised

multiple times and a 12-clade system was also introduced (Holterman *et al.*, 2006; Van Megen *et al.*, 2009).

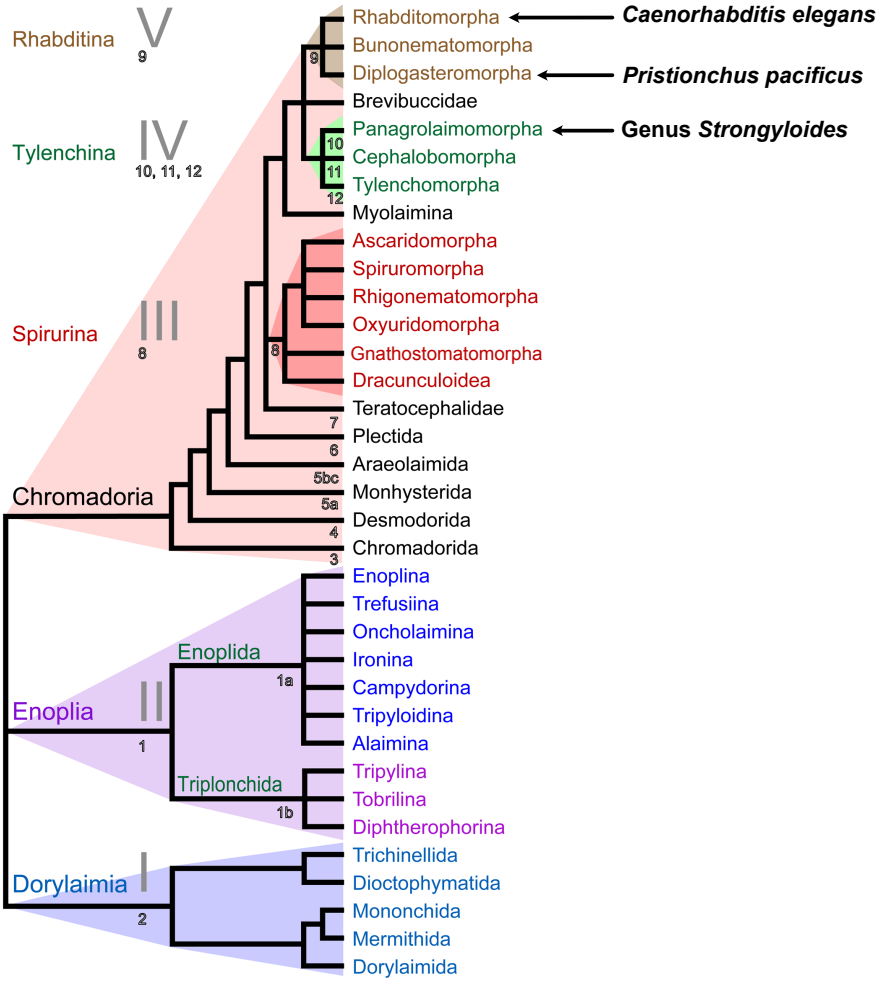

Figure 1 – Phylogeny of phylum Nematoda based on SSU sequences, modified from (Blaxter, 2011), used under the Creative Commons Attribution License.

In the five-clade phylogeny based on molecular sequences, clades III (Spirulina), IV (Tylenchina) and V (Rhabditina) (suborders) belong to the order Rhabditida. Later, the branching order of clades within Rhabditida was resolved as clades III and (IV and V) (Blaxter and Koutsovoulos, 2015).

Free-living model nematodes

Several nematodes have been developed as model organisms in basic and applied research. *Caenorhabditis elegans* and *Pristionchus pacificus,* which belong to clade V, infraorders *Rhabditomorpha* and *Diplogasteromorpha,* respectively (Blaxter, 2011) (see Figure 1), are two of the most used model nematodes at present (Meneely, Dahlberg and Rose, 2019; Sommer, 2025). Both these nematodes are free-living, have short generation times and are easy to culture in laboratory conditions (Sommer, 2006; Corsi, Wightman and Chalfie, 2015). One of the major advantages of using free-living species as model systems is that they can be cultured in the absence of a host throughout their entire life cycle. *C. elegans* was first introduced as a genetic model by Brenner in 1974 (Brenner, 1974). It is now well-established and is one of the best studied model organisms (Corsi, Wightman and Chalfie, 2015; Meneely, Dahlberg and Rose, 2019). *P. pacificus* has initially been developed as a satellite model for *C. elegans* for comparative studies (Hong and Sommer, 2006) but later gained importance as an independent model system (Prabh *et al.*, 2018; Sieriebriennikov *et al.*, 2018). Both of these model systems provide comparative insights into other nematodes, such as *Strongyloides,* in terms of evolution, development and state-of-the-art genetic and genomic tools.

Genus *Strongyloides*

The genus *Strongyloides* is found within clade IV, in the family *Strongyloididae,* along with the genus *Parastrongyloides*. *Strongyloides* is a vertebrate parasitic genus containing about 50 species (Speare, 1989). These parasitic nematodes have complex but fascinating life cycles, including a free-living adult generation and a parasitic generation.

While the more inaccessible parasitic generation can provide morphological features such as the shape of the stoma and ovary for species characterisation, the more accessible free-living generation is rather limited in this area (Little, 1966). Owing to these difficulties in the early days, some *Strongyloides* species were characterised based on the host they were found in (Little, 1966), adding to the theatrical confusions in the species descriptions I will come to discuss later. Therefore, the taxonomic classification within the genus is based on molecular phylogenies. A representative phylogeny of *Strongyloides* spp. is shown in Figure 2.

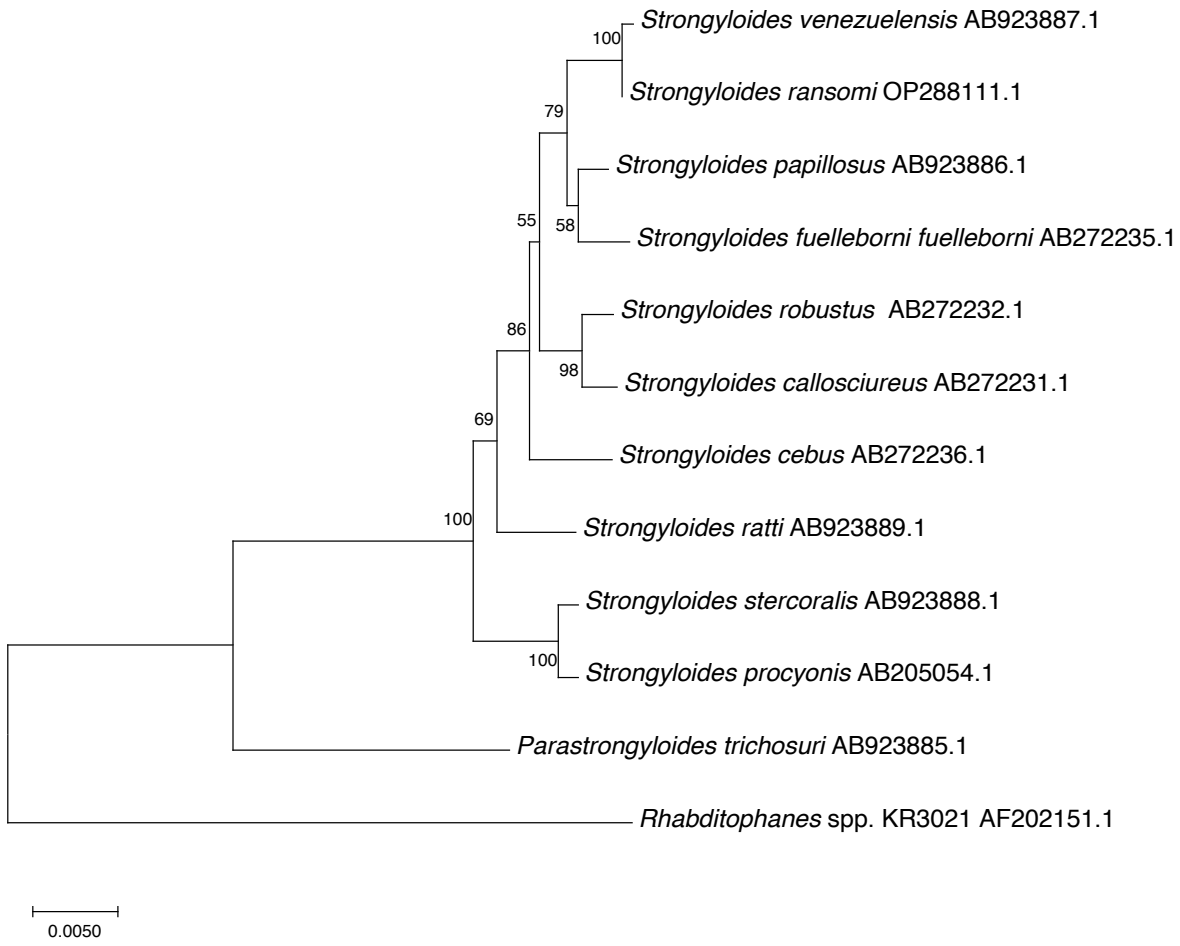

Figure 2 – Phylogenetic relationship of *Strongyloides* species based on 18S SSU sequences. This tree was generated using the *Strongyloides* species from (Hino *et al.*, 2014) and further extended with additional species.

Within the genus *Strongyloides*, *Strongyloides stercoralis*, *Strongyloides fuelleborni* and *Strongyloides ratti* will be discussed in this thesis along with *C. elegans* and *P. pacificus*.

The life cycle of *Strongyloides* parasites is captivating for the curious mind and will be introduced below using the life cycle of the human parasite *S. stercoralis* (Figure 3).

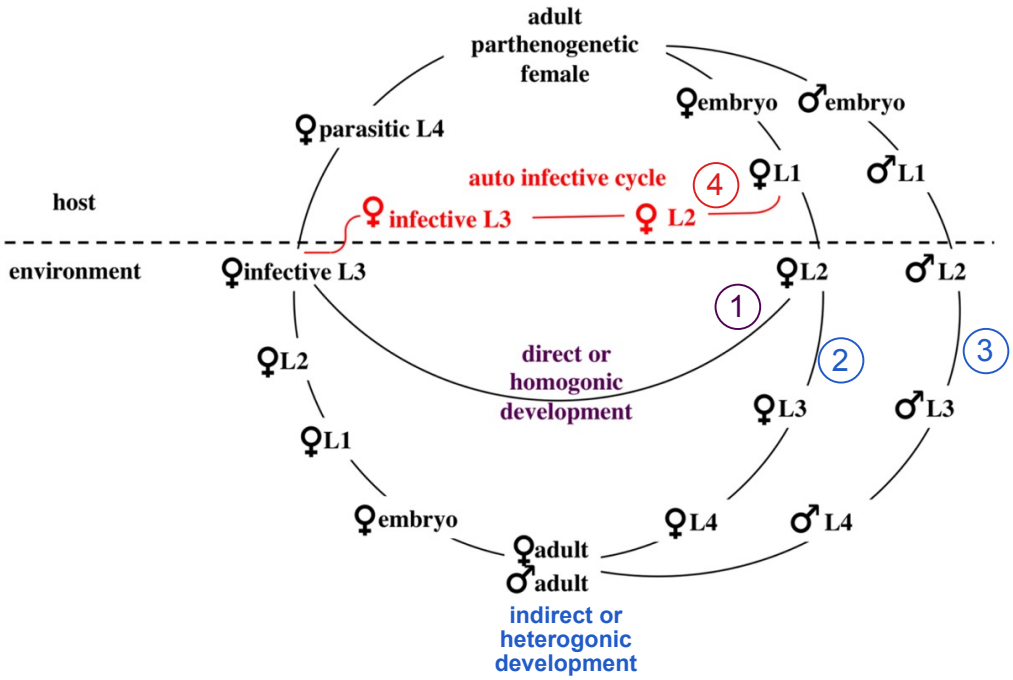

Figure 3 - The life cycle of *S. stercoralis*. For more explanation see text. The figure was reproduced from (Jaleta et al., 2017) under the Creative Commons Attribution License.

The host is infected by the infective third-stage larvae (iL3s), which are all females (Viney and Lok, 2015). They invade a new host percutaneously, migrate through the circulatory system and the respiratory system of the host and eventually establish themselves as adult parasites in the small intestine (Tindall and Wilson, 1988). iL3 is 0.4-0.8mm in body length and 13-20µm in width, has a filariform oesophagus that is about half of the body length (Little, 1966).

The parasitic adult females are also filariform worms, with 2-5mm in length and 30-80µm in width (Little, 1966). They reproduce by parthenogenesis, and depending on the species, the eggs (40-85µm in length) produced by the parasitic female or hatched larvae (L1) are released to the environment with the host's faeces (Viney and Lok, 2015). The L1 has multiple developmental options as illustrated in Figure 3: (1) they may become female, leave the host as first-stage larvae and develop into iL3 in the environment and search for a new host (direct/homogonic development); (2) they may become female, leave the host as first-stage larvae, and develop into free-living, non-infective third-stage larvae and subsequently develop into adult females (indirect/heterogonic development); (3) they may become male and develop into free-living adult males (indirect/heterogonic cycle). Only *S. stercoralis* has the additional developmental option (4) where they may become female, and develop into iL3

within the host and re-infect the same host individual (autoinfective cycle) (Viney and Lok, 2015).

Both free-living adults are rhabditiform and males are about 0.8-1.1mm in length and 38-55µm in width, with the female being distinctly larger than the male within the species (0.8-1.7mm in length and 40-85mm in width) (Little, 1966). They mate and reproduce sexually in the environment and all their progeny are females and develop into iL3s (Viney and Lok, 2015).

The first mention of a parasite belonging to the genus *Strongyloides* was the sheep parasite described as *Trichosoma papillosum* in 1856 (Wedl, 1856) and later reclassified as *S. papillosus* in 1911 (Ransom, 1911). But it wasn't until 20 years later that the genus got much attention with the discovery of *S. stercoralis,* the type species for the genus (Ransom, 1911). *S. stercoralis* is a human parasite first found in troops returning from modern-day Vietnam to France by Louis Normand in 1876 (Normand, 1876). What Normand found were eel-like worms in the faeces; thus his colleague Bavay named those worms as *Anguillula stercoralis* ("anguillula" meaning eel, "stercus" meaning dung) (Bavay, 1876). These eel-like worms were larvae which developed into free-living female and male adults. During autopsies, Normand found these larvae in multiple organs of the deceased soldiers as well, namely the stomach, intestine, pancreatic duct, bile duct, hepatic ducts and the gallbladder (Bavay, 1876). In 1876, Normand discovered two other life stages, the parasitic adult worms and the filariform larvae resulting from the direct development. However, Bavay described these as a different species in 1877 and gave the name *A. intestinalis* (Bavay, 1877a, 1877b). Later, in 1878, Grassi and Parona discovered that eggs of *A. intestinalis* hatched in the intestine were identical *to A. stercoralis* found in faeces (Grassi and Parona, 1879), leading Grassi to usher in a new genus called *Strongyloides* in 1879 (Grassi, 1879).

Since the first appearance of these worms, there has been much debate, from the higher taxon level down to the spelling of the name. It is incredible that since its introduction to the world, *S. stercoralis* has had 11 names until the current name was settled in 1902 (Stiles and Hassall, 1902; Speare, 1986).

Strongyloides infection in humans

Causative agents

Human infection is mainly caused by *S. stercoralis*. However, *S. fuelleborni fuelleborni* and *S. fuelleborni kellyi* can also infect humans (Olsen *et al.*, 2009; Nutman, 2017).

S. fuelleborni is commonly considered the 'egg-producing' *Strongyloides*, as eggs are found in the faeces instead of larvae. This species is the predominant *Strongyloides* in Old World non-human primates (NHP). *S. fuelleborni* were found in *Pan troglodytes* (chimpanzees) and *Papio cynocephalus* (yellow baboons) in Africa and were first described by von Linstow in 1905 (von Linstow, 1905). Later, another *Strongyloides* species was described for NHP as *S. simiae* which was found in *Macaca* spp. in Asia by Hung and Hoppli in 1923 (Hung, S.L.Hoppli, 1923). Later Sandground (Sandground, 1925), Goodey (Goodey, 1926), Premvati (Premvati, 1959) and Little (Little, 1966) showed that the morphological characters used to differentiate *S. simiae* from *S. fuelleborni* were 'assumed', thus invalid. Hence, the worms identified as *S. simiae* are in fact *S. fuelleborni*.

In 1971, Kelly described a *Strongyloides* spp. in humans in Papua New Guinea (PNG) that resembled *S. fuelleborni* (Kelly and Voge, 1973). Viney later split the species into two subspecies based on comparison of *S. fuelleborni* from Africa and PNG. He proposed the *S. fuelleborni* from Africa to be named as *S. fuelleborni fuelleborni* and from PNG as *S. fuelleborni kellyi* (Viney, Ashford and Barnish, 1991). However, the taxonomic status of *S. fuelleborni kellyi* remains unclear (Zhao *et al.*, 2025). For the remainder of this thesis, *S. fuelleborni* always refers to *S. fuelleborni fuelleborni.*

The Asian and African *S. fuelleborni* are monophyletic but divide into two sub-clades as Asian and African, based on geography, in genetic phylogeny analysis (Hasegawa *et al.*, 2010; Thanchomnang *et al.*, 2017; Janwan *et al.*, 2020; Bradbury *et al.*, 2021; de Ree *et al.*, 2024). The only other *Strongyloides* that infects primates is *S. cebus* and is the *Strongyloides* of the New World NHP of South America which is considered a different species (Little, 1966). *S. cebus* is not reported to infect humans so far. Recently, based on mitochondrial protein sequences, Ko (Ko *et al.*, 2023) showed that *S. cebus* is quite distant from the other two

primate *Strongyloides; S. stercoralis* and *S. fuelleborni* suggesting its independent evolution in South America.

Natural *S. stercoralis* infections were found in dogs, cats and NHPs (Sandground, 1925; Faust and Kagy, 1933; Hasegawa *et al.*, 2010; Labes *et al.*, 2011; Riggio *et al.*, 2013; Wulcan *et al.*, 2019). Experimentally, *Meriones unguiculatus* (Mongolian gerbils) can be successfully infected by *S. stercoralis,* to show all life cycle choices, including the autoinfective cycle (Nolan *et al.*, 2002). Gerbils are not considered natural hosts of *S. stercoralis* and to establish a stable infection, quite a high number (at least several hundred) of iL3s are required.

The zoonotic potential of *S. stercoralis*

S. stercoralis, found in dogs, sparked debate regarding its zoonotic potential since its first description. Initially, Fülleborn found worms morphologically indistinguishable from those of humans in Japan and China (Fűlleborn and Schilling-Torgau, 1911; Fülleborn, 1914). Later, Ware and Ware reported the same from a dog in India (Ware and Ware, 1923). However, Brumpt regarded worms found in the dogs were distinct enough from those of the man to be considered a different species; *S. canis* (Brumpt, 1922). Brumpt's opinion was based on (1) the geographical range differences in the human and dog infections, (2) difficulties with establishing lasting infections in dogs using strains from humans, and (3) the occurrence of only direct life cycle in natural dog infection or both direct and indirect life cycles in dogs infected with human strains.

Then, over the next 70 years, a series of cross-infection studies flooded the literature [reviewed by (Bradbury and Streit, 2024)], yet none were able to unequivocally elucidate the species specificity or non-thereof of the worms found in humans and in dogs [for examples, see (Grassi, 1879; Brumpt, 1922; Sandground, 1925, 1926, 1928; Fülleborn, 1927; Augustine and Davey, 1939; Galliard, 1939, 1951a, 1951b; Sandosham, 1952; Georgi and Sprinkle, 1974; Grove and Northern, 1982; Grove, Heenan and Northern, 1983; Mansfield *et al.*, 1996)].

Both dogs and humans were experimentally infected with different *S. stercoralis* isolates using greatly varying experimental procedures, making comparison difficult. Dogs infected with human strains from different geographical origins either were refractory or self-cured within

3-13 weeks or remained patent for more than 3 months. Immunosuppressed dogs were more susceptible to the patent infection. Human infection with dog strains has been attempted twice (Sandground, 1925; Augustine and Davey, 1939). Both times, strains were from the United States of America (USA). In the first case, the infection was patent, but the larval excretion only lasted 4 days (Sandground, 1925), and in the second case, the infection was refractory (Augustine and Davey, 1939).

Ramachandran used molecular tools to study the worms in humans and in dogs for the first time in 1997, based on polymerase chain reaction (PCR) amplification and restriction enzyme digestion (Ramachandran, Gam and Neva, 1997). Since then, a PCR-sequencing-based phylogenetic analysis system has been established to study *Strongyloides* species. Several hypervariable regions (HVR) in SSU were identified to differ between and within *Strongyloides* species; HVR I and HVR IV, and part of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*-1) were used to recognise variation within and among species (Hasegawa *et al.*, 2009, 2010). *S. stercoralis* phylogeny based on the above showed separation based on host species instead of geography, unlike *S. fuelleborni*, which phylogenetically groups according to geography and not according to the host species.

Recently, based on SSU HVR I, HVR IV, *cox*-1, major sperm protein domain (MSPD) and whole genome sequence (WGS) phylogeny, two distinct types were identified within *S. stercoralis* as the 'human and dog shared' type/clade and the 'dog only' type/clade by Jaleta and Nagayasu in 2017 (Jaleta *et al.*, 2017; Nagayasu *et al.*, 2017). The 'dog only' type has so far been reported from Cambodia, Myanmar and Australia and has the SSU HVR IV haplotype B (Jaleta *et al.*, 2017; Beknazarova *et al.*, 2019). Haplotypes A and E were found in worms belonging to the 'human and dog shared' type from Japan, Myanmar, Cambodia, China, Thailand, Laos, Uganda, Tanzania, Central African Republic (CAR), Australia and Switzerland in humans and dogs; in St Kitts in cats and Tanzania in chimpanzees (Jaleta *et al.*, 2017; Nagayasu *et al.*, 2017; Basso *et al.*, 2019; Beknazarova *et al.*, 2019; Wulcan *et al.*, 2019; Zhou *et al.*, 2019; Aupalee *et al.*, 2020).

The same host being infected with multiple haplotypes has also been reported (Schär *et al.*, 2014; Nagayasu *et al.*, 2017). Using such multi-locus sequence typing (MLST) with different

loci combinations (SSU MSPD, *cox*-1) (even different lengths) from single worm DNA extractions made comparison between studies rather difficult. In 2020, Barratt and Sapp introduced a machine learning (ML) method to use clustered overlapping but different/even incomplete loci combinations of MLST data to calculate phylogenetic distance for *S. stercoralis* and *S. fuelleborni*. Employing this method, they proposed that *S. stercoralis* is more of a species complex comprising seven clusters. Clusters 4 and 5 were overrepresented in dogs, and the rest contained worms isolated from human, dog, cat and chimpanzee (Barratt and Sapp, 2020).

Taken together, these studies suggest that dogs carry types of *S. stercoralis* exclusively found in dogs (dog only) and others that occur in both dogs and humans (human and dog shared). However, it is not proven that the 'human and dog shared' worms found in humans and dogs are really the 'same' or just so similar that they cannot be separated using current molecular taxonomy. Further, the analyses by (Barratt and Sapp, 2020) as well as the fact that some experimental infections of dogs with human isolates were not successful (see above), suggest that there might also exist types that can infect humans but not dogs. It should also be noticed that there is a clear sampling bias towards Southeast Asia and Australia, and towards humans as hosts, compared to dogs, cats or NHPs.

Prior to our study in Bangladesh (de Ree et al. 2024), which is part of this thesis, co-occurrence of *cox*-1 sequences (mitochondrial genome) from 'human and dog shared' type with HVR IV sequences (nuclear genome) from 'dog only' type or vice versa had never been observed. Therefore, it was suggested that the two types do not interbreed despite the co-occurrence in the same dog (Barratt *et al.*, 2019; Beknazarova *et al.*, 2019). This assumed inability to interbreed and being exclusively found in dogs has prompted suggestions to recognise the 'dog only' type as *S. canis* as originally proposed by Brumpt (Brumpt, 1922; Jaleta *et al.*, 2017; Barratt *et al.*, 2019).

The zoonotic potential of *S. fuelleborni*

S. fuelleborni infections in humans are mostly reported from Africa (Barratt and Sapp, 2020). In 1972, Pampiglione had shown the ability of human to human transmission of *S. fuelleborni*

by infecting a human with *S. fuelleborni* larvae obtained from another human (Pampiglione and Ricciardi, 1972). Later, Hera and Patel showed indication of human-to-human transmission in urban and peri-urban areas in Zambia, where the transmission took place in the absence of an NHP host (Hira and Patel, 1980). In 2016, based on the differences in *cox*-1 and HVR-IV sequences, Hasegawa showed indications of human-adapted lineages in the Central African Republic (CAR) (Hasegawa et al., 2016). *S. fuelleborni* genotypes similar to that of in NHP was reported in humans who have close contact with NHP in CAR (gorilla trackers. NHP: Bulindi and Tanzania) (Hasegawa et al., 2016), humans returning from Tanzania (Japanese mammalogist, who participated in a field survey in Tanzania) (Hasegawa et al., 2010) and from the Democratic Republic of the Congo (DRC) (a Belgian research student collecting faeces and urine from Pan paniscus) (Potters et al., 2020).

In Asia, human *S. fuelleborni* infection was thought to be extremely rare and was only reported in humans in close contact with NHPs. Labes reported *S. fuelleborni* in a human caretaker working with Bornean orangutans in Indonesia (Labes *et al.*, 2011), while in Thailand, reports came in by Thanchomnang in a human living near a monkey park and by Janwan in the owners of pig-tailed macaques (Thanchomnang *et al.*, 2017; Janwan *et al.*, 2020). However, our study in Bangladesh suggests that *S. fuelleborni* in Asia may play a more important role as a human parasite (de Ree *et al.*, 2024).

Disease Manifestation in Humans

Strongyloides worm infection in humans causes the disease strongyloidiasis (Nutman, 2017). The predominant causative agent of the infection, *S. stercoralis*, is estimated to infect 600 million people worldwide (Buonfrate *et al.*, 2020), yet the correct number is expected to be even higher.

At the early stages of the infection, iL3s enter the host, migrate through the circulatory and the respiratory systems and establish themselves in the small intestine. The infection at this stage is usually light, often asymptomatic due to low worm burden, and the symptoms, if present, are often nonspecific and go undiagnosed. Symptoms can include gastrointestinal-related symptoms such as abdominal discomfort, abdominal pain, diarrhoea, constipation, and nausea; respiratory symptoms such as coughing and wheezing; dermatological

symptoms such as irritation at the site of penetration, itching and urticarial rashes known as 'larva currens'; and other observations such as splenomegaly and transaminitis (Freedman, 1991; Angheben *et al.*, 2011; Greaves *et al.*, 2013; Nutman, 2017; Yeung *et al.*, 2022)**.**

Normally, parasitic infections only last for as long as the parasite lives. In the case of *S. stercoralis,* however, persisting chronic infection is possible (up to 75 years has been reported) due to the autoinfective life cycle (Figure 3) (Leighton and Macsween, 1990; Robson, Beeching and Gill, 2009; Prendki *et al.*, 2011). In chronic strongyloidiasis, only about half of the patients become symptomatic (Salvador *et al.*, 2019; Buonfrate *et al.*, 2021). As a result of the chronic asymptomatic status of the disease combined with issues related to diagnosis mentioned below, the number of strongyloidiasis cases worldwide is speculated to be much higher than 600 million (Autier *et al.*, 2021). The clinical symptoms of strongyloidiasis vary depending on the worm burden, and in the chronic state relatively low numbers of worms are maintained in the host. Most of the symptoms of chronic strongyloidiasis are similar to those described above for the early stage infection (Ming *et al.*, 2019; Buonfrate *et al.*, 2021). Additionally, recurring gastrointestinal symptoms may mimic inflammatory bowel disease, and the 'larva currens' can appear in the perianal, thigh and abdominal regions as well due to the auto-infective cycle (Arthur and Shelley, 1958; Gomez-Hinojosa *et al.*, 2020; Buonfrate *et al.*, 2021). In asymptomatic strongyloidiasis patients, the infection may be visible in the form of mild eosinophilia (Kunst *et al.*, 2011; Buonfrate *et al.*, 2021).

In immunocompromised patients, the altered immune response may lead to acceleration of the autoinfection cycle, leading to excessive worm burden and the development of the hyperinfection syndrome with more severe respiratory and gastrointestinal symptoms (Keiser and Nutman, 2004; Buonfrate *et al.*, 2013; Geri *et al.*, 2015).

Widespread dissemination of larvae beyond the lungs and intestine into other organs such as the liver, brain, heart and urinary tract results in disseminated strongyloidiasis (Keiser and Nutman, 2004; Salluh *et al.*, 2005). The larvae penetrating the intestinal lumen can carry bacteria from the intestinal lumen, causing secondary bacterial sepsis and multiorgan failure (Salluh *et al.*, 2005). Patients may also develop meningitis caused by translocated gastric

bacteria and show symptoms of altered mental status (Keiser and Nutman, 2004). Both hyperinfection syndrome and disseminated strongyloidiasis, if not diagnosed in time and left untreated, is fatal in most cases (Salluh *et al.*, 2005; Buonfrate *et al.*, 2013; Geri *et al.*, 2015).

Diagnosis

The asymptomatic nature and the presentation of nonspecific symptoms make the diagnosis challenging. The other hurdle in diagnosis is the lack of a gold standard diagnostic technique. In *S. stercoralis* infection larvae are excreted with faeces (Figure 3). Most standard faecal-based techniques that are used to detect helminth infections such as the one recommended by the WHO; Kato-Katz (World Health Organisation, 2006) mainly detect eggs and have low sensitivity. Low worm burden resulting in few larvae in faeces and intermittent larval excretion inherently contribute to the low sensitivity of these faecal-based techniques. The Agar plate method (specifically Kogar agar) and Baerman technique are currently the most sensitive faecal-based methods but are not 100% reliable (Intapan *et al.*, 2005; Knopp *et al.*, 2008; Requena-Méndez *et al.*, 2013; Polanco, Gutiérrez and Arias, 2014; Requena-Mendez *et al.*, 2014).

Serological techniques have demonstrated better sensitivity, but can show cross-reactivity with other helminth infections (Bisoffi *et al.*, 2014; Weitzel *et al.*, 2024). Another drawback is the lack of sensitivity of serological techniques in immunocompromised patients (Hall, Salibindla and Luckett, 2024). Several molecular-based techniques exist but lack accuracy (Buonfrate *et al.*, 2017, 2018) although can be useful in monitoring treatment efficacy in clinical settings (Wammes *et al.*, 2023).

Eosinophilia has been observed in patients with acute and chronic strongyloidiasis (Angheben *et al.*, 2011; Mitchell *et al.*, 2017). Although it is a nonspecific indicator and cannot be used as the sole criterion for diagnosis, unexplained eosinophilia acts as a warning sign for strongyloidiasis (Sudarshi *et al.*, 2003; Page and Speare, 2016; Ashiri *et al.*, 2025). However, eosinophilia is less common in patients with hyperinfection and disseminated stages (Rojas *et al.*, 2023).

The most tragic hurdle in diagnosing strongyloidiasis in clinical settings and the high mortality resulting consequently, is the lack of awareness of such a life-threatening disease among medical professionals. Surveys conducted among practicing physicians and medical trainees revealed a lack of awareness of strongyloidiasis and a lack of consideration for parasitic screening, as well as opting to use steroids to treat symptoms (Boulware *et al.*, 2007; De l'Étoile-Morel *et al.*, 2022).

Medication

The broad-spectrum anthelmintic, ivermectin is the drug of choice to treat strongyloidiasis (Gordon *et al.*, 2024). In nematodes, it binds to glutamate-gated chloride channels of pharyngeal muscles, motor nerves, female gonad and excretory/secretory pores; inhibiting pharyngeal pumping, motility, egg/microfilaria release and the ability to suppress host immunosuppression, respectively. The binding of ivermectin to the iron channels increases the influx of chloride ions, leading to subsequent hyperpolarisation, paralysis and the death of the parasite (Ikeda, 2003; Martin, Robertson and Choudhary, 2020; Mathachan, Sardana and Khurana, 2021; Sulik *et al.*, 2023).

Ivermectin is more effective than the alternative drug albendazole and at least as effective as thiabendazole, with a better safety profile than thiabendazole (Henriquez-Camacho *et al.*, 2016). Albendazole is still the second choice of treatment if ivermectin is unavailable or not recommended (Buonfrate *et al.*, 2022). A new alternative drug, moxidectin, approved for treating *Onchocerca volvulus* is currently being tested for efficacy and safety in treating strongyloidiasis (Sprecher *et al.*, 2024). Moxidectin also belongs to the same family as ivermectin, with small structural differences.

Despite the heavy global burden, there were no global public health control programs or guidelines for strongyloidiasis until the WHO introduced the "WHO guideline on preventive chemotherapy for public health control of strongyloidiasis" in 2024 (World Health Organisation, 2024). Here, mass drug administration (MDA) of ivermectin is recommended for strongyloidiasis control in endemic areas. Ivermectin has been used in mass drug administration programs before for onchocerciasis and lymphatic filariasis in humans for

decades and there is growing concern over developing drug resistance to ivermectin, as this is already a serious concern in veterinary settings (Prichard, 2007).

Global distribution of strongyloidiasis and transmission routes

The most common route of transmission of strongyloidiasis is the skin penetration of the host by the infective larvae (iL3) that lives in the environment (Figure 3) (Viney and Lok, 2007). Strongyloidiasis is commonly known as a neglected tropical disease (NTD) found in tropical and sub-tropical regions where the warm, moist climates favour the growth and survival of soil-transmitted helminths (STH) (Schär *et al.*, 2013). The disease is also associated with poor hygiene and rural communities with low socioeconomic status (Beknazarova, Whiley and Ross, 2016). The majority of the infections, amounting to the 600 million estimated global infections, occur in Southeast Asia, Africa and the Western Pacific regions (Buonfrate *et al.*, 2020). In Australia, a country with a high socioeconomic status, the infections are found among remote and indigenous communities living in low socioeconomic settings (Fisher, McCarry and Currie, 1993; Einsiedel *et al.*, 2013).

However, strongyloidiasis prevalence is also reported in temperate climates such as North America and Europe (Buonfrate et al., 2020). *Strongyloides* prevalence has been reported in several states of the USA. Hospitalisations with strongyloidiasis in the USA were associated with low socioeconomic status with non-white ethnicity, male sex and advanced age (Inagaki, Bradbury and Hobbs, 2022). A recent study in Texas suggested ongoing autochthonous transmission within the community (Singer *et al.*, 2020).

In Europe, most reports are concentrated in the Southern and Eastern European countries. Albeit low, historically, local transmission was known in Europe mainly in association with elderly people and agricultural work (Buonfrate *et al.*, 2016; Ottino *et al.*, 2020). Most of these reports are from Spain, Italy and France. Autochthonous transmission has also been reported in recent years in Spain, eastern Slovakia and Portugal (Román-Sánchez *et al.*, 2003; Duvignaud, Pistone and Malvy, 2016; Štrkolcová *et al.*, 2017; Pinto *et al.*, 2021).

While in USA, Europe and Australia, strongyloidiasis prevalence is largely attributed to immigrants or refugees from endemic areas, cases were also seen in returning military

personnel and travellers from endemic countries (Robson, Beeching and Gill, 2009; Angheben *et al.*, 2011; Buonfrate *et al.*, 2012, 2016; Asundi *et al.*, 2019; Ming *et al.*, 2019; Martinez-Pérez *et al.*, 2020; Shield *et al.*, 2021; Inagaki, Bradbury and Hobbs, 2022).

The estimated global pooled prevalence of *S. stercoralis* in the zoonotic host candidate, dogs, is 6%, with a pooled prevalence of 5% in Asia, 21% in Africa, 2% in North America, 2% in South America, 6% in Oceania, and 2% in Europe (Eslahi *et al.*, 2022; Gorgani-Firouzjaee *et al.*, 2022). The genotype of the 'dog only' type has been found so far in Australia, Cambodia, Myanmar, Grenada and Bangladesh, while the potential zoonotic genotype, belonging to the 'human and dog shared' type has been found worldwide including the temperate regions (Barratt *et al.*, 2019; Basso *et al.*, 2019; Nosková *et al.*, 2023; de Ree *et al.*, 2024). Like in humans, local transmission within Europe and imported strongyloidiasis in dogs has been reported (Eydal and Skírnisson, 2016; Hall *et al.*, 2020; Unterköfler *et al.*, 2022; Nosková *et al.*, 2024). In dogs, transmammary transmission of *S. stercoralis* has also been observed (Shoop *et al.*, 2002).

Sexual transmission of strongyloidiasis among men who have sex with men (MSM) has been reported in the USA and Europe. This has been in association with oral-anal sex, anilingus, scatophiliac practices with receptive coprophagia, and various other practices, all of which facilitate person-to-person transmission of the infective larvae. These reports have also been associated with foreign travel and sexual partners from endemic regions, along with HIV (Sorvillo *et al.*, 1983; Chessell *et al.*, 2024; Develoux *et al.*, 2025).

Strongyloidiasis has also been reported in relation to solid-organ and hematopoietic cell transplant and associated high mortality. It can present both as a donor-derived infection and as an acceleration of the chronic infection due to the immunocompromised status of the recipient (Kim *et al.*, 2016; Abrantes *et al.*, 2023). Travel history to or residence of endemic areas were commonly observed in both doners and recipients. (Abad *et al.*, 2022). Patients co-infected with human T cell lymphotropic virus 1 (HTLV-1) have also shown tendency to develop hyperinfection syndrome (Ye, Taylor and Rosadas, 2022).

Due to the possibility and the nature of person-to-person transmission and local transmission mentioned above, it is clear that the absence of foreign travel history does not exclude the possibility of strongyloidiasis. Taken together, it can also be derived that the increasing prevalence in temperate climate regions, including USA and Europe and the potential zoonosis facilitated by the increasing global warming, strongyloidiasis, no longer remains a concern only of the tropical and sub-tropical regions but rather a global threat.

However, it is also important to highlight that current knowledge of strongyloidiasis is very limited. Many questions remain unanswered, including the genetic reasoning behind disease manifestation and advancement (Al-Jawabreh *et al.*, 2024). The decision whether to develop into infective filariform larvae in an immunocompromised host will determine the host's life. Most genomic/molecular taxonomic studies of *S. stercoralis* are concentrated in East and Southeast Asia [Cambodia (Schär *et al.*, 2014; Jaleta *et al.*, 2017), Laos (Laymanivong *et al.*, 2016), Myanmar (Nagayasu *et al.*, 2017), China (Zhou *et al.*, 2019), and Thailand (Aupalee *et al.*, 2020)], Australia (Beknazarova *et al.*, 2019) and Japan (Kikuchi *et al.*, 2016). *S. stercoralis* isolated from most of these locations showed extensive genetic variability. Many worms isolated from some countries showed closer relatedness to some worms from another continent than to worms from the same country (Aupalee *et al.*, 2020). An exception to this was the *S. stercoralis* in Guangxi, China, which were phylogenetically closely related to each other and appeared to reproduce predominantly, if not exclusively, asexually (Zhou *et al.*, 2019). This diversity in human *S. stercoralis* genotypes/species could explain the varied virulence and shed light on the aforementioned 'decision-making'. To better understand the genetic structure, it is important to expand the study area further toward the west.

Strongyloides **tools and resources**

Genomes

Molecular epidemiologic and phylogenetic studies are highly dependent on genome sequences. The first (in 1998) and most complete genome assembly of a multicellular organism to date belongs to *C. elegans* (The C. elegans Sequencing Consortium, 1998) and is about 100.3 Mb in size (NCBI GCF_000002985.6) and *P. pacificus* has a somewhat larger genome of 158.5 Mb (NCBI GCA_000180635.4). The only available *Strongyloides* genomes, *S.*

ratti (43.9 Mb) (NCBI GCA_963264645.1), *S. venezulensis* (56Mb) (NCBI GCA_963280985.1), *S. papillosus* (58.2Mb) (NCBI GCA_005656395.1) and *S. stercoralis* (44.4 Mb) (NCBI GCA_029582065.1), were all published by the *Strongyloides* genome consortium in 2016 along with two outgroup species, *Parastrongyloides trichosuri* and *Rhabditophanes diutinus* (KR2031) (Hunt *et al.*, 2016). For *S. fuelleborni*, so far, only the mitochondrial genome is available (Ko *et al.*, 2023).

Analysis of the *Strongyloides* genomes and the outgroup species has shown conserved synteny across species (Hunt *et al.*, 2016; Kounosu *et al.*, 2023). The contiguity of the *Strongyloides* genome assemblies varies, and the assemblies are continuously being improved (Al-Jawabreh *et al.*, 2023; Kounosu *et al.*, 2023). In terms of the karyotype, *C. elegans* and *P. pacificus* have five autosomes and one X chromosome, while *S. ratti* and *S. stercoralis* have two autosomes and one X chromosome. *S. papillosus* and *S. venezuelensis* have two chromosomes, one of which is presumed to be the result of the fusion of the X chromosome with one of the autosomes (Nemetschke *et al.*, 2010; Hunt *et al.*, 2016). At the time of writing this thesis, the four genome assemblies of *Strongyloides* have the following assembly status. The assembly of *S. venezuelensis* have chromosome-length scaffolds (NCBI GCA_963280985.1). *S. ratti* genome assembly has two autosomal scaffolds, and the X chromosome is in two scaffolds (NCBI GCA_963264645.1), whereas in *S. stercoralis,* the two autosomes are in chromosome-level scaffolds, and the X chromosome is in six scaffolds (NCBI GCA_029582065.1). The *S. papillosus* genome remains highly fragmented, comprising 79 scaffolds (NCBI GCA_005656395.1).

While the *S. ratti* and *S. stercoralis* genomes are less than half the size of the *C. elegans* genome, the protein-coding contents (~18-22Mb) are comparable with other nematode genomes (Hunt *et al.*, 2016). According to Hunt, the smaller size of the *Strongyloides* genomes is attributed to the significant loss of introns and shorter intergenic regions in these genomes. *S. ratti* and *S. stercoralis* have about 21% and 22% GC content, respectively, making them the most AT-rich genomes among known nematodes (Hunt *et al.*, 2016).

In *Strongyloides* spp. the parasitic female reproduces by mitotic parthenogenesis; therefore, the parasitic (adult parasitic female and iL3) and the adult free-living females are genetically

identical. Hence, the differences between these two stages are due to differential gene expression between the stages. Transcriptomic comparisons between the parasitic (parasitic female and iL3) and free-living stages of *Strongyloides* species have identified key gene families upregulated in the parasitic stages compared to the free-living stages (Hunt *et al.*, 2016, 2018; Baskaran *et al.*, 2017). Most prominent among these gene families, CAP proteins (that have an immunomodulatory role) and astacin proteins (that is associated with tissue migrations of nematode infective larvae) are markedly expanded in *Strongyloides* spp. and are distinctively upregulated between different species, perhaps suggesting their unique use of parasitic mechanisms in infecting their hosts.

Genetic tools

Genetic tools are of paramount importance in studying the biological functions of genes. Being the model nematodes, *C. elegans* and, to a somewhat lesser extent, *P. pacificus* have well-established molecular genetic toolkits as reviewed in (Sommer, 2025) that other free-living and parasitic nematodes comparatively still lack. Despite being parasitic, the sexually reproducing free-living generation of the *Strongyloides* spp. has allowed the adaptation of tools from *C. elegans* for genetic manipulation (Lok *et al.*, 2017).

Transgenesis

Protocols for microinjection of DNA into the syncytial gonad of the adult hermaphroditic worms allow introducing transgenes, which can then be passed to the next generation in the form of extrachromosomal arrays (Kimble *et al.*, 1982; Stinchcomb *et al.*, 1985; Fire, 1986; Mello *et al.*, 1991; Mello and Fire, 1995). Because of the similarity between the gonadal anatomy of *C. elegans* hermaphrodites, *P. pacificus* hermaphrodites and the adult free-living *Strongyloides* females, attempts for the adaptation of methods described for *C. elegans* for these species have been made, but with limited success. While the DNA could be successfully introduced, the transgenes were silenced rapidly. For *P. pacificus* this problem was solved by the co-injection of fragmented genomic DNA (Schlager *et al.*, 2009).

In *S. stercoralis* and *S. ratti,* tissue-specific transgenic expression was achieved in the F1 progeny but not in the subsequent generations (Lok and Massey, 2002; Li *et al.*, 2006, 2011; Junio *et al.*, 2008).

The chromosomal integration of the transgene using piggyBac transposase has been shown to generate stable transgenic lines in *S. stercoralis* and *S. ratti* (Lok, 2012; Shao *et al.*, 2012). This method has recently been optimised to overcome the low transgenic rate from F2 generation onwards compared to the previous protocol (Patel *et al.*, 2024). Both these methods have drawbacks. The extrachromosomal array contains many copies of the transgene and are not fully heritable, leading to mixed (transgenic and non-transgenic) progeny and also to mosaic animals (Mello *et al.*, 1991). The insertion of the transgene using piggyBac transposase is semi-random, thus, the expression of the transgene can be variable due to the position where the transgene is inserted and the copy number variation. It should also be noted that due to the randomness of the transgenic integration, the transgene can be inserted into a genic region, causing gene inactivation.

To overcome these problems, recently CRISPR/Cas9 based methods, have been developed, which allow both generating knockout mutants through non-homologous end joining that result in small insertions or deletions as well as targeted chromosomal integration of single-copy transgenes into precise locations of the genome using homology-directed repair with a template DNA. Among nematodes, these methods were first and most completely established for *C. elegans* (Kim, Hong and Chen, 2022). In *P. pacificus* CRISPR/Cas9 based methods are also routinely used for the generation of knockout mutations (Witte *et al.*, 2015) and for the targeted insertion of small pieces of DNA, such as epitope tags (Lightfoot *et al.*, 2019). Introduction of larger pay loads has so far not been successful. Successful CRISPR/Cas9 based generation of knockout mutants and insertion of transgenes such as fluorescent proteins has been reported for *S. stercoralis* and *S. ratti* (Gang *et al.*, 2017, 2020; Bryant *et al.*, 2018; Cheong *et al.*, 2021; Wang *et al.*, 2021). However, these methods are not routine as they are in the model nematodes. Inserting large DNA fragments (>3kb) using CRISPR/Cas9 however, has been challenging even in *C. elegans* (Ghanta, Ishidate and Mello, 2021). Also, establishing mutant/transgenic lines in *Strongyloides* spp. requires host passages. Further, the number of possible target sites is smaller in *Strongyloides* spp.

compared with *C. elegans*, because the CRISPR/Cas9 editing system requires a 5'-NGG-3' motif close to the desired editing site (Jinek *et al.*, 2012). Due to the AT-richness of the genome of *S. ratti* and *S. stercoralis* (Hunt *et al.*, 2016), 5'-NGG-3' occur less frequently in *Strongyloides* spp. than in *C. elegans*.

RNA interference (RNAi)

Obtaining homozygous mutant worms and establishing transgenic lines of these obligatory parasites using CRISPR/Cas9 system requires several passages through the host (Lok and Unnasch., 2005; Ward, 2015). After the first passage, the mutant adult females and males must be crossed, and their progeny iL3 are used to infect a host again. Each host passage would take about 3 weeks to obtain iL3 of the next generation (own lab experience). It should also be noted that for a successful infection of the *S. stercoralis* alternative host gerbil, at least a few hundred iL3s are required, as mentioned previously [own lab experience, (Nolan *et al.*, 2002)]. The number of host animals required for such experiments increases drastically with each gene of interest and with each attempt that may or may not be successful. Establishing and maintaining transgenic lines in host animals is therefore no small feat in terms of the 'operational cost'. On the other hand, permanently mutating essential genes could result in lethal phenotypes, hindering studies of such genes. Therefore, a method which does not require establishing mutant lines in hosts to study gene function is desirable.

RNA interference (RNAi) is one such method where genes can be temporarily downregulated, thus eliminating the need for host passage. In this method, genes are transcriptionally and post-transcriptionally downregulated in a sequence-specific manner (Hammond, Caudy and Hannon, 2001; Sharp, 2001; Agrawal *et al.*, 2003; Grishok, 2005). RNAi is a naturally existing mechanism in eukaryotes that has been developed as a tool. The natural functions of this mechanism are to protect the genome from the activity of mobile genetic elements [e.g.: viruses and transposons (Tabara *et al.*, 1999)] and to regulate endogenous gene expression (Fischer, 2010).

RNAi was first discovered in *C. elegans* in 1998 (Fire *et al.*, 1998). In *C. elegans*, double-stranded RNAs (dsRNA), identical in sequence to part of the targeted gene, are applied to initiate the process in the experimental setting. RNAi has a cascading mechanism requiring many components, which has been deciphered in *C. elegans*. The applied dsRNAs are processed into small interfering RNAs (siRNA), by the RNase III type enzyme Dicer (Knight and Bass, 2001). Then the siRNAs are loaded onto the primary argonaute protein (RDE-1) and form the RNA-induced silencing complex (RISC) (Tabara *et al.*, 1999; Parrish and Fire, 2001; Steiner *et al.*, 2009). Here, the strand that does not direct the silencing is removed, and the other strand binds with and causes degradation of the endogenous mRNA to achieve gene downregulation. Later, it was shown that siRNAs can be directly applied to obtain the same end result (Elbashir *et al.*, 2001). Applying dsRNA or siRNA can be done in several ways in nematodes, such as injection (Fire *et al.*, 1998), feeding (Timmons and Fire, 1998), electroporation (Issa *et al.*, 2005; Geldhof *et al.*, 2006) and soaking (Tabara, Grishok and Mello, 1998).

In nematodes, plants and fungal systems, there exists an intermediary amplification pathway that enhances the RNAi mechanism, which generates secondary siRNAs using RNA-dependent RNA polymerase (RdRP) (Cogoni and Macino, 1999; Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Smardon *et al.*, 2000; Sijen *et al.*, 2001; Pak and Fire, 2007). In plants, RdRP generates double-stranded secondary 21/24 nt siRNA that are then processed by dicer, while in nematodes, single-stranded 22G siRNA (22 nt long and have a G at their 5' end) with characteristic 5’-PPP are directly generated without dicer processing (Baulcombe, 2007; Chen and Rechavi, 2022). Argonaut proteins are key components in both initial and secondary RNAi pathways. In nematodes, argonaut proteins are greatly expanded, and a nematode-specific argonaut group known as worm argonautes or WAGOs has been identified, onto which the 22G siRNAs are loaded (Yigit *et al.*, 2006; Gu *et al.*, 2009; Buck and Blaxter, 2013). Later, based on sequence similarity, WAGOs were shown in *P. pacificus* as well (Hoogstrate *et al.*, 2014). In all four *Strongyloides* species with a genome sequence, WAGO-like genes have been identified and are suspected to be associated with parasitism (Holz and Streit, 2017; Hunt *et al.*, 2018). Apart from *C. elegans*, as a tool within nematodes, RNAi appears to work reliably in many plant-parasitic nematodes but gave inconsistent results in animal-parasitic nematodes (Maule *et al.*, 2011). Until recently, it was believed

that *Strongyloides* is refractory to RNAi (Viney and Thompson, 2008). In 2019, Dulovic showed RNAi-induced gene silencing in *S. ratti* using a soaking protocol and siRNAs (Dulovic and Streit, 2019). However, this protocol requires 48hrs to 96hrs of soaking, and the mRNA levels are reduced to only about 30%. Hence, the development of more straightforward and more effective methods is still desirable.

In 2020, Shukla showed that poly(UG)-tailed RNAs serve as a step in the intermediary amplification loop in the *C. elegans* RNAi pathway (Shukla *et al.*, 2020). Injection of poly(UG)-tailed RNA can be used to initiate downregulation of genes. This method is of particular importance as it triggers the RNAi pathway quite downstream and eliminates the requirement of initiation using long dsRNA or primary siRNAs, eliminating potential refractory effects due to biological differences in different systems.

The process by which the secondary siRNA and the amplification loop achieve gene downregulation is believed to be as follows (Figure 4). The nucleotidyltransferase RDE-3 recognises the 3' ends of mRNAs that were cleaved by primary or secondary siRNA or piRNAs (Chen *et al.*, 2005; Preston *et al.*, 2019) and adds 3' poly(UG) repeats. The poly(UG) tail is recognised by RdRP, which generates secondary siRNAs using this poly(UG)-tailed RNA as a template. Then these secondary siRNAs associate with WAGOs to bind with and cleave homologous target RNA. The cleaved 3' end is then again recognised by RDE-3 that adds a new poly(UG) tail, which will again be recognised by RdRP. This iterative amplification loop thus can maintain the silencing within and even across a few generations in *C. elegans* (Shukla *et al.*, 2020).

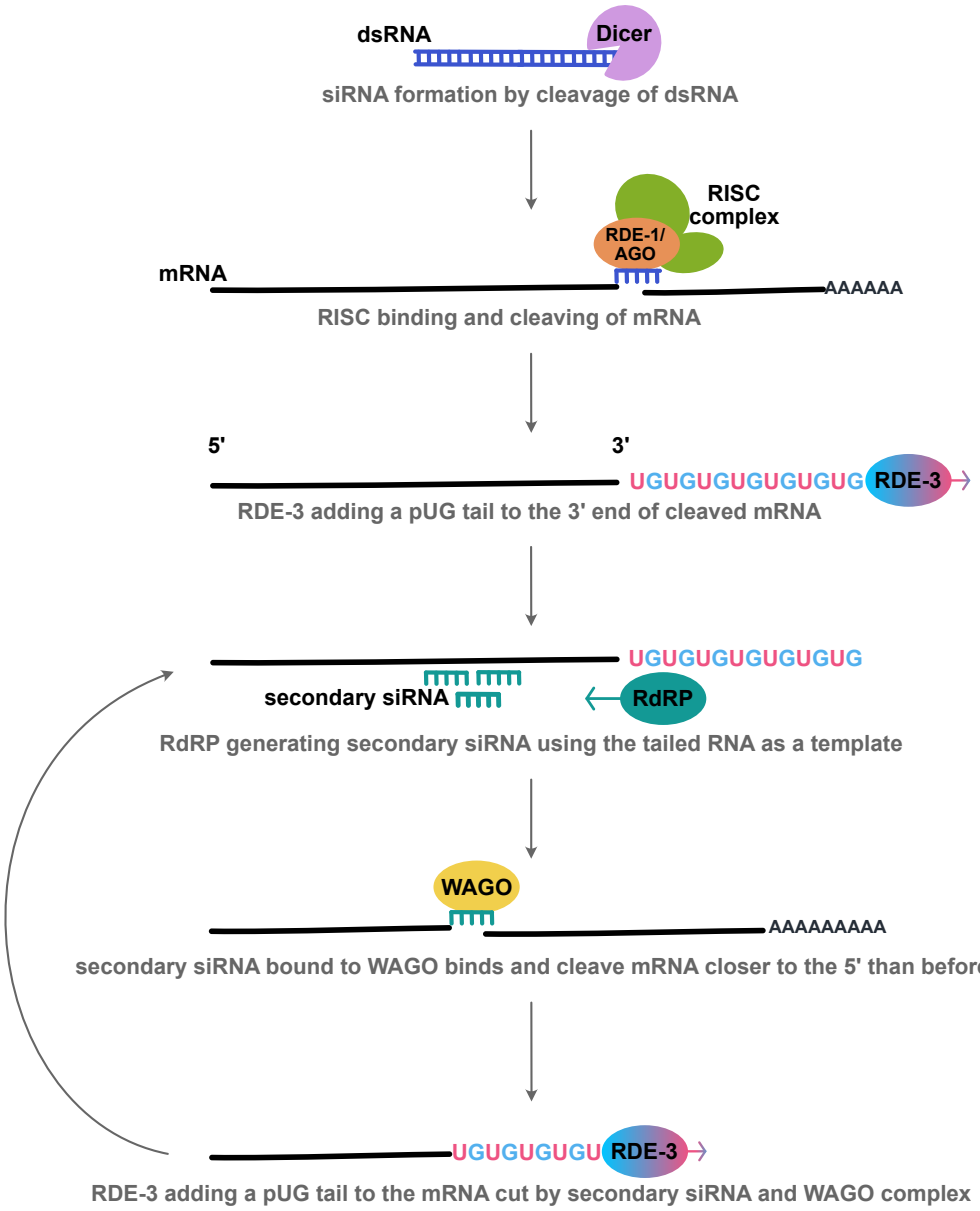

Figure 4 – Working model for RNAi pathway with poly(UG) tailed RNA-dependent siRNA amplification.

Establishing the injection of poly(UG)-tailed RNAs as a method for gene knockdown in *Strongyloides* would be highly appealing. Free-living adults could be injected with the poly(UG)-tailed RNA and the effect could be analysed in the progeny of each mother separately. A prerequisite for such an experimental approach is that the mechanism exists in *Strongyloides*. Adapting methods described for *C. elegans* for other species may be difficult in particular for the phylogenetically distant *Strongyloides*. Therefore, adapting and

optimising such a protocol first in a more closely related nematode to *C. elegans* can be used as a stepping stone to explore if the approach can be extended to nematodes other than *C. elegans*. *P. pacificus* is ideal in this situation, as it is in the same clade as *C. elegans* and equipped with more established lab protocols than *Strongyloides*, along with an easy-to-work free-living life cycle (Pires-daSilva, 2013). In *P. pacificus,* RNAi using dsRNA has been shown to work in some cases, but not in others (Cinkornpumin and Hong, 2011; Adams *et al.*, 2019). Thus, this model system could also benefit from such a method.

Thesis aims

There are two main aims of this thesis. The first is to enhance the current understanding of the *Strongyloides* species responsible for human infections, as well as their potential zoonotic threat, by expanding the sampling area to the west of Southeast Asia. Second, it aims to explore if a novel RNAi method described for *C. elegans* can be used in other nematodes, in particular *Pristionchus pacificus* and *Strongyloides* spp.

Genetic variation and zoonotic potential of *S. stercoralis* and *S. fuelleborni* in Asia

S. stercoralis has been described as the predominant *Strongyloides* species infecting humans worldwide. Its zoonotic potential has been debated for a long time. Different types of *S. stercoralis* have been identified, with some being shared between humans and dogs and others occurring only in dogs and there might also be types occurring in humans but not in dogs. However, so far, the studies on which this finding is based were concentrated in East Asia, Southeast Asia and Australia. *S. fuelleborni* is the second species of *Strongyloides* known to infect humans. In Asia, *S. fuelleborni* infections were considered to be restricted to a very few zoonotic cases (from monkeys) while in Africa there is some evidence for human-to-human transmission of this species. Given the global distribution of strongyloidiasis, there is a clear need to expand the understanding of the genetic structure/diversity of the human-infecting *Strongyloides* species. It is also important to find out if these species are also present in potential zoonotic hosts, e.g. free-roaming dogs and monkeys in the region.

I aimed to expand the geographic range for which molecular genetic information about the *Strongyloides* spp. infecting humans is available. I approached this by collecting and characterising different wild isolates from humans, dogs and monkeys in Iran, Bangladesh and Sri Lanka through field work, lab work and in silico. These results will help to evaluate epidemiological parameters such as the danger of spreading drug resistance, potential drug susceptibility issues due to strain differences, and evaluate control strategies such as MDA, given the potential for zoonotic transmission.

Establishing a gene downregulation method in *P. pacificus* and *Strongyloides*

Current knowledge of strongyloidiasis is very limited, and many questions remain unanswered, including the genetic reasoning behind disease manifestation and advancement, along with the fascinating genetic switches, especially of *S. stercoralis*. To better understand such topics, tools to manipulate genes and their function are essential. The best toolkit among nematodes belongs to the model organism *C. elegans,* and the methods described for *C. elegans* are constantly being adapted to other nematode systems. RNAi is a tool of exceptional importance for *Strongyloides*, particularly *S. stercoralis,* due to the limitations and difficulty in establishing permanent mutant lines in the host species, as it allows temporary gene knockdown. Differences in the performance of RNAi when applied to other nematodes, using methodology as it was described for *C. elegans,* have been noted and were attributed to biological differences.

Recently, in *C. elegans* it has been shown that application of poly(UG)-tailed RNAs, which are an intermediate product of the RNAi amplification machinery, can be used to knockdown gene function. Since this method allows triggering the RNAi cascade quite downstream, it might be suitable for other species where activation of the RNAi machinery at the top, by application of long dsRNA does not work reliably. The aim was to test if the amplification mechanism involving poly(UG)-tailed RNA is also present and might be used for experimental gene knockdown in other nematodes, especially the free-living nematode *P. pacificus* and the parasitic *Strongyloides* spp.

Results and discussion

To present the results of aim 1 that include unpublished data in a continuous form, and to present the published data first, the results for aim 2 are presented first in this section.

Poly(UG)-tailed RNAs are involved in the control of thousands of genes predominantly in the germline in *Pristionchus pacificus*

Veroni de Ree, Dorothee Harbecke, Hanh Witte, Christian Rödelsperger, Catia Igreja, Adrian Streit

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Synopsis

In *C. elegans*, poly(UG)-tailed RNA has been identified as an intermediate product of the downstream amplification step of the RNAi pathway. Injecting such tailed single stranded RNA (ssRNA) leads to RNAi-mediated gene silencing. This method bypasses the need for the processing of double-stranded RNA into siRNAs. Such a method is desirable for experimental gene knockdown in nematodes where long dsRNA-mediated RNAi does not work reliably or not at all, such as the satellite model organism *P. pacificus* and *S. stercoralis*. In this manuscript, we explore this opportunity for these two taxa.

Naturally existing poly(UG)-tailed RNAs throughout the genome were identified in *P. pacificus* using custom RNA sequencing, while in *Strongyloides,* the poly(UG)-tailed RNAs found were comparatively not convincing. Injecting such tailed RNA targeting two endogenous genes (*eud-1, dpy-1*) and a transgene (*daf-1*::TurboRFP) into the gonads of *P. pacificus* worms led to gene-specific knockdown and to newly generated poly(UG)-tailed RNAs that were shorter than the injected ones. Using poly(UG)-tailed RNAs that were tagged by two point mutations, we showed that the endogenous as well as the injected RNAs serve as substrates for the formation of new tailed RNAs.

Knocking out the one-to-one ortholog of *rde-3,* the gene that encodes the polymerase that adds the poly(UG)-tails, in *P. pacificus* by CRISPR/Cas9 showed maternal effect lethality. In *rde-3* mutants that were the progeny of heterozygous mothers, poly(UG)-tailed RNAs appeared reduced but were still detectable, presumably due to the maternal contribution of RDE-3 activity, which allowed these mutants to reach adulthood.

Unlike in *C. elegans*, in *P. pacificus,* gene silencing was not strictly dependent on the presence of a poly(UG) tail, as poly(AC)-tail or non-tailed RNA also induced gene-specific knockdown, albeit comparatively less efficiently than poly(UG)-tailed RNA. To rule out the possible dsRNA contamination, that can occur during the *in vitro* synthesis of the tailed RNAs, was responsible for the silencing effect, the experiments in *C. elegans* were done in a *rde-1* mutant background. In *C. elegans*, *rde-1* mutants cannot respond to long dsRNA and show increased expression of transgenes. *P. pacificus* has three *rde-1*-like genes. We knocked them out individually and in combination. Consistent with *C. elegans*, we observed an increase in the expression of the *daf-1*::TurboRFP transgene in the triple mutant. Different from *C. elegans*, the *P. pacificus* triple *rde-1* mutants were resistant to poly(UG)-tailed RNA induced silencing. All other mutant combinations we tested were still susceptible to silencing by poly(UG)-tailed RNA as well as non-tailed RNA.

In order to find out if poly(UG)-tailed RNAs naturally exist, we performed customized 3'-end RNA sequencing and found poly(UG)-tailed transcripts derived from thousands of genes. Our results suggest that the poly(UG)-tailed RNA-dependent RNAi enhancement mechanism described in *C. elegans* is conserved in *P. pacificus*, a nematode that is estimated to be phylogenetically separated from *C. elegans* by about two hundred million years, but still belongs to the same major clade of nematodes (clade V). Next, we asked if the mechanism is also conserved in the much more phylogenetically distant (clade IV) nematode *S. stercoralis*. Following the injection of poly(UG)-tailed RNAs into *S. stercoralis*, we failed to detect the formation of new shorter poly(UG)-tailed RNAs, and in the 3'-end sequencing experiments, we observed only so few poly(UG)-tailed RNAs for *S. stercoralis* and *S. ratti* that we doubt were real. Although a negative result, we think that the mechanism is either absent or works much less efficiently in *S. stercoralis* and in *S. ratti,* compared with *C. elegans* and *P. pacificus*.

Own contribution

Adrian Streit, Catia Igreja and I designed the experiments. Dorothee Harbecke and Hanh Witte performed injections and Hanh Witte determined the phenotype in the *dpy* experiments. Christian Rödelsperger performed the Illumina sequence analysis. All other experiments and analysis were performed by me. I and Adrian Streit wrote the manuscript with input from the other authors. I consider my contribution to this study to be approximately 75%.

Molecular taxonomic and genomic analysis of *Strongyloides stercoralis* and *Strongyloides fuelleborni* in Iran, Bangladesh and Sri Lanka

This part consists of two published manuscripts and a section describing unpublished results.

Synopsis

Strongyloidiasis is a neglected tropical disease caused by soil transmitted nematodes in the genus *Strongyloides*. Among about 50 species within the genus *Strongyloides*, *S. stercoralis* is the main contributor for the human disease, with about 600 million people currently infected. *S. fuelleborni* can also infect humans and is the predominant *Strongyloides* in old world non-human primates. Based on DNA sequence information, two main clades of *S. fuelleborni* have been proposed, one in Africa and the other in Asia. The majority of human *S. fuelleborni* infections have been reported from Africa. In Asia, it is believed that the few human *S. fuelleborni* infections are restricted to individuals with close contact to non-human primates, indicating that most, if not all, human *S. fuelleborni* cases in Asia are zoonotic.

S. stercoralis is a parasite of humans, and at least some non-human primates (NHP), dogs and cats. Dogs have also been used as experimental hosts for *S. stercoralis*. Recently, two genetically distinct populations were discovered in dogs; ('dog only' and 'human and dog shared' types); one of which is shared with humans, demonstrating the possibility of dogs being a zoonotic reservoir for strongyloidiasis. For *S. stercoralis* with molecular taxonomic information, there is a strong sampling bias towards East Asia, Southeast Asia and Australia.

In order to extend the geographic range of sampling and evaluate the zoonotic potential of *Strongyloides* spp., human, dog and monkey-derived *Strongyloides* spp. were collected from Iran, Bangladesh and Sri Lanka. Worms collected were subjected to molecular taxonomic and genomic analysis based on nuclear (HVR-I and HVR-IV) and mitochondrial (*cox*-1) markers, supplemented with WGS. The results are included in this thesis in the form of two published papers (Iran and Bangladesh) and a chapter with yet unpublished data (Sri Lanka).

***Strongyloides stercoralis* genotyping in a human population in southwestern Iran**

Molouk Beiromvand, Alireza Ashiri, **Veroni de Ree**, Dorothee Harbecke, Christian Rödelsperger, Adrian Streit, Abdollah Rafiei

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In Iran, human samples from patients referred to hospitals in the Khuzestan province for different reasons but found to be infected with *Strongyloides* spp. were analysed. All the sequences belonged to the 'human and dog shared' type based on the *cox*-1 mitochondrial sequences. However, some of these sequences grouped closely with existing sequences from Southeast Asia, showing that this *S. stercoralis* population shares much of the genetic diversity with the population in Southeast Asia, and others made an Iran-specific cluster. In the nuclear genome, all worms had the previously described SSU HVR IV haplotype A, which is indicative for the 'human and dog shared' type. At the SSU HVR I we found the previously described haplotypes I, II and III. However, several worms were heterozygous for two different haplotypes at this locus, something that had been only very rarely observed in Southeast Asia. We also determined WGS for a number of worms and this is the first study that reported WGS of *S. stercoralis* in Iran.

Taken together, this study suggests that in Khuzestan province of Iran, there is an *S. stercoralis* population of the 'human and dog shared' type that shares much of the genetic diversity with the population in Southeast Asia but also has a contribution from a different genetic source. There is indication for different subpopulations, which, however, do inter-breed at least occasionally.

Own contribution

I performed bioinformatic analyses with help and guidance from Christian Rödelsperger and provided input for the manuscript writing. I consider my contribution to this study to be approximately 25%.

Genomic analysis of *Strongyloides stercoralis* and *Strongyloides fuelleborni* in Bangladesh

Veroni de Ree, Tilak Chandra Nath, Priyanka Barua, Dorothee Harbecke, Dongmin Lee, Christian Rödelsperger, Adrian Streit

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Field studies were carried out in two locations of Bangladesh: Sylhet and Dhaka. For comparison, in addition to *Strongyloides* spp., hookworms were also collected. Human hookworm infection is estimated to be 406-480 million globally and is mostly attributed to two closely related species, *Necator americanus* and *Ancylostoma duodenale* (CDC, 2024). Despite the phylogenetic distance, hookworms share the same soil-transmission route of infection with *Strongyloides,* leading to coinfection. All hookworms found in Bangladesh were *Necator americanus*. Out of 18 different *cox*-1 haplotypes found, 12 were newly identified in this study.

In contrast to the expectation based on previous publications about human *S. fuelleborni* infections in Asia, we noticed a rather high incidence of human *S. fuelleborni* infections in Bangladesh. Therefore, *S. fuelleborni* may play a more prominent role in human strongyloidiasis in Asia than previously thought.

Both ‘dog only’ and ‘human and dog shared’ type *S. stercoralis* were found in Bangladesh. 25 different *cox*-1 haplotypes were identified of which 22 were new. All the samples had the previously described SSU HVR-IV haplotype A or a new haplotype, now called V. At the SSU HVR-I we found the previously described haplotypes I(28), II(26), III(1) and V(1). Based on whole genome sequencing, no indications were found for genetic isolation from the

Southeast Asian populations. Previously, the ‘dog only’ type had only been found in Southeast Asia and Australia. Thus, our study extended the range where this type is known to occur further west.

In this study, for the first time, a genomically ‘dog only’ type worm was found in a human sample. More interestingly, two worms from a dog sample showed a nuclear genome of the ‘dog only’ type but a mitochondrial genome of the ‘human and dog shared’ type. The introgression of the ‘human and dog shared’ mitochondrial genome into the ‘dog only’ population suggests that rare interbreeding between the two S*. stercoralis* types does occur. Consequently, exchange of genetic properties, for example, drug resistance, between the two types is conceivable.

Heterozygosity analysis using WGS data from this and previous studies showed very high apparent heterozygosity in the worms belonging to the ‘dog only’ type. Strangely, this was also observed on the X chromosome in males, which have only one X. Upon further analysis, we attributed this finding to structural differences (e.g. duplication and translocations) between the reference genome and the genomes of our ‘dog only’ samples. The reference genome is of ‘human and dog shared’ type. Therefore, a reference genome suitable for ‘dog only’ type is desirable for future analysis.

Own contribution

I performed the field sample collection with Tilak Chandra Nath and Priyanka Barua. I did the laboratory analysis with the help from Dorothee Harbecke. I and Christian Rödelsperger did the bioinformatics analysis. I and Adrian Streit wrote the manuscript with the input from the other authors. This international collaboration was supervised by Tilak Chandra Nath, Priyanka Barua, Dongmin Lee and Adrian Streit. I consider my contribution to this study to be approximately 75%.

Molecular taxonomic study of *Strongyloides spp.* in Sri Lanka (preliminary unpublished results)

Abstract

In Sri Lanka, human samples were isolated from two different locations, Nuwara Eliya and Anuradhapura. Despite the predicted high prevalence, we did not identify any human *S. stercoralis* cases in these sampling areas, suggesting that recent control measures had been successful. We also obtained samples from monkeys and dogs. All the *Strongyloides* samples isolated from monkeys belonged to *S. fuelleborni*. Interestingly, *S. fuelleborni* was also found in two dog samples within the University premises of Rajarata University of Sri Lanka. Although transient infection in dogs or passing through upon coprophagy cannot be ruled out at this point, the possibility of dogs as an additional host for *S. fuelleborni* should be further investigated. Presence of *S. fuelleborni* in the faeces of ever free-roaming monkeys and dogs within the University premises, including the student dormitory area and swimming pool, raises concerns of possible exposure of the University community to *S. fuelleborni* contamination within the University premises.

Based on the mitochondrial sequences, *S. fuelleborni* from Sri Lanka clustered together with the Asian clade samples and did not mix with the African clade. While some samples from Sri Lanka clustered together with samples from other Asian countries, there appear to be 2 additional Sri Lankan-specific sub-clusters. Taken together, *S. fuelleborni* from Asia appears to belong to different mitochondrial subgroups.

At the nuclear level, we observed SSU haplotypes (HVR-I and HVR-IV) so far only found within Asia and Africa co-occur in the same worm, yet at the *cox*-1 level, clustering with the worms from the Asian clade.

The only *S. stercoralis* sample from Sri Lanka was found co-infected with *S. fuelleborni* in a dog and clustered with the 'human and dog shared' *S. stercoralis* samples in terms of mitochondrial sequences.

Introduction

Prevalence and control measures of STH

The overall prevalence of STH in Sri Lanka has dropped dramatically over the last 100 years. In an island-wide survey, conducted in 1924-25, a 90.5% prevalence of hookworms was reported (Chellappah, 1938). By the turn of the century, a survey done with school children in 2003 reported a 6.9% cumulative prevalence for STH (Pathmeswaran *et al.*, 2005) and 1.2% prevalence for hookworms. According to the latest island-wide prevalence survey we are aware of, carried out in 2017, STH prevalence in Sri Lankan school children was 0.97% (Ediriweera *et al.*, 2019).

However, a much higher prevalence compared to the overall national prevalence has been steadily reported in the plantation sector over the years. A survey in 1992-93 reported 89.7% and 86.2% prevalence in children and in women, respectively (Sorensen *et al.*, 1996), whereas a later survey in 2009 reported 29.0% prevalence in the plantation sector (Gunawardena *et al.*, 2011). The survey in 2017 (Ediriweera *et al.*, 2019) continued to report a much higher prevalence in the plantation sector. STH prevalence in the Central Province was reported as 0.42% overall, but in the plantation community, it was 9.02% with the hookworm prevalence being 1.18%. The study area in this chapter was in Nuwara Eliya, which is in the Central Province, and the study subjects belong to a plantation community.

Mass drug administration as a control measure has been employed in Sri Lanka since 1930 (Chellappah, 1938). Later, the mass deworming efforts have been more directed towards targeted populations such as the plantation sector, where a major deworming effort started in 1994 with a biannual single dose of 500mg mebendazole (Ismail *et al.*, 2003) and was discontinued due to lack of funds in 2005 (Gunawardena *et al.*, 2011). Based on the 2003 national survey and the 2009 survey in the plantation sector, another deworming program was carried out from 2013 to 2018, including the plantation sector, with the same medication strategy as before for this population (Ediriweera *et al.*, 2019).

The aforementioned surveys were general STH surveys focused on ascariasis, trichuriasis and hookworm infections. Strongyloidiasis in Sri Lanka is not reported in these surveys, which is not surprising as the detection method used in these surveys is mainly Kato-Katz, which

mainly detects eggs in the faeces and is likely to miss *S. stercoralis,* which sheds hatched larvae, not eggs (Knopp *et al.*, 2014).

Prevalence of strongyloidiasis

The first strongyloidiasis case in Sri Lanka was reported in 1983 when two patients with watery diarrhoea were diagnosed (Wijesundera, Senanayake and Ratnatunge, 1983). Since then, *Strongyloides* prevalence in community surveys has been reported as follows. In 1986 and 1989 in Jaffna: 0.5% [single case in (Nageswaran *et al.*, 1986)] and 1.6% [(Ramadas and Ramadas, 1989) as mentioned in (Weerasekera *et al.*, 2024)], respectively, in 1992-93 in the plantation sector: 0.0% [(Sorensen *et al.*, 1996) as mentioned in (Weerasekera *et al.*, 2024)], in 2006 in the plantation sector: 0.9%, in 2010 among inmates of psychiatric institutions: 0%, in 2012 in Jaffna: 0%, in 2018 in the plantation community and in Colombo: 0% [as reviewed in (N. K. Jayakody *et al.*, 2024)].

However, contrary to the trend with STH in general, there has been an increasing number of case reports of strongyloidiasis in the recent past in Sri Lanka (Morel, Ekanayake and Abeykoon, 2007; Rodrigo *et al.*, 2012; Gunathilaka *et al.*, 2020; Karunatilaka *et al.*, 2021; Morel *et al.*, 2022). All of these reports, except for (Rodrigo *et al.*, 2012), are about immunocompromised patients. A recent cross-sectional study conducted during 2021-23 in immunocompromised patients showed a prevalence of 0.6% for direct smear and culture methods, 11.4% for the PCR-based method and 16.4% for serological methods used in the study, including over 100 patients in each method (Weerasekera *et al.*, 2024).

According to the 2017 island-wide survey, the Anuradhapura district was recognised as a low-prevalence area for STH in general, but a pilot study in 2023 showed 5% prevalence of *S. stercoralis* in the school children (N. Jayakody *et al.*, 2024).

A study conducted in Kandy from 2010-11 showed 11.1% of dogs being infected with *Strongyloides* spp. (domestic-0%, semi-domestic- 20%, stray-13.3%) (Perera, Rajapakse and Rajakaruna, 2013). Another study in 2019 showed *Strongyloides* spp. prevalence in 26% of dogs and in 2% of humans in Lunugala tea estate (Bandaranayaka, Rajapakse and Rajakaruna, 2019). A study in 2022 showed a combined 22.7% prevalence of *Strongyloides* spp. in dogs for two locations in Kandy (Amarasingha *et al.*, 2024).

However, according to the global prevalence estimation study by Buonfrate (Buonfrate *et al.*, 2020), the estimated strongyloidiasis prevalence for Sri Lanka is >10-15% for 2017 as predicted by the best statistical model.

In Sri Lanka, there are 5 non-human primate species, including 3 monkey species: purple-faced langur (*Trachypithecus vetulus* – endemic with four subspecies; *T.v. vetulus, T.v. nestor, T.v. philbricki, T.v. monticola*), toque macaques (*Macaca sinica* - endemic with 3 subspecies; *M.s. aurifrons, M.s. sinica, M.s. opisthomelas*) and Grey Langurs (only found *Semnopithecus priam thersites;* the endemic subspecies) and 2 loris species: *Loris tardigradus* (endemic with 2 subspecies; *L.t. tardigradus* and *L.t. nycticeboides*) and *Loris lydekkarianus* (with 2 out of 4 subspecies found in the island and are endemic; *L.l. nordicus* and *L.l. grandis* (Nahallage *et al.*, 2008; Nekaris and De Silva Wijeyeratne, 2009).

A survey in 2016-17 sampled around 10 species of wild mammals in four national parks in Sri Lanka (Wilpattu, Horton, Udawalawe and Wasgamuwa). They found *Strongyloides* in a Grey Langur and in an unknown carnivore in Udawalawe national park and in Asian Palm Civets (nocturnal mammal) in Wasgamuwa national park (Sepalage and Rajakaruna, 2020). *Strongyloides* was also reported from other animals in the Wasgamuwa national park, namely for Asian Elephants, Bears and Civets (Hewavithana, Wijesinghe and Udagama, 2022).

A study conducted in 2018-19 showed 14.3% overall *Strongyloides* spp. prevalence in toque macaques (*M.s. sinica* and *M.s. aurifrons*) (Thilakarathne *et al.*, 2021). This study also included samples from free-roaming monkey troops within the University of Peradeniya. A more elaborate study in 2019 sampled all 3 toque macaque species from all 3 different climate zones covering urban, suburban and wild areas where they are found in Sri Lanka (Fernando, Udagama and Fernando, 2021). The highest overall parasite prevalence was due to *Strongyloides* infection. Interestingly, urban and suburban monkeys had a higher prevalence of *Strongyloides* than wild monkeys.

Materials and methods

Ethics statement

Both human and animal faecal sample collection and processing were approved by the Ethics Review Committee of the Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka (ERC/2023/11). Participants volunteered in the study and gave informed written consent. In the case of children, the guardian of the child gave written consent. Interested participants were given information verbally in a group setting, followed by a one-on-one interaction regarding the study, where a written information sheet was also given, along with the opportunity to ask questions. All the interactions with the potential participants were done in their preferred language (Sinhalese or Tamil). Then the participants were instructed on how to properly collect the sample and handed a sample collection kit. Returning the sample was entirely voluntary.

Study area, sample collection and processing

Study site selection

We selected the plantation sector as one of the two sites in the sample collection based on the literature. As explained in the introduction, the plantation sector continued to give higher general STH prevalence compared to the rest of the Island. Although few studies in the plantation sector reported zero or very low prevalence for *S. stercoralis*, generally, *S. stercoralis* prevalence in Sri Lanka is understudied (N. K. Jayakody *et al.*, 2024). The Anuradhapura area was selected as the second site, also based on the literature, as a recent pilot study countered the predicted prevalence in the national survey by reporting higher *S. stercoralis* prevalence in the area (N. Jayakody *et al.*, 2024).

Sample collection

Human faecal samples were collected in two areas in Sri Lanka, Nuwara Eliya, along with dog samples, and Anuradhapura. More dog and monkey samples were collected from the premises of the Faculty of Technology of the Rajarata University of Sri Lanka. Sample collection was done in June 2024.

In Nuwara Eliya, samples were collected from the residents of the Dessford Tea Estate, Nanuoya. A total of 200 sample collection kits were distributed, and 125 samples were received, of which 117 were processed. 5 samples from free-roaming dogs in the neighbourhood were also collected. In Anuradhapura, 9 samples were collected from school children. In the University premises, 29 monkey samples and 8 dog samples were collected.

The sample collection kit handed to the participants included a diagrammatic instruction on how to collect the samples in both languages, a pair of gloves to wear during the collection, a tissue paper to catch the faecal sample, a labelled sample collection jar with a spoon and a screw-top lid, a piece of news paper to cover the jar with faeces for privacy, and a Ziplock bag to safely bring back the jar. The next day, the sample jars were collected. Samples from both humans and animals were mixed on site with approximately equal volumes of charcoal to facilitate air exchange, and water was added if needed to moisturise the sample well without it becoming watery. The samples from Nuwara Eliya, and Anuradhapura were then transported to the Faculty of Technology of Rajarata University of Sri Lanka on the same day of collection. The samples collected in the University premises were also treated the same way as described above.

Sample processing

All the samples were processed at the Faculty of Technology of the Rajarata University of Sri Lanka, where they were incubated for 48 hours and remoisturized on a need-to basis. Samples were analysed using the Baermann technique and the sediment was observed under a stereo dissecting microscope for the presence of worms (Zhou, Harbecke and Streit, 2019). Worms were then individually preserved in 80% ethanol and brought back to the Max Planck Institute for Biology, Tübingen for further analysis.

Single-worm lysis was done as described in (Zhou, Harbecke and Streit, 2019) and the SSU HVR-I and *cox*-1 genotyping was done using the primers listed in (Zhou *et al.*, 2019). For SSU the HVR-I primers RH5401 and RH5402 were used and for *cox*-1, the primers ZS6985 and ZS6986 were used. Although the primers used for *cox*-1 genotyping were designed for *S. stercoralis*, they successfully amplified the *S. fuelleborni* sequence in (de Ree *et al.*, 2024). Sequencing of the PCR products and sequence analysis were also done as described in (de Ree *et al.*, 2024).

Whole genome sequencing

Library preparation for 150bp paired-end Illumina sequencing was done as described in (Beiromvand *et al.*, 2024), except that 12-18µl of lysate were used as starting material instead of 10µl, and the bead cleanup after the pooling was skipped. For 30 single worms (of 35 attempted), the library preparation was successful, and 2.2nM from each sample was pooled and submitted for sequencing to the in-house sequencing facility of the Max Planck Institute for Biology, Tübingen, which uses an Illumina NexSeq 2000 instrument.

cox-1 tree

Sequences from whole genome sequencing were aligned to the *S. fuelleborni* whole mitochondrial sequence arrangement A (OL505577.1). Read alignment was done using the bioinformatic pipelines described in (Beiromvand *et al.*, 2024), and the resulting SAM files were converted to BAM files. Then the alignments were loaded to IGV and the consensus sequences were extracted and saved as .dna-files (de Ree *et al.*, 2024), which were then loaded onto SnapGene software (from Dotmatrics; available at snapgene.com) and the same 552bp of *cox*-1 sequences as used in our previous studies (Beiromvand *et al.*, 2024; de Ree *et al.*, 2024) were extracted. The 24 successfully extracted WMIT sequences allowed identifying the *cox*-1 sequence for 12 more worms.

The extracted *cox*-1 sequences along with sequences from PCR were used to generate Neighbour Joining (NJ) trees with 1000 bootstrap values, using MEGA12 (Kumar *et al.*, 2024) after aligning them with the MUSCLE function in the software.

SSU hypervariable regions (HVR-I and HVR-IV)

GenBank entry AB272235.1 was used as the reference in this analysis. Read alignment and consensus sequence extraction were done as described above for *cox*-1 sequences. Sequences were checked manually using IGV and SnapGene software (from Dotmatrics; available at snapgene.com) to determine the haplotypes for HVR-I and HVR-IV.

Results and discussion

Anuradhapura and Nuwara Eliya

All 9 human samples from Anuradhapura and 114 of the 117 human samples from Nuwara Eliya, were negative for any worm. In the three positive samples, based on the 431bp SSU HVR-I fragment, we found worms that were 100% identical with GenBank entry EU196004 (*Auanema rhodensis*, samples SL079 and SL038), worms whose best BLAST hit (84% identity) was GenBank entry OR632674 (*Tokorhabditis artipennis*, samples SL019 and SL038) and worms whose best BLAST hit (84% identity) was MG669838 (*Litoditis* spp., sample SL038).

All these taxa are not parasites (Kanzaki *et al.*, 2017) and we assume that they were contaminants that arrived in the stool sample after deposition. We had noticed *Tokorhabdhitis* spp. in human samples before in Bangladesh (de Ree *et al.*, 2024). *Tokorhabdhitis artipennis* has been found on dung beetles (Kanzaki *et al.*, 2021; Ragsdale *et al.*, 2022). Dung beetles can colonise faeces very quickly, sometimes within seconds (Mohr, 1943).

4 out of 5 dog samples collected from Nuwara Eliya were positive for worms other than *Strongyloides*. Molecular analysis, based on the same 431bp SSU HVR-I fragment, confirmed that 3 of them (SL161, SL163 and SL164) were infected with *Ancylostoma caninum* (100% identity to AJ920347). SL161 also had worms with 99-100% identity to *Ancylostoma* spp. MZ681936. The fourth dog sample (SL165) was contaminated with nematodes presumably of the genus *Caenorhabditis* (98% identical with MH590240, *Caenorhabditis briggsae*), which are free-living nematodes.

As outlined in the introduction, we expected the prevalence of STHs in general and of *S. stercoralis* to be rather high in the chosen study area. Nevertheless, we do believe that the failure of detecting *S. stercoralis* in our samples represents a low worm burden in Nuwara Eliya and is not the result of improper handling of the samples. All samples from Nuwara Eliya (human and dog) were treated the same. 4 out of 5 dog samples contained nematodes, indicating that culture and diagnostic procedures, in particular the transportation, were appropriate. Also, the fact that 3 human samples contained free-living nematodes indicates that the conditions were suitable for nematode survival. The fairly high return rate of the

samples in this study of 62.5% represents a good representative sample of the community, and it indicates a high degree of awareness and interest in engaging in such studies within the community. Recently, there have been continued and increasing efforts to elevate living standards in the plantation sector along with education in the community. Our study suggests that these measures were effective, at least in this particular tea estate community. Given the diagnostic difficulties with detecting low-level *S. stercoralis* infections with the Bearman method, from our results, it cannot be concluded that this parasite is really fully eliminated from the study site. From Anuradhapura, the sample size was too low to come to any conclusion. It should also be noted that this study was designed to isolate *S. stercoralis* individuals for molecular genotyping in order to extend the geographic range from which *S. stercoralis* molecular genetic/genomic information is available, and it was not optimised to assess the prevalence of *S. stercoralis*. Therefore, only diagnostic techniques that allow the isolation of live individual worms (i.e. Baermann funnels) were used. Given the limited number of surveys and the very different estimates of *S. stercoralis* prevalence in Sri Lanka (Buonfrate *et al.*, 2020; N. K. Jayakody *et al.*, 2024), large-scale epidemiological studies utilising multiple diagnostic methods would be very desirable, preferably in the context of more general STH prevalence studies.

University premises

On the premises of the Rajarata University of Sri Lanka, numerous monkeys and dogs roam freely. They live in close proximity to the student dormitories and sports facilities (e.g. the swimming pool). We collected freshly deposited faeces from these animals. Based on morphological and molecular taxonomic (HVR-I and/or *cox*-1) analyses, we found 16 out of 29 monkey samples to be positive for *Strongyloides* and for 15 of them the molecular analysis strongly suggested that these worms belonged to the species *S. fuelleborni*. In the remaining sample, the number of worms was very low and the molecular analysis failed.

In 3 out of 8 dog samples, we found nematodes. Both *cox-1* sequences from sample SLD010 were 90% identical with GenBank entry LC72833.1, which is from *Gurtia* spp. Both other dog samples (samples SLD005 and SLD009) were positive for *S. fuelleborni* based on HVR-I sequences (100% identical with GenBank entry AB677955.1) and cox-1 sequences (see Figure 5). One (sample SLD005) was also positive for *Ancylostoma* spp. (99% identity to the

GenBank entry MZ681936.1), and one worm with a 98% identity to the GenBank entry OM976832.1 (*Panagrolaimus* spp.). Based on the *cox*-1 sequence, one of two sequenced *Strongyloides* spp. of dog SLD005 was *S. stercoralis*, while the other one was *S. fuelleborni* (see Figure 5).

We performed single-worm short-read WGS. Since there is no reference genome for *S. fuelleborni* available and WGS read data were published for only a rather small number of *S. fuelleborni* individuals (Ko *et al.*, 2023; de Ree *et al.*, 2024), we decided to make the read data from the single-worm whole genome sequencing from this study publicly available in order to facilitate future analysis and comparisons (will be published with the manuscript). For this publication, we decided to limit our analyses to the SSU HVR-I*,* SSU HVR-IV and *cox*-1 sequences as those markers were used more widely in previous studies globally.

In total, *cox*-1 sequences from 38 individual worms, representing 14 monkey samples and two dog samples, were used to generate the NJ tree shown in Figure 5. Identical sequences were included in the tree only once, unless they were derived from different host species. There were 12 different *cox*-1 sequences identified in monkeys and two different sequences in dogs, of which one sequence was found in both host species. Figure 5 shows a phylogeny based on *cox-1* of all *Strongyloides* spp. found in this study, along with selected sequences from the databases for species identification.

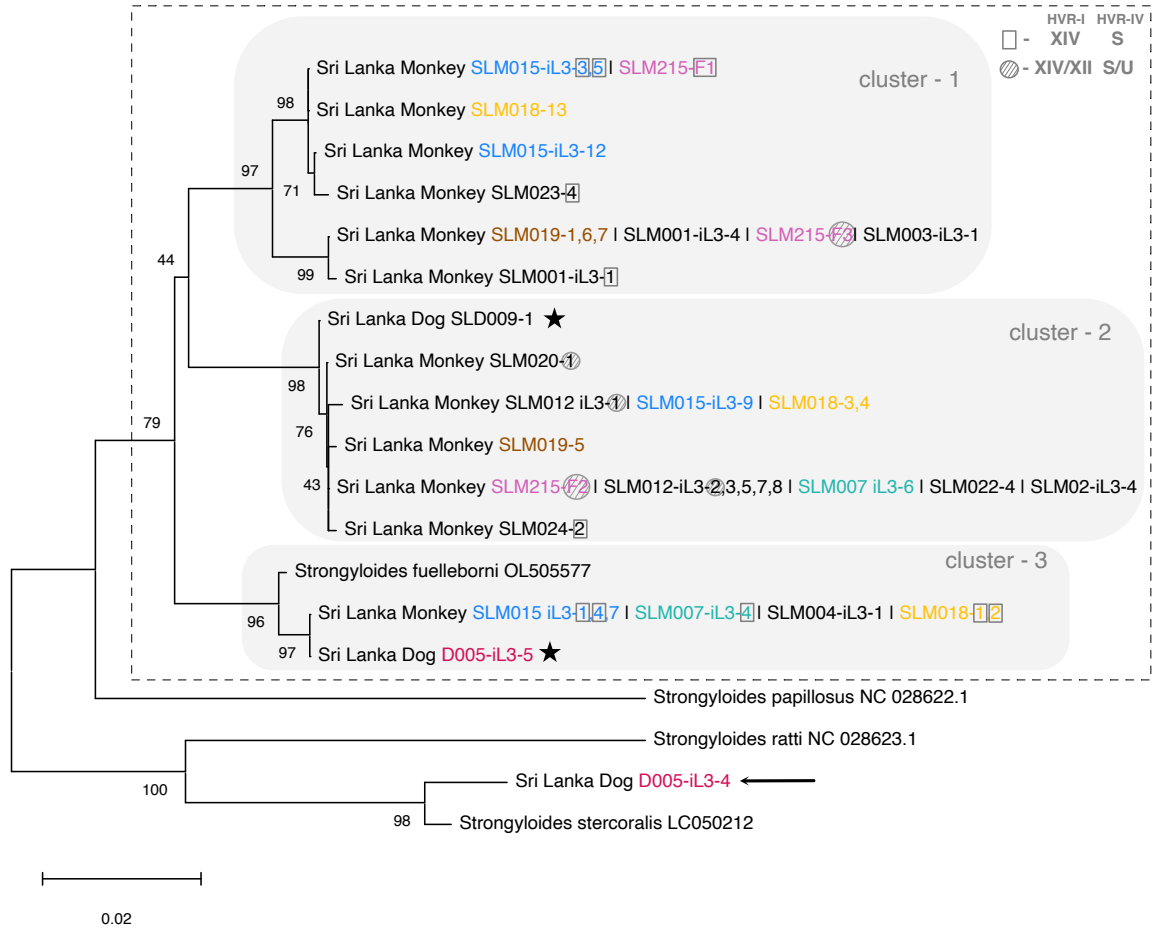


Figure 5 – Neighbour Joining tree of partial *cox*-1 sequences (552bp). The *Strongyloides* spp. *cox*-1 sequences found in this study in Sri Lanka are included. The only sequence found in monkeys and dogs is included twice (in cluster 3). To our knowledge, no *Strongyloides* spp. *cox*-1 sequences from Sri Lanka have been reported before. Worm identifiers written in the same colour are from the same host individual. Asterisks represent the *S. fuelleborni* found in dog samples. The only *S. stercoralis* worm found in this study is indicated by an arrow. For the published sequences, GenBank accession numbers are given. Worms of which both SSU HVR-I and HVR-IV haplotypes were identified are marked in the figure.

The *S. fuelleborni* samples from Sri Lanka clustered with the reference *S. fuelleborni* sequence (OL505577) as expected (Figure 5). These *S. fuelleborni* samples fell into three well-supported (bootstrap) separate clusters. The three clusters do not represent worms from the different monkey species that exist in Sri Lanka, because different worms from the same host individual can be seen in different clusters (worms written in the same colour in Figure 5 were isolated from the same host).

In order to see where the Sri Lankan samples fall within the global phylogeny of *S. fuelleborni* and *S. stercoralis*, selected *S. fuelleborni* and the one *S. stercoralis* sequence from Sri Lanka were used to generate a NJ tree with selected published sequences (Figure 6, an extended version of the figure with all the samples from Sri Lanka and more published data can be seen in Supplement Figure 1). It was made sure that, for each Sri Lanken sequence, the best non-Sri Lankan BLAST hit was included. The representative sequences from cluster 3 of Figure 5, cluster clearly with some sequences from Asia and has 100% identity with MT479211.1 from Thailand. The representative sequences from clusters 1 and 2 also fall within the previously proposed (Barratt and Sapp, 2020) Asian clade of *S. fuelleborni* (although not with very high bootstrap support), but they do not group with high support with any of the published sequences. Therefore, these two clusters, for the moment, appear Sri Lanka-specific. The one *S. stercoralis* sequence we found from a dog in Sri Lanka falls within the previously proposed (Jaleta *et al.*, 2017; Nagayasu *et al.*, 2017) human and dog shared cluster, very close to a sequence previously found in Myanmar (Figure 6).

The SSU HVR-I haplotype for 22 worms representing 12 monkeys was determined. 15 worms had the haplotype XIV and 7 were heterozygous for the haplotypes XIV and XII [for SSU HVR haplotype nomenclature see (Richins *et al.*, 2025)]. The SSU HVR-IV haplotype for 16 worms representing 9 monkeys was determined. 11 worms had the haplotype S and 5 were heterozygous for the haplotypes S and U. From the worms, for which both SSU haplotypes were determined (marked in Figure 6), 11 had HVR-I haplotype XIV and HVR-IV haplotype S. These worms originated from 6 different monkeys. The other 5 worms were heterozygous for the HVR-I haplotypes XIV and XII, and HVR-IV haplotypes S and U. These 5 worms were derived from 3 monkeys. In one monkey sample (M215), both types were observed (XIV, S and XIV/XII, S/U). In the dog samples, we only managed to obtain the HVR-I for 2 worms (in SLD005) of which both had haplotype XIV.

It is important to note that HVR-I haplotype XII and the recently published HVR-IV haplotype U had so far only been found in Africa, while HVR-I haplotype XIV and HVR-IV haplotype S had only been found in Asia (Barratt and Sapp, 2020; Richins *et al.*, 2025). To our knowledge, this is the first observation of *S. fuelleborni* that appear to be hybrids between the Asian and the African clades. This observation must be taken with care. The number of *S.*

fuelleborni for which nuclear markers have been sequenced is not very large and our observation relies only on one marker, namely the SSU.

We noticed that extracting the SSU HVR sequences and the *cox-1* sequences from whole genome sequencing was not always as straight forward as one would expect for multi copy loci. We noticed that in some samples the read coverage dropped dramatically in the HVRs or parts of the *cox-1* locus, such that from some worms some sequences could not be extracted. One possible reason might be that in these samples there was a sequence present (either homozygous or heterozygous) that was just too different from the reference sequences used (AB272235.1 for the SSU and OL505577 for *cox-1*) for the reads to align. This may also have led to missing additional hybrids. Therefore, high-quality de novo genome assemblies for *S. fuelleborni* from different geographic locations would be highly desirable.

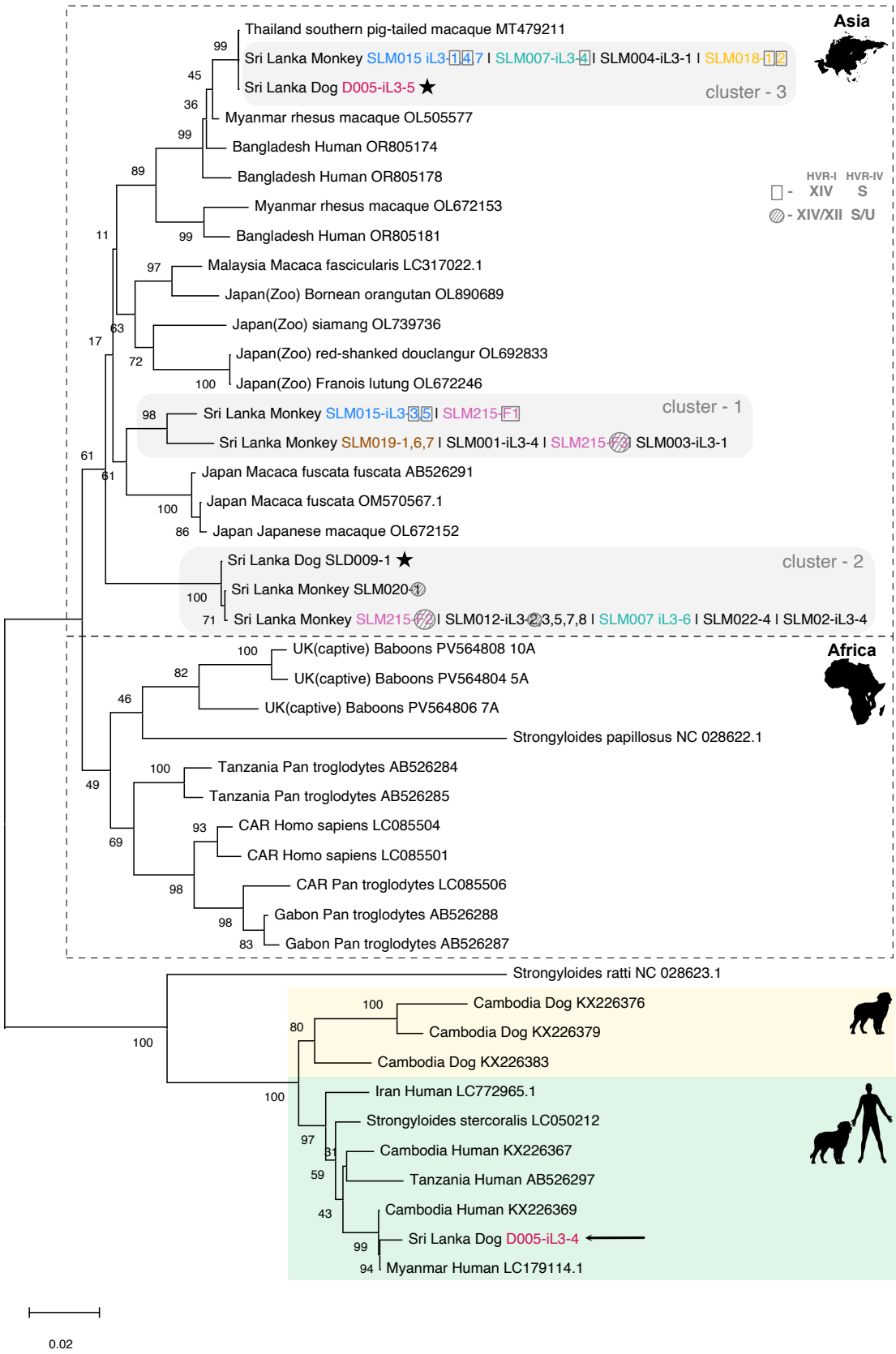


Figure 6 – NJ tree of selected *Strongyloides cox*-1 sequences from Sri Lanka from Figure 5, with selected published *Strongyloides* sequences. Worm identifiers written in the same colour are from the same host individual (the colour scheme is the same as in Figure 5).

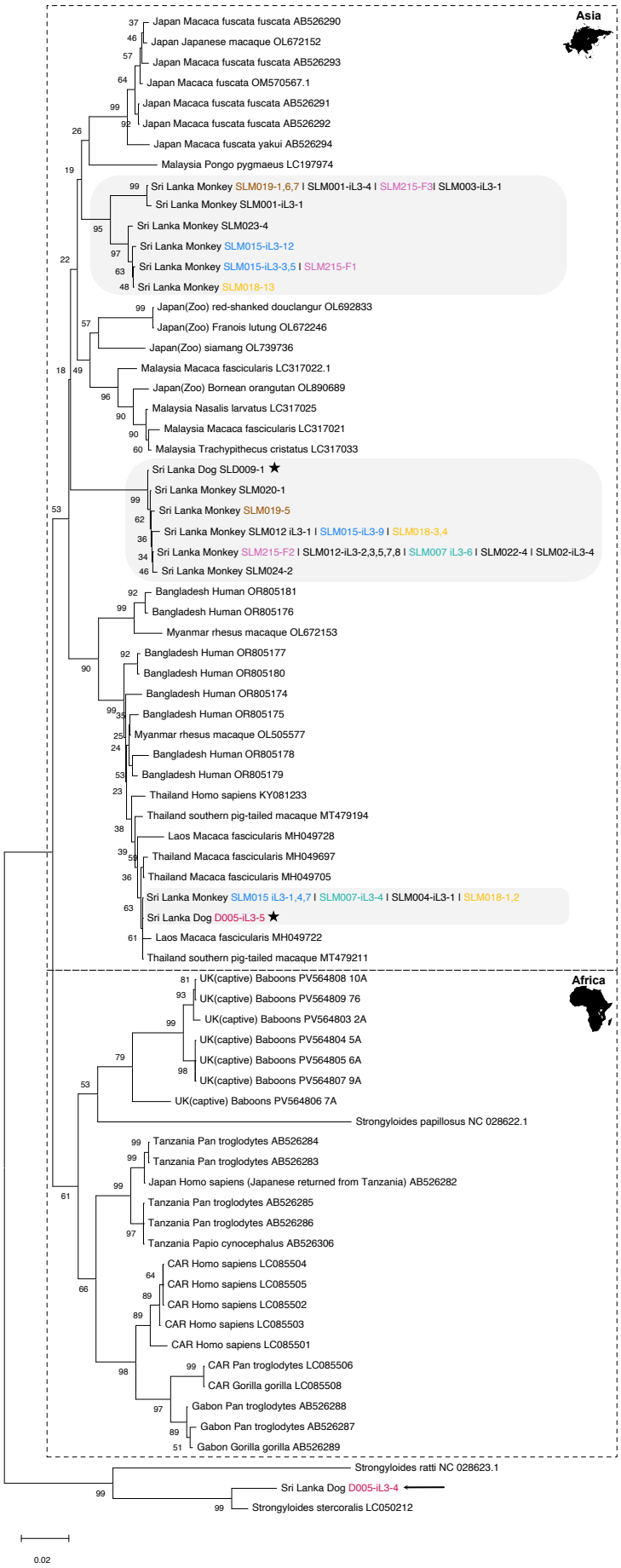
Asterisks represent the *S. fuelleborni* found in dog samples. Worms from this study of which both SSU HVR-I and HVR-IV haplotypes were identified are marked in the figure. The only *S. stercoralis* worm found in Sri Lanka is indicated by an arrow. For the published sequences, the country of origin (CAR = Central African Republic, UK = United Kingdom), the host and the GenBank accession numbers are given.

To our knowledge, this is the first report of *S. fuelleborni* in dog samples. Currently, we cannot tell if these dogs were really fully infected with S*. fuelleborni*. Our findings might represent a transient infection or even passing through of larvae upon coprophagy. However, even if the dogs were not solidly infected with *S. fuelleborni,* they still may contribute to the soil contamination and transmission of *S. fuelleborni*. This observation, therefore, warrants further investigation into considering dogs as possible alternative hosts for *S. fuelleborni*.

It should also be highlighted that many studies from Sri Lanka reporting *Strongyloides* prevalence in dogs and monkeys are reporting *Strongyloides* spp. prevalence mostly based on egg counts. One such study (Perera, Rajapakse and Rajakaruna, 2013) was also considered for the global *S. stercoralis* prevalence prediction studies for dogs (Eslahi *et al.*, 2022). However, since *S. fuelleborni* sheds eggs, while already hatched larvae are passed in the faeces in *S. stercoralis*, the authors of these reports most likely detected *S. fuelleborni*.

It is important to notice that our monkey and dog samples were collected from the University premises, which also include student hostels. The university premises are surrounded by forest and rich in greenery with ample amounts of large fruit trees that attract monkey troops. There are also free-roaming dogs within the university premises. In this large setting, both monkey and dog faeces are not actively cleared from public areas such as paths, the university pool and the immediate vicinity of hostels where students may come into frequent contact with these faeces. Given the recent increase in reports of *S. fuelleborni* infections in humans in Asia, it might be necessary to check the students for infection with *S. fuelleborni*, especially since some of the Sri Lankan *S. fuelleborni* samples clustered together with the *S. fuelleborni* samples found in humans in Bangladesh (Figure 6 and Supplement Figure 1).

Supplements


Asia
Japan Macaca fuscata fuscata AB526290
Japan Japanese macaque OL672152
Japan Macaca fuscata fuscata AB526293
Japan Macaca fuscata OM570567.1
Japan Macaca fuscata fuscata AB526291
Japan Macaca fuscata fuscata AB526292
Japan Macaca fuscata yakui AB526294
Malaysia Pongo pygmaeus LC197974
Sri Lanka Monkey SLM019-1,6,7 I SLM001-iL3-4 I SLM215-F3I SLM003-iL3-1
Sri Lanka Monkey SLM001-iL3-1
Sri Lanka Monkey SLM023-4
Sri Lanka Monkey SLM015-iL3-12
Sri Lanka Monkey SLM015-iL3-3,5 I SLM215-F1
Sri Lanka Monkey SLM018-13
Japan(Zoo) red-shanked douclangur OL692833
Japan(Zoo) Franois lutung OL672246
Japan(Zoo) siamang OL739736
Malaysia Macaca fascicularis LC317022.1
Japan(Zoo) Bornean orangutan OL890689
Malaysia Nasalis larvatus LC317025
Malaysia Macaca fascicularis LC317021
Malaysia Trachypithecus cristatus LC317033
Sri Lanka Dog SLD009-1 ★
Sri Lanka Monkey SLM020-1
Sri Lanka Monkey SLM019-5
Sri Lanka Monkey SLM012 iL3-1 I SLM015-iL3-9 I SLM018-3,4
Sri Lanka Monkey SLM215-F2 I SLM012-iL3-2,3,5,7,8 I SLM007 iL3-6 I SLM022-4 I SLM02-iL3-4
Sri Lanka Monkey SLM024-2
Bangladesh Human OR805181
Bangladesh Human OR805176
Myanmar rhesus macaque OL672153
Bangladesh Human OR805177
Bangladesh Human OR805180
Bangladesh Human OR805174
Bangladesh Human OR805175
Myanmar rhesus macaque OL505577
Bangladesh Human OR805178
Bangladesh Human OR805179
Thailand Homo sapiens KY081233
Thailand southern pig-tailed macaque MT479194
Laos Macaca fascicularis MH049728
Thailand Macaca fascicularis MH049697
Thailand Macaca fascicularis MH049705
Sri Lanka Monkey SLM015 iL3-1,4,7 I SLM007-iL3-4 I SLM004-iL3-1 I SLM018-1,2
Sri Lanka Dog D005-iL3-5 ★
Laos Macaca fascicularis MH049722
Thailand southern pig-tailed macaque MT479211
Africa
UK(captive) Baboons PV564808 10A
UK(captive) Baboons PV564809 76
UK(captive) Baboons PV564803 2A
UK(captive) Baboons PV564804 5A
UK(captive) Baboons PV564805 6A
UK(captive) Baboons PV564807 9A
UK(captive) Baboons PV564806 7A
Strongyloides papillosus NC 028622.1
Tanzania Pan troglodytes AB526284
Tanzania Pan troglodytes AB526283
Japan Homo sapiens (Japanese returned from Tanzania) AB526282
Tanzania Pan troglodytes AB526285
Tanzania Pan troglodytes AB526286
Tanzania Papio cynocephalus AB526306
CAR Homo sapiens LC085504
CAR Homo sapiens LC085505
CAR Homo sapiens LC085502
CAR Homo sapiens LC085503
CAR Homo sapiens LC085501
CAR Pan troglodytes LC085506
CAR Gorilla gorilla LC085508
Gabon Pan troglodytes AB526288
Gabon Pan troglodytes AB526287
Gabon Gorilla gorilla AB526289
Strongyloides ratti NC 028623.1
Sri Lanka Dog D005-iL3-4
Strongyloides stercoralis LC050212
0.02

Supplement Figure 1 – NJ tree of *Strongyloides cox*-1 sequences from Sri Lanka, with more published *S. fuelleborni* sequences. Worm identifiers written in the same colour are from the same host individual. The colour scheme is the same as in Figure 5. Asterisks represent the *S. fuelleborni* found in dog samples. The only *S. stercoralis* worm found in Sri Lanka is indicated by an arrow. For the published sequences, the country of origin (CAR = Central African Republic, UK = United Kingdom), the host and the GenBank accession numbers are given.

Own contribution

I initiated this international collaboration and organized the field trip with Adrian Streit, Kosala Weerakoon and Ajith Rathnayake. I conducted the sample collection with, Lakshitha Kumara, W.A.A.H. Kalhari, H.K.S. De Zoysa, Adrian Streit and Kosala Weerakoon. Sample processing in the field was done by me, with Lakshitha Kumara, W.A.A.H. Kalhari and Adrian Streit. I was involved in the molecular analysis for which I got help from Dorothee Harbecke and Sandra Gyarteng in our laboratory. I performed the bioinformatic analysis under the guidance of Christian Rödelsperger. This work is being prepared as a manuscript for submission, and I wrote the manuscript with input from Adrian Streit and other authors. I consider my contribution to this study to be approximately 80%.

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Publications

This thesis includes two peer reviewed manuscripts and one manuscript submitted to the Bioarchive. Supplementary materials can be found online.

***Strongyloides stercoralis* genotyping in a human population in southwestern Iran**

Molouk Beiromvand, Alireza Ashiri, **Veroni de Ree**, Dorothee Harbecke, Christian Rödelsperger, Adrian Streit, Abdollah Rafiei

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Genomic analysis *of Strongyloides stercoralis* and *Strongyloides fuelleborni* in Bangladesh

Veroni de Ree, Tilak Chandra Nath, Priyanka Barua, Dorothee Harbecke, Dongmin Lee, Christian Rödelsperger, Adrian Streit

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Poly(UG)-tailed RNAs are involved in the control of thousands of genes predominantly in the germline in *Pristionchus pacificus*

Veroni de Ree, Dorothee Harbecke, Hanh Witte, Christian Rödelsperger, Catia Igreja, Adrian Streit

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RESEARCH

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Strongyloides stercoralis genotyping in a human population in southwestern Iran

Molouk Beiromvand[1,2*], Alireza Ashiri[1,2], Veroni de Ree[3], Dorothee Harbecke[3], Christian Rödelsperger[3], Adrian Streit[3*] and Abdollah Rafiei[1,2]

Abstract

Background Strongyloidiasis is a neglected tropical disease (NTD) that is caused mainly by *Strongyloides stercoralis*, with an estimated 600 million people infected worldwide, and in fewer cases by *Strongyloides fuelleborni fuelleborni* and *Strongyloides fuelleborni kellyi*. A number of studies have been conducted on the genetic diversity of *S. stercoralis* in East and Southeast Asia; however, there is very limited corresponding information from West Asian countries, including Iran.

Methods For *Strongyloides* worms collected from patients in southwestern Iran, the hypervariable regions I (HVR-I) and IV (HVR-IV) of the nuclear 18S ribosomal DNA (rDNA) locus (*SSU*) and a fragment of the subunit 1 mitochondrial cytochrome *c* oxidase gene (*cox-1*) were sequenced. For a subset of the worms, whole-genome sequencing data were generated.

Results The *cox-1* sequences of 136 worms isolated from 23 patients indicated that all isolates were *S. stercoralis*. Among the *cox-1* sequences, 33 polymorphic sites and 13 haplotypes were found. The phylogenetic analysis demonstrated that some sequences clustered fairly closely with sequences from humans and dogs from other parts of the world, while others formed a separate, Iran-specific group. Among 64 *S. stercoralis* analyzed, we found three of the previously described *SSU* HVR-I haplotypes, with haplotype II being the most frequent haplotype. In contrast to Southeast Asia, where *S. stercoralis* heterozygous for different haplotypes at the HVR-I locus are rare, we found 20 worms to be heterozygous for two different HVR-I haplotypes, 18 of which fell into the Iran-specific *cox-1* cluster. *SSU*-heterozygous worms also showed elevated heterozygosity at the whole-genome level.

Conclusions We conclude that the *S. stercoralis* population from the Khuzestan province shares much of the genetic diversity with the population in Southeast Asia, but there is an indication of additional genetic input. There appears to be some population structure with different subpopulations, which however do interbreed at least occasionally.

Keywords Strongyloidiasis, *Strongyloides stercoralis*, Iran, Molecular taxonomy, Whole-genome sequencing

*Correspondence:
Molouk Beiromvand
beiromvandm@gmail.com
Adrian Streit
adrian.streit@tuebingen.mpg.de
Full list of author information is available at the end of the article

Background

As one of the most common neglected tropical diseases (NTDs) [1], strongyloidiasis is an intestinal infection affecting about 600 million individuals, predominantly in tropical and subtropical areas [2, 3]. The disease is caused by *Strongyloides stercoralis* and, to a much lesser extent, *Strongyloides fuelleborni fuelleborni* and *Strongyloides fuelleborni kellyi*. In addition to humans, *S. stercoralis* can infect certain animals, including non-human primates (NHPs), cats, and dogs [2, 4, 5]. *Strongyloides stercoralis* is a soil-transmitted helminth with a unique life cycle alternating between free-living and parasitic cycles [6]. Moreover, its internal autoinfection cycle lets it persist in the host's body for decades [6]. Most patients with *S. stercoralis* are asymptomatic [7]. However, several gastrointestinal manifestations, including abdominal pain, diarrhea, and constipation, have been observed in acute and chronic infections [8]. Also, it has been shown that hyperinfection and disseminated strongyloidiasis, which may result from a loss of control of the autoinfection cycle predominantly in immunocompromised patients, is fatal in 85–100% of cases [9]. A lack of early diagnosis and effective treatment can significantly increase *S. stercoralis*-related mortality and morbidity [10].

Strongyloidiasis is usually diagnosed using traditional and routine laboratory methods, such as the Baermann technique or agar plate culture (APC), followed by light microscopy. However, these methods are somewhat limited because of their low sensitivity in mild and chronic infections, and their time-consuming culture process [11]. Moreover, detection of *S. stercoralis* larvae is not always possible, especially in chronic strongyloidiasis, due to intermittent and low egg-laying rates [6]. Therefore, advanced techniques such as molecular and serological methods are sometimes used to overcome the limitations of traditional parasitological methods. However, even these techniques may not have enough sensitivity in acute cases, and they tend to have specificity issues because false-positive results may arise due to cross-reactions with other nematodes [5].

Several studies have investigated the diagnosis, treatment, and epidemiology of *S. stercoralis* in different areas [12–14], and a number of studies distributed over several decades have investigated the zoonotic properties of this parasite, yielding controversial results [15]. However, only a few of these studies included genetic/genomic investigations of this nematode, and the studies that did were geographically heavily biased towards East Asia and Australia [14, 16–19]. Therefore, a comprehensive genomic analysis of this nematode is needed, including its genotypes in various hosts from different areas [2].

Ramachandran et al. [20] were the first to investigate *Strongyloides* using a molecular genetics approach by analyzing a part of its genome from the gene of 18S ribosomal RNA (rRNA) to the gene of 28S rRNA using the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method [20]. Hasegawa et al. [21] subsequently investigated the 18S ribosomal DNA (rDNA) of this nematode to find the sequences that could be used for species–specific diagnosis. This study introduced four hypervariable regions (HVRs), HVR-I to HVR-IV, in the 18S rRNA gene as diagnostic markers for genotyping *Strongyloides* spp. [21]. Since different isolates of *S. stercoralis* from humans, dogs, and chimpanzees tended to show little or no variation in these HVRs, Hasegawa et al. [21] proposed a 722-base-pair (bp) fragment of the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox-1*) as a suitable genotyping marker for detecting intraspecific variation [21]. Several recent studies have investigated the HVR-I, HVR-IV, *cox-1*, and whole-genome sequences of *Strongyloides* spp. isolated from Southeast Asia, Japan, China, and Australia to identify intraspecific variations [14, 16, 17, 22].

Iran is an endemic area for *S. stercoralis* [23]. However, molecular genetic/genomic information about *S. stercoralis* in Iran is extremely limited. While a limited number of *cox-1* and partial 18S sequences derived from *S. stercoralis* from Iran are available in GenBank (search for "cytochrome OR 18S AND *Strongyloides* AND Iran" on the GenBank website [https://www.ncbi.nlm.nih.gov/nuccore] on October 14, 2022, and [24]), to our knowledge, no full-genome information about *S. stercoralis* from Iran has been reported. Therefore, the present study analyzed the HVR-I and HVR-IV of the nuclear 18S rDNA locus (*SSU*), *cox-1*, and the whole-genome sequences of the isolates of *S. stercoralis* collected from the human population of Khuzestan province, located in southwestern Iran.

Methods

Study area and sample collection

Khuzestan province, which is located in southwestern Iran near the border of Iran and Iraq, has hot and sometimes humid summers, especially in the south, and cold and dry winters. The province has an area of 63,238 km^2 and a population of over 4.7 million individuals [25].

Twenty-three patients who had been referred to hospitals in the southern counties of Khuzestan province, including Abadan, Khorramshahr, and Ahvaz, for different reasons but found to be infected with *Strongyloides* spp. were enrolled in the present study (Fig. 1). The patients with strongyloidiasis were first diagnosed using direct smear examination with a light microscope under ×100 and ×400 magnification. Then, their infections were confirmed by observing the morphological characteristics of *S. stercoralis* cultured using the APC method

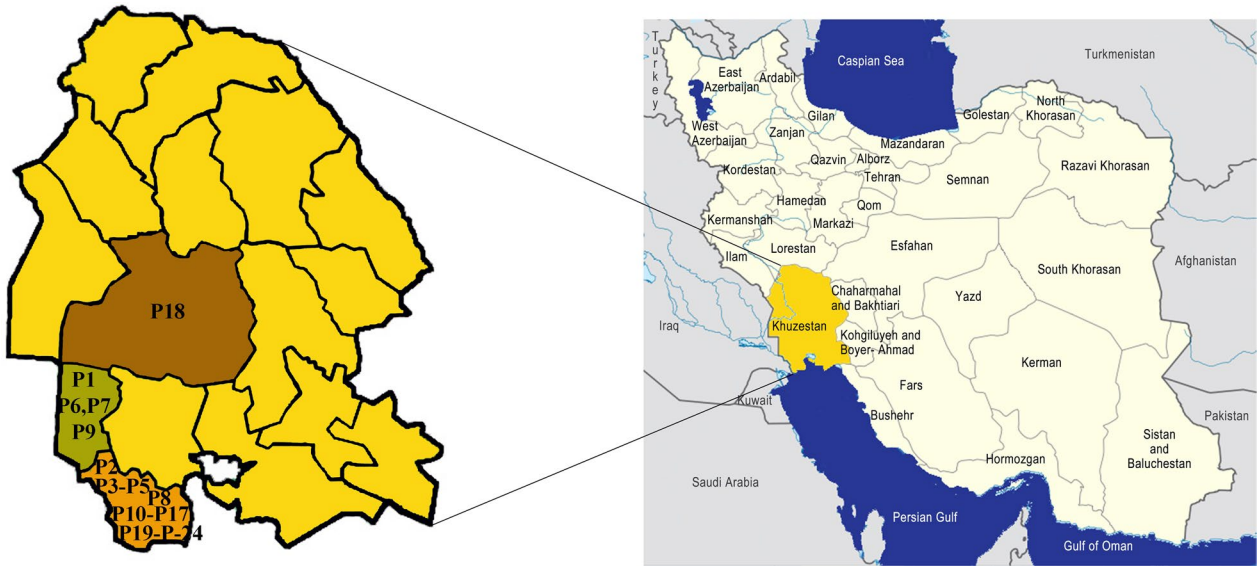

Fig. 1 Map of the sampling area. The origin of the patients (P[number]) is indicated

[26]. After that, the infective larvae and adult worms were transferred to 1.5-ml tubes containing 80% ethanol and stored at −80 °C. The samples were then sent to the Department of Integrative Evolutionary Biology, Max Planck Institute for Biology, Tübingen, Germany, for molecular analyses.

Lysis of *S. stercoralis* adult worms and larvae

The samples containing *S. stercoralis* worms and larvae fixed in ethanol were transferred to a watch glass and rinsed at least three times with tap water. Afterward, each adult worm or larva was picked and transferred to a PCR tube containing 10 µl of PCR water. The samples were then frozen in liquid nitrogen and thawed at room temperature; this process was carried out three times. Afterward, 10 µl of 2× lysis buffer (20 mM Tris–HCl with a pH of 8.3, 100 mM KCl, 5 mM $MgCl_2$, 0.9% NP-40, 0.9% Tween 20, and 240 µg/ml proteinase K) was added to each tube. The tubes were then incubated at 65 °C for 2 h. The resulting lysate was stored at −20 °C until further examination.

PCR amplification of *SSU* and *cox-1*

Three molecular markers (18S HVR-I, 18S HVR-IV, and *cox-1*) were amplified using the primers described by Zhou et. al. [19] (Table 1). A fragment of the nuclear *SSU* gene of *S. stercoralis*, 18S rRNA (HVR-I), with a length of about 862-bp was amplified using 10 µl of Thermo Scientific DreamTaq Green PCR Master Mix (2×), 0.25 µM of each primer, 8 µl of nuclease-free water, and 1 µl of template DNA in a final volume of 20 µl. The PCR program included an initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 20 s,

Table 1 Sequences, annealing temperatures, and product size of PCR primers used in the current study[a]

Target gene	Primers	Nucleotide sequences (5′–3′)	Annealing temp (°C)	Product length
18S rRNA (HVR-I)	Forward (RH5401)	AAACATGAAACCGCGGAAAG	52	862 bp
	Reverse (RH5402)	CATTCTTGGCAAATGCTTTCG		
	Sequencing (RH5403)	AGCTGGAATTACCGCGGCTG		
18S rRNA (HVR-IV)	Forward (18SP4F)	GCGAAAGCATTTGCCAA	57	712 bp
	Reverse (18SPCR)	ACGGCCGGTGTGTAC		
	Sequencing (ZS6269)	GTGGTGCATGGCCGTTC		
cox-1	Forward (ZS6985)	GGTGGTTTTGGTAATTGAATG	47	837 bp
	Reverse (ZS6986)	ACCAGTYAAACCACCAATAGTAA		
	Sequencing (ZS6990)	GGTTGATAAACTATAACAGTACC		

[a] All primers were taken from Zhou et. al. [19]

annealing at 52 °C for 15 s, extension at 72 °C for 90 s, and final extension at 72 °C for 5 min [22].

In addition, a 712-bp fragment of the nuclear *SSU* gene, the 18S rRNA (HVR-IV), was amplified using 10 µl of Thermo Scientific DreamTaq Green PCR Master Mix (2×), 0.2 µM of each primer, 7.2 µl of nuclease-free water, and 2 µl of DNA template in a final volume of 20 µl. The program consisted of one cycle at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 15 s, extension at 72 °C 90 s, and final extension at 72 °C for 10 min. Also, an 837-bp fragment of the mitochondrial *cox-1* gene was amplified using 10 µl of Thermo Scientific DreamTaq Green PCR Master Mix (2×), 0.2 µM of each primer, and 1 µl of the DNA template adjusted to 20 µl with nuclease-free water. The PCR program was as follows: initial denaturation at 95 °C for 30 s, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 47 °C for 15 s, extension at 68 °C for 90 s, and final extension at 68 °C for 5 min.

Sequencing

For sequencing, 1 µl PCR product was mixed with 1 µl of sequencing primer (Table 1) and 8 µl of nuclease-free water and submitted for Sanger sequencing to Genewiz, Leipzig, Germany. The Sanger sequencing data were analyzed using SeqMan Pro version 17.3 (Lasergene package; DNASTAR, Inc., Madison, WI, USA) and were compared with the sequences previously deposited in GenBank at the National Center for Biotechnology Information (NCBI). The accession numbers and the corresponding references are given in Fig. 2. The number of polymorphic sites, average number of pairwise nucleotide differences (K), nucleotide diversity (Pi), and haplotype (gene) diversity (Hd) were calculated using DnaSP version 6. The trees were constructed using MEGA 7 with the neighbor-joining method, and evaluated with 1000 bootstrap repetitions. The evolutionary distances were computed using the Kimura 2-parameter method. The use of the different models resulted in essentially the same tree topology.

Whole-genome sequencing

The protocol for library construction for Illumina whole-genome sequencing was based on a previously described protocol [22]. This protocol was modified according to suggestions by Kohta Yoshida. The modified protocol is described below (for buffer compositions see [22]):

DNA clean-up

A total of 10 µl of single worm lysate was added to a mixture of 10 µl of nuclease-free water, 16 µl of PEG8000/NaCl, and 4 µl of Sera-Mag beads in PEG8000/NaCl. The tube's contents were mixed and left at room temperature for 10 min. The tubes were then placed on a magnetic rack for 5 min at room temperature, the supernatant was discarded, and the remaining content (beads) was washed twice with 200 µl of 80% ethanol while the tube was on the magnet. Afterward, the beads were left to dry for a few minutes until all the remaining ethanol had evaporated. The tubes were removed from the magnetic rack, and 9 µl of Tris–HCl (10 mM, pH = 8.0) was added to the beads. Following complete mixing through pipetting, the tube was incubated for 10 min at room temperature. The samples were then placed on a magnetic stand for 5 min. Finally, 7 µl of the supernatant was transferred to a new tube without disturbing the beads.

DNA tagmentation

The DNA tagmentation was performed as follows: To the 7 µl supernatant, which included the DNA, 5 µl of the Tn5 reaction solution (consisting of 2 µl of water, 2 µl of 5× [tris(hydroxymethyl)methylamino]propanesulfonic acid–dimethylformamide [TAPS-DMF] buffer, and 1 µl of 25× diluted [in glycerol/dialysis buffer] Tn5 [taken from the Nextera DNA Library Prep Kit, Cat. No. FC-121-1030]) was added and mixed by pipetting. The resulting mixture was incubated twice for 7 min at 55 °C in a PCR machine with a lid temperature of 75 °C with mixing by finger tapping between the two incubation steps.

PCR amplification and adapter extension

To the mixture from the previous step (12 µl), 10 µl of 5× Q5 buffer (High-Fidelity DNA Polymerase, New England Biolabs), 2 µl of deoxynucleotide triphosphates (dNTPs) (10 mM each), 2 µl of i5 barcoded Nextera primer (5 µM), 1 µl of i7 barcoded Nextera primer (5 µM), 0.5 µl of Q5 Hi-Fi polymerase, and 39.5 µl of water were added and thoroughly mixed. Afterward, the mixture underwent the following steps in a thermal cycler: one cycle at 72 °C for 4 min, one cycle at 98 °C for 30 s, 14 cycles of denaturation at 98 °C for 15 s, annealing at 67 °C for 20 s, extension at 72 °C for 90 s, and cooling to 4 °C.

Quality check and size selection

For checking the DNA quality and length, 18 samples which included 10 adult worms and eight larvae were randomly selected, and 5 µl of PCR reaction mix was evaluated by gel electrophoresis on a 1.5% agarose 1× tris-acetate-ethylenediaminetetraacetic acid (TAE) gel at 70 V for 5 min and 100 V for 25 min. Afterward, the DNA molecules between 600 and 300 bp were enriched using Sera-Mag beads following the published protocol [22].

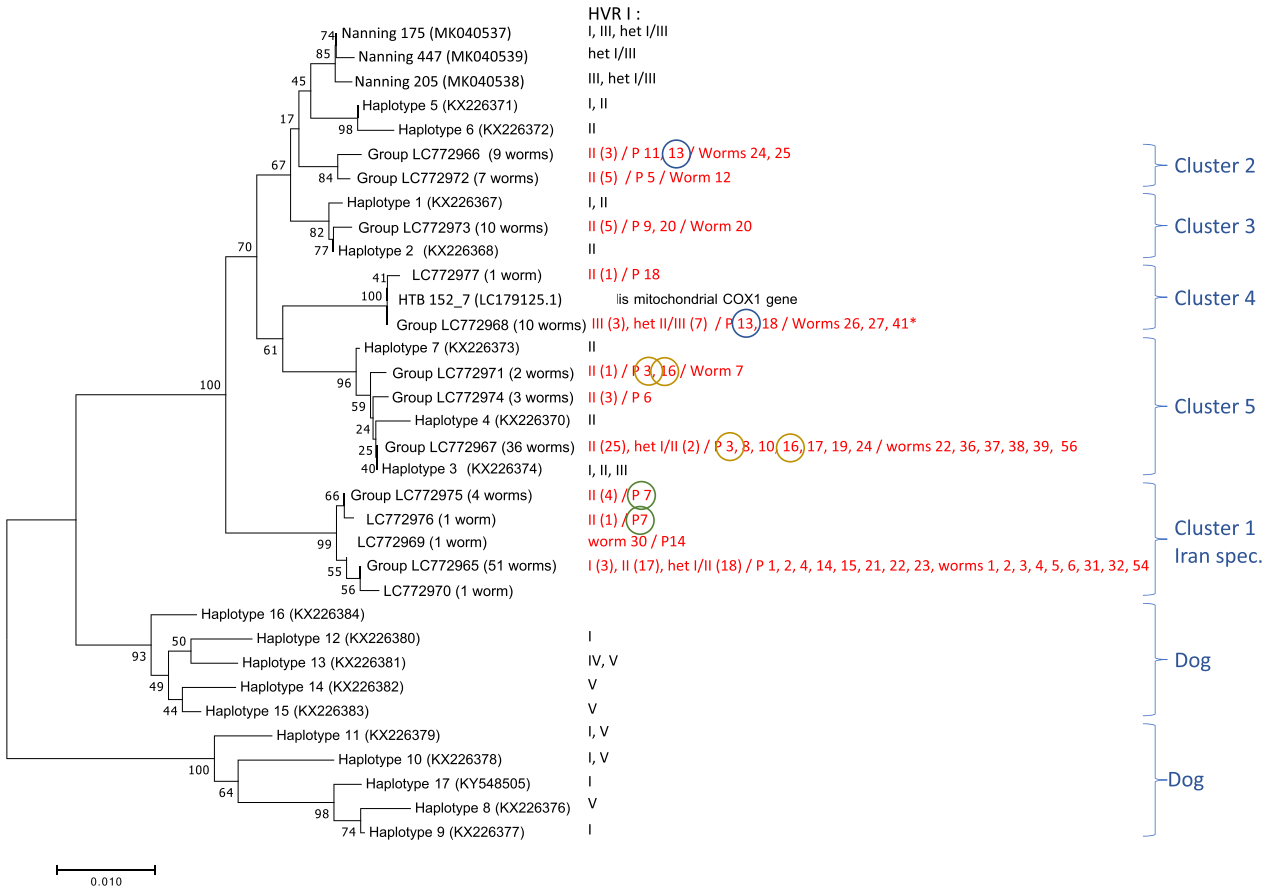

Fig. 2 Neighbor-joining tree based on 552 bp of the mitochondrial *cox-1* gene. In total, 136 *Strongyloides stercoralis* worms from Iran are represented. For comparison, published sequences are included. Each haplotype is included in the tree only once, except for the one case where a haplotype (LC772967) was identical to a previously reported haplotype (KX226374 [14]). To the right, the nuclear *SSU* HVR-I haplotypes (nomenclature according to [14, 32] that were present among the bearers of the particular *cox-1* haplotype are indicated, if known. Values in parentheses indicate the number of worms with this haplotype (note that the *SSU* HVR-I haplotype is not known for all the worms for which the *cox-1* sequence was determined). Results from this study are in red. For these also, the patients with worms with this haplotype and the worms selected for whole-genome sequencing are indicated. Patients with worms of more than one *cox-1* sequence are circled. The blue brackets show clusters. The two dog clusters are from [14]. Scale bar denotes 0.01 changes per nucleotide site. *Note that *SSU* HVR-I haplotypes II and III differ by only one nucleotide (TTT in haplotype II and TAT in haplotype III). Distinguishing homozygous III and heterozygous II/III is therefore not obvious. All three whole-genome-sequenced worms of this group turned out to be heterozygous, although only one of them had been scored as heterozygous based on the HVR-I sequencing alone. Labels: Samples starting with "Nanning" are from [19], samples starting with "Haplotype" are from [14], HTB 152_7 is from [17], samples starting with "Group" are sequences from this study and were found in multiple worms (the number of worms with the particular haplotype is indicated in parentheses), and plain accession numbers are from this study and were found in only one worm

Quantification of concentration and size

The DNA concentration in all samples was assessed using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and the fragment lengths were determined on an Agilent 2100 Bioanalyzer, following the manufacturers' instructions.

Pooling and concentration adjustment

Based on the DNA concentration determined above, 60 fmol of each library was pooled. The pooled volume was measured, and then 1.2× volume of Sera-Mag beads was added to the sample. The pooled sample was incubated for 10 min at room temperature and 5 min on a magnetic stand. After removing the supernatant, the beads were washed twice with 1 ml 80% ethanol while the tube was on the magnet, and the beads were left to dry for a few minutes. The tube was removed from the magnetic stand and the beads were resuspended in 18 µl Tris–HCl (10 mM, pH = 8.0) and incubated for 10 min at room temperature. The tube was then placed on a magnetic stand for 5 min. Finally, 16 µl of the supernatant was transferred to a new tube without disturbing the beads.

The concentration was measured using a Qubit (Thermo Fisher Scientific) and adjusted to 2.5 nM, and then submitted to the Max Planck Institute for Biology in-house sequencing facility for sequencing on an Illumina NextSeq 2000 instrument.

Analysis of whole-genome sequencing data

Between 3.6 and 8.4 million read pairs were sequenced (2 × 150 bp) per sample, resulting in theoretical coverage of 20–60 times for the *S. stercoralis* genome (43 Mb). All reads were uploaded to the European Nucleotide Archive under the study accession PRJEB64686. The BWA-MEM program (version 0.7.17-r1188, default parameters) was used to align raw reads against the *S. stercoralis* genome from WormBase ParaSite (version WBPS11) [27–29]. Since in this genome assembly the *SSU* locus is not fully represented, the reads were also aligned to the 18S sequence (AF279916) in order to confirm the *SSU* HVR-I and HVR-IV haplotypes. The samtools program (version 1.18, view, sort, index, and rmdup commands) was run to generate binary alignment files and to remove duplicate reads, and initial variant calls were generated by combining the mpileup, bcftools view (version 0.1.17-dev), and vcfutils.pl varFilter (-D1000-w0 parameters) commands of the samtools program (version 0.1.18) [27]. Heterozygous sites were defined based on a positive consensus quality (FQ) value in the variant calling file. Variant positions with quality scores > 20 were pooled and genotyped in the alignment files of the current and previous studies using samtools [16, 19]. Variant positions that were genotyped in all samples were used for constructing a neighbor-joining tree with the help of the phangorn library in R [30] and for performing a principal component analysis (PCA) using the Eigensoft smartpca program (version 8.0.0) [31]. At this step, two samples with fewer than 30,000 genotyped variant positions were discarded. For heterozygosity analysis, we considered only samples where at least 85% of all variant positions could be genotyped with a quality score > 20.

Results and discussion

Among the 23 examined *Strongyloides*-positive patients, ranging in age from 40 to 92 years and with a mean age of 70.2 years, 18 (78.3%) were male and the remaining five (21.7%) were female. The majority of patients (18/23) were from Abadan County and its dependent cities and rural regions, and the rest were residents of Khorramshahr (4/23) and Ahvaz counties (1/23), southwestern Iran. In total, 50 adult worms and 106 larvae were isolated from the patients and subjected to molecular analysis.

cox-1 Haplotypes

The PCR amplification and sequencing of *cox-1* was successful for 136 worms. The obtained sequences all had database entries derived from *S. stercoralis* as the best BLAST (Basic Local Alignment Search Tool) hits with very low e-values and, upon phylogenetic analysis, grouped with previously published *S. stercoralis* sequences (see Fig. 2, Additional file 1, for accession numbers and references), indicating that all isolates were *S. stercoralis.* Among the 136 *S. stercoralis cox-1* sequences, 33 polymorphic sites and 13 haplotypes were found (Table 2). The average number of pairwise nucleotide differences (552 bp) and nucleotide diversity among the 13 haplotypes were 11.949 and 0.02165, respectively (for pairwise comparisons see Additional file 2). To evaluate the phylogenetic relationships among the haplotypes from this study and with isolates from different geographical locations, a phylogenetic tree was constructed using the neighbor-joining method (Fig. 2, Additional file 1: Fig. S1). All sequences grouped with the sequences from the human and dog parasitic cluster of *S. stercoralis* [14, 17, 32]. Within this cluster, some sequences grouped fairly closely with sequences from other parts of the world, while others formed a separate Iran-specific group (Fig. 2). The closest database entry to this group is MK049075.1, which is derived from a worm isolated in the Khuzestan province. Since in this database entry the full fragment considered in this study is not available, this sequence is not included in Fig. 2. The most prevalent haplotype outside of the Iran-specific group (LC772967, 36 worms from seven different patients) was identical to haplotype 3 (KX226374) previously reported from humans and from dog-derived worms in Cambodia [14]).

SSU Haplotypes

Overall, we identified *SSU* HVR-I haplotypes I, II, and III [14, 17, 32], and we mapped the *SSU* haplotypes onto the *cox-1* tree. Haplotypes I and II were present in both major parts of the tree, with haplotype II being the most frequent haplotype, observed in 64 isolates. Haplotype III was only found in all 10 worms (isolated from two patients) of cluster 4 of which the *SSU* HVR-I sequence was determined. Our results showed that haplotype II was distributed over the entire *cox-1* phylogeny. In contrast to earlier reports from Southeast Asia [14, 17], which had reported no or very few heterozygous worms, we found 27 heterozygous individuals (13 adults and 14 larvae) among our samples (Table 2). Interestingly, 18 of them fell into the Iran-specific *cox-1* cluster (Fig. 2, Additional file 1: Fig. S1). In order to gain further insight into the nature of this Iran-specific cluster, we performed

Table 2 *Strongyloides stercoralis* with the different *cox-1* haplotypes from 23 patients

cox-1 Haplotype[a]	Patient number, location[b]	*S. stercoralis* individuals with this sequence[c]	Number (%) of worms with this *cox-1* haplotype
LC772965 (cluster 1, Iran-spec.)	1 Khorramshahr 2 Abadan 4 Abadan **14** Abadan **15** Abadan 21 Abadan 22 Abadan 23 Abadan	A1-P1.adult (het)[d] [Worm 1], B1-P1.adult (het)[d] [Worm 2], C1-P1.adult (I or het), D1-P1.adult (het)[d] [Worm 3], E1-P1.larva, F1-P1.larva (het), G1-P1. larva[d] A2-P2.adult (het)[d] [Worm 4], B2-P2.adult (het)[d] [Worm 5], C2-P2.adult (het)[d] [Worm 6], D2-P2.adult (het), E2-P2.larva (het), F2-P2.larva (het), G2-P2. larva (het) A4-P4.adult (II), B4-P4.larva (II), C4-P4.larva (II), D4-P4.larva (II), E4-P4.larva (II), F4-P4.larva (II) B12-P14.adult (II)[d] [Worm 31], C12-P14.adult (II)[d] [Worm 32], D12-P14.adult (II), E12-P14.larva (II), G12-P14.larva (II) B1-P15.larva (II), C1-P15.larva (het), D1-P15.larva, E1-P15.larva, F1-P15.larva, G1-P15.larva A7-P21.larva, B7-P21.larva, C7-P21.larva, D7-P21.larva (I), E7-P21.larva (I), F7-P21. larva, G7-P21.larva A8-P22.larva (het), B8-P22.larva (het), C8-P22.larva (het), D8-P22.larva (het), F8-P22. larva (het), G8-P22. larva (I) A9-P23.adult (II)[d] [Worm 54], B9-P23.adult (II), C9-P23.adult (II), D9-P23.adult (II), E9-P23.larva[d], F9-P23.larva, G9-P23. larva (II)	51 (37.5)
LC772966 (cluster 2)	11 Abadan **13** Abadan	A10-P11.adult (II)[d] [Worm 24], B10-P11.adult (II)[d] [Worm 25], C10-P11.larva, D10-P11.larva, G10-P11.larva, G11-P13.larva, F10-P11.larva (II) D11-P13.larva, E11-P13.larva	9 (6.62)
LC772967 (cluster 5)	**3** Abadan 8 Abadan 10 Abadan **16** Abadan 17 Abadan 19 Abadan 24 Abadan	B3-P3.adult (II), C3-P3.adult (II), D3-P3.adult (II), E3-P3.adult (II), F3-P3.larva (II), G3-P3.larva (II) B8-P8.larva, C8-P8.larva, D8-P8.larva A9-P10.adult (het)[d] [Worm 22], B9-P10.larva (het) A2-P16.adult (II)[d] [Worm 36], C2-P16.adult, D2-P16.adult (II)[d] [Worm 37], E2-P16. larva (II), F2-P16.larva (II), G2-P16.larva A3-P17.adult (II)[d] [Worm 38], B3-P17.adult (II)[d] [Worm 39], C3-P17.adult (II), D3-P17.adult (II), E3-P17.larva (II), F3-P17.larva B5-P19.larva (II), C5-P19.larva (II), D5-P19.larva (II), E5-P19.larva (II), F5-P19.larva (II), G5-P19.larva (II) A10-P24.adult (II)[d] [Worm 56], B10-P24.larva (II), C10-P24.larva, D10-P24.larva, E10-P24.larva, F10-P24.larva (II)[d], G10-P24.larva (II)	36 (26.47)
LC772968 (cluster 4)	**13** Abadan **18** Ahvaz	A11-P13.adult (het II/III)[d] [Worm 26], B11-P13.adult (het II/III)[d] [Worm 27], C11-P13.larva (III), F11-P13.larva (het II/III) A4-P18.adult (III), B4-P18.adult (het II/III)[d] [Worm 41], C4-P18.adult (het II/III), D4-P18.larva (het II/III), F4-P18.larva (III), G4-P18.larva (het II/III)	10 (7.35)
LC772969 (cluster 1, Iran-spec.)	**14** Abadan	A12-P14.adult (II)[d] [Worm 30]	1 (0.74)
LC772970 (cluster 1, Iran-spec.)	**15** Abadan	A1-P15.larva[d]	1 (0.74)
LC772971 (cluster 5)	**3** Abadan **16** Abadan	A3-P3.adult (II)[d] [Worm 7] B2-P16.adult	2 (1.47)
LC772972 (cluster 2)	5 Abadan	A5-P5.adult (II)[d] [Worm 12], B5-P5.adult (II), C5-P5.adult (II), D5-P5.adult (II), E5-P5. larva, F5-P5.larva (II), G5-P5.larva	7 (5.14)
LC772973 (cluster 3)	9 Khorramshahr 20 Abadan	9-P9.adult (II)[d] [Worm 20], 10-P9.larva, 11-P9.larva, 13-P9.larva[d], 14-P9.larva, 15-P9. larva A6-P20.adult (II), B6-P20.adult (II), C6-P20.adult (II), D6-P20.adult (II)	10 (7.35)
LC772974 (cluster 5)	6 Khorramshahr	A6-P6.larva (II), C6-P6.larva (II), D6-P6.larva (II)	3 (2.2)
LC772975 (cluster 1, Iran-spec.)	**7** Khorramshahr	A7-P7.adult (II), C7-P7.larva (II), E7-P7.larva (II), F7-P7.larva (II)	4 (2.94)
LC772976 (cluster 1, Iran-spec.)	**7** Khorramshahr	B7-P7.adult (II)	1 (0.74)
LC772977 (cluster 4)	**18** Ahvaz	E4-P18.larva	1 (0.74)

Table 2 (continued)

[a] GenBank accession number; the cluster (cf. Fig. 2) is given is parentheses[b]Patients with *S. stercoralis* worms with different *cox-1* haplotypes are in bold. Note that only patient 13 had worms with *cox-1* haplotypes from different clusters[c]The codes are composed as follows: [coordinates on the sequencing plate]—[patient code]. [developmental stage] ([haplotype at the nuclear HVR-I if known]), het: heterozygous for haplotypes I and II; het II/III: heterozygous for haplotypes II and III

[d] For these worms the *SSU* HVR-IV was confirmed to be haplotype A. [Worm XY] indicates that this worm was used for whole-genome analysis with this name (cf. Figs. 3, 4, 5). For a sortable table with all the information for each worm, see Additional file 3

whole-genome sequencing on selected individuals (see below).

For the 24 worms, we sequenced the whole genome, plus for an additional seven *S. stercoralis* from seven patients we also determined the *SSU* HVR-IV sequence (for two of these worms the *cox-1* sequence could not be determined, so they are not in Table 2 and Additional file 3). They all showed haplotype A (cf. 14). This haplotype has been previously reported from humans, dogs, and chimpanzees [14, 16, 32]. Three worms (worms 26, 27, 41) showed a mixed signal, with roughly half of the reads being G and the other half A at position 210 according to Barratt et al. [32], which corresponds to position 1454 in AF279916.

Whole-genome comparison

For 24 worms we managed to sequence the entire genome with coverage above the threshold specified in the Methods section. They were isolated from 14 different patients and represented all the major *cox-1* clusters

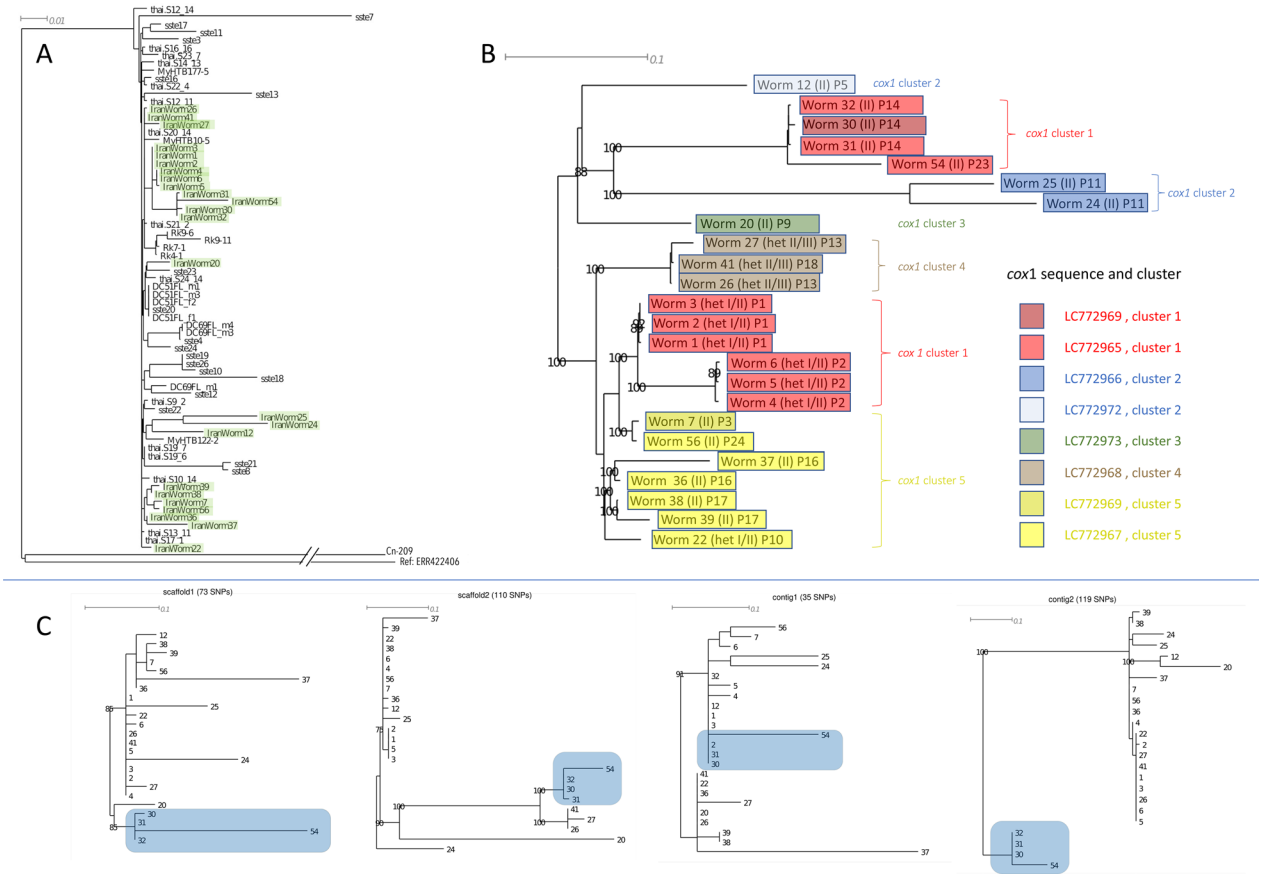

Fig. 3 Whole-genome neighbor-joining trees of *Strongyloides stercoralis*. **A** Comparison of the Iranian samples with published sequences from Asia. Cn-209 is an individual from a possibly asexual population in Southern China [19], ERR422406 is one individual of the USA-derived reference isolate [35]. Samples starting with Rk are from Japan [36], new Iranian samples are highlighted in green, and all other samples are from Southeast Asia and are the same as in Fig. 2 of [16]. **B** Iranian samples only. The corresponding *cox-1* haplotypes are color-coded. The *SSU* HVR-I haplotype is indicated. PX: patient [number]. **C** Neighbor-joining trees based on the four largest contigs (genome assembly from Hunt et al. [35]). The resolution is very limited, but examples (one highlighted in blue) with different topology are visible, indicating at least occasional meiotic recombination

(Fig. 2, Additional file 1: Fig. S1). In a neighbor-joining cladogram, all sequences fell into the Southeast Asian cluster when compared with the findings of Zhou et. al. [19] (cf. Fig. 4 A of this reference) or Aupalee et al. [16] (cf. Fig. 2 of this reference) (Fig. 3A). However, the resolution within this cluster is low because of the rather small number of variable positions that could be genotyped in all samples (1034 SNPs). Figure 3B shows an unrooted tree with only the sequences from this study. The *SSU* HVR-I haplotypes, the *cox-1* haplotypes and clusters, and the patient of origin are mapped onto the tree. The worms in the Iran-specific *cox-1* cluster appear to fall into two separable groups with respect to their nuclear genome. One group contains the worms with the *SSU* HVR-I haplotype II and the other group the ones that are heterozygous at this locus. Worms from the same patient tend to have very high genomic similarity, both in the biparentally inherited nuclear (whole) genome, which can undergo changes through recombination, and in the only maternally inherited, non-recombining, mitochondrial genome. This indicates that they might have shared common ancestors within the last few generations. Possible cryptic species diversity is an issue in parasitic nematodes in general [33] and in *S. stercoralis* in particular [14, 17, 22, 34]. In an attempt to look for recent recombination, we reconstructed trees based on the four largest genomic contigs [35] separately (Fig. 3C). Although the resolution power of these trees is very limited due to the small number of markers, it appears that the four trees differ from each other, indicating that the relationship of the worms differs across the genome. This indicates recent meiotic recombination and suggests that the worms we studied belong to the same and not to multiple cryptic species.

Next, we performed a principal component analysis (PCA) with our sequences and selected published sequences. In this analysis, the Iranian samples also fell within the range of the ones from Southeast Asia and were clearly separated from the sequences reported from South China by Zhou et al. [19] and the reference isolate from the USA [35]. Since these sequences dominated this analysis, we repeated the PCA, excluding them (Fig. 4). Also, in this analysis, the majority of the Iranian samples were very close to those from Southeast Asia, although slightly separated in PC2. However, the samples with a *cox-1* haplotype in the Iran-specific cluster and haplotypes I and II heterozygous *SSU* HVR-I (worms 1, 2, 3, 4, 5, 6) formed a group clearly separated from the rest; in addition, the other haplotype I and II heterozygous *SSU* HVR-I heterozygous worm (worm 22) as well as all three haplotypes II and III heterozygous worms (worms 26, 27, 41) were well separated from the rest in PC2.

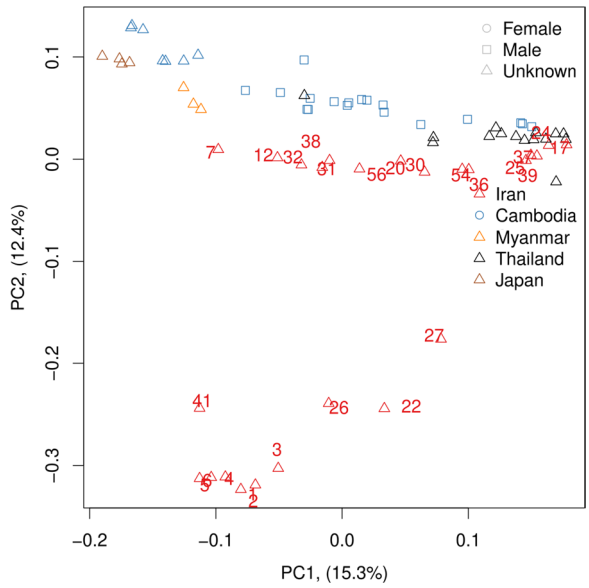

Fig. 4 Principal component analysis of whole-genome sequences from Iran and published sequences from Asia. The geographical origin is color-coded. The numbers with the Iranian numbers are the worm numbers (cf. Additional file 1: Fig. 1 and Figs. 2 and 3). The samples from Myanmar and Japan are from [36], the samples from Thailand are from [16], and the samples from Cambodia are from [14]. Note that the sequences from [19] (from Southern China) and the sequences from [35] (from the USA) were not included because they are so different that they dominated the PCA such that no other differences were visible in PC1 and PC2

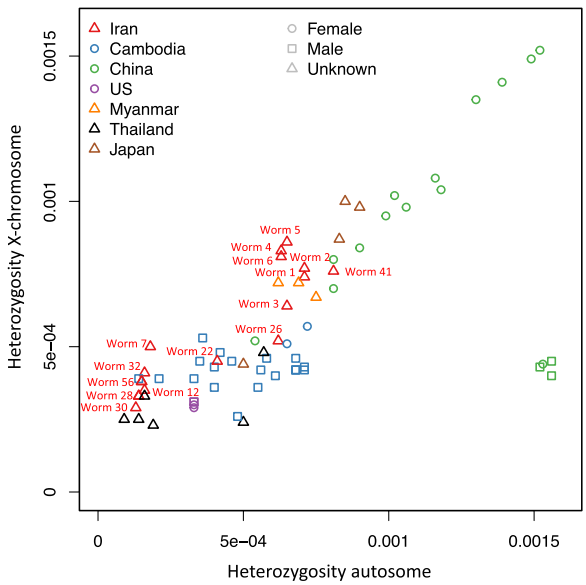

Fig. 5 Heterozygosity plot of whole-genome sequences from Iran and published sequences from Asia and the USA (reference isolate [35])). The samples from Myanmar and Japan are from [36], the samples from Thailand are from [16], the samples from China are from [19], and the samples from Cambodia are from [14]. The *X*-axis shows the heterozygosity on the autosomes, the *Y*-axis shows the heterozygosity on the X chromosome. Note that males have only one X chromosome and cannot be heterozygous on this chromosome. The X-chromosomal heterozygosity measured for males is therefore a measure for the error in this measure caused for example by genome assembly and annotation errors

Finally, we analyzed the heterozygosity in our samples (Fig. 5). The Iranian samples fell within the range of the ones from Southeast Asia here as well. Note that many of the samples included for comparison were males, which have only one X chromosome. Therefore, only the autosomal heterozygosity should be compared. However, the Iranian samples formed two distinct groups on either end of the range in Southeast Asia with the worms that were separate from the others in the PCA (Fig. 4) and were heterozygous at the *SSU* HVR-I, showing higher heterozygosity (note that these two analyses are not independent, but heterozygosity may have been a factor in the PCA).

Conclusions

From all these results we conclude that the *S. stercoralis* population from the Khuzestan province shares much of the genetic diversity with the population in Southeast Asia. However, the presence of an Iran-specific *cox-1* cluster (representing a matrilineage) and the high heterozygosity in the nuclear genome of some individuals indicate a contribution from an additional genetic source. There appears to be some population structure with different subpopulations, which however do interbreed at least occasionally.

Abbreviations

NTD	Neglected tropical disease
HVR	Hypervariable region
cox-1	Subunit 1 of mitochondrial cytochrome *c* oxidase gene
APC	Agar plate culture
PCR–RFLP	Polymerase chain reaction–restriction fragment length polymorphism

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-023-06103-6.

Additional file 1: cox-1 neighbor-joining tree with all worms from this study listed separately. For comparison, published sequences were included. The tree was constructed using MEGA 7 with the neighbor-joining method and evaluated with 1000 bootstrap repetitions. The evolutionary distances were computed using the Kimura 2-parameter method (using different models resulted in essentially the same tree topology). Scale bar denotes 0.01 changes per nucleotide site. Nomenclature: [worm identifier]-P [patient number].[developmental stage] ([nuclear *SSU* HVR-I haplotype according to [14, 32]]). het: heterozygous for haplotypes I and II, het II/III: heterozygous for haplotypes II and III. Clusters (cf. Fig. 2) are indicated in blue. The worms selected for whole-genome sequencing are indicated in red. Note that not all whole-genome sequencing fulfilled the inclusion quality criteria for all analyses. Therefore, not all the indicated worms are included in Figs. 3, 4 and 5. *These sequences from [14] were found in humans and in dogs and are therefore listed twice. $^{+}$Note that *SSU* HVR-I haplotypes II and III differ only by one nucleotide (TTT in haplotype II and TAT in haplotype III). Distinguishing homozygous III and heterozygous II/III is therefore not obvious. All three whole-genome-sequenced worms of this group turned out to be heterozygous although one of them had been scored as homozygous for III based on the HVR-I sequencing alone.

Additional file 2: Estimates of evolutionary divergence between the different *cox-1* sequences.

Additional file 3: Sortable Excel table with all available information for each worm. For nomenclature see legends to Table 2.

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Author contributions

MB and ASt designed the study. AA and AR collected the samples. MB performed the molecular analysis with the help of DH under the supervision of ASt during a research stay in the laboratory of ASt. VdR, ASt, and CR performed the bioinformatics analysis of the data. MB wrote the manuscript with input from ASt and the other authors. MB coordinated the project. The manuscript was read and approved by all authors.

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Availability of data and materials

All data supporting the findings of this study are available within the article and/or its supplementary materials or were deposited in publicly available databases.

Declarations

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (Approval No. IR.AJUMS.MEDICINE.REC.1398.039).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. [2]Department of Parasitology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. [3]Department of Integrative Evolutionary Biology, Max Planck Institute for Biology Tübingen, Tübingen, Baden-Württemberg, Germany.

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Publisher's Note

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Genomic analysis of *Strongyloides stercoralis* and *Strongyloides fuelleborni* in Bangladesh

Veroni de Ree[1], Tilak Chandra Nath[2], Priyanka Barua[3], Dorothee Harbecke[1], Dongmin Lee[4], Christian Rödelsperger[1], Adrian Streit[1]*

1 Department of Integrative Evolutionary Biology, Max Planck Institute for Biology Tübingen, Tübingen, Germany, **2** Department of Parasitology, Sylhet Agricultural University, Bangladesh, **3** Department of Zoology, University of Dhaka, Bangladesh, **4** International Parasite Resource Bank, Chungbuk National University, Seowon-gu, Korea

* adrian.streit@tuebingen.mpg.de

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Data Availability Statement: All data are contained in the manuscript or the supplemental material or were submitted to publicly accessible databases, namely NCBI GenBank accession numbers OR810937-54 (Necator americanus cox-1 sequences), OR805174-81 (Strongyloides fuelleborni cox-1 sequences), OR804688-712 (Strongyloides stercoralis cox-1 sequences) or the European Nucleotide Archive accession number PRJEB70604 (whole genome Illumina sequences).

Abstract

Background

About 600 million people are estimated to be infected with *Strongyloides stercoralis*, the species that causes most of the human strongyloidiasis cases. *S. stercoralis* can also infect non-human primates (NHPs), dogs and cats, rendering these animals putative sources for zoonotic human *S. stercoralis* infection. *S. fuelleborni* is normally found in old world NHPs but occasionally also infects humans, mainly in Africa. Dogs in southeast Asia carry at least two types of *Strongyloides*, only one of which appears to be shared with humans ("dog only" and "human and dog" types). For *S. stercoralis* with molecular taxonomic information, there is a strong sampling bias towards southeast and east Asia and Australia.

Methodology/Principle findings

In order to extend the geographic range of sampling, we collected human and dog derived *Strongyloides* spp. and hookworms from two locations in Bangladesh and subjected them to molecular taxonomic and genomic analysis based on nuclear and mitochondrial sequences. All hookworms found were *Necator americanus*. Contrary to earlier studies in Asia, we noticed a rather high incidence of *S. fuelleborni* in humans. Also in this study, we found the two types of *S. stercoralis* and no indication for genetic isolation from the southeast Asian populations. However, we found one genomically "dog only" type *S. stercoralis* in a human sample and we found two worms in a dog sample that had a nuclear genome of the "dog only" but a mitochondrial genome of the "human and dog" type.

Conclusions/Significance

S. fuelleborni may play a more prominent role as a human parasite in certain places in Asia than previously thought. The introgression of a mitochondria haplotype into the "dog only" population suggests that rare interbreeding between the two *S. stercoralis* types does occur and that exchange of genetic properties, for example a drug resistance, between the two types is conceivable.

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Author summary

More than 600 million people are infected with the nematode intestinal parasite *Strongyloides stercoralis*. Dogs can also carry *S. stercoralis*. In southeast Asia different genetic types that either infect only dogs or humans and dogs were described. *Strongyloides fuelleborni*, (normally found in old-world monkeys) can also infect humans, mainly in Africa. We collected *Strongyloides* spp. and hook worms, from humans and a dog in Bangladesh and analysed their nuclear and mitochondrial genomes. All hookworms were *Necator americanus*, one of the two major human hookworm species. Contrary to the general belief that human infections with *S. fuelleborni* are extremely rare in Asia, we found multiple such cases, suggesting that *S. fuelleborni* plays a more important role as a human parasite than previously thought also in Asia.

We found the two expected genetic types of *S. stercoralis*. For the first time, we found a genomically "dog only" type worm in a person and we found two worms with nuclear genomes of the "dog only" type but mitochondrial genomes of the "human and dog" type. This suggest that rare interbreeding between the two types occurs, such that exchange of genetic properties, such as a drug resistance, between the two types is conceivable.

Introduction

Strongyloidiasis is one of the soil-transmitted helminthiasis (STH), which are recognized as neglected tropical diseases (NTDs, https://www.who.int/health-topics/neglected-tropical-diseases#tab=tab_1). However, although clearly more prevalent in tropical and sub-tropical areas, the disease is not limited to these regions. It should probably be regarded as a disease of socioeconomically disadvantaged people rather than strictly as a tropical disease [1,2]. Until recently, strongyloidiasis was often neglected, even in comparison with other STHs [3] but over the last few years there has been increasing interest in this disease [1,4,5]. The estimate of the number of people currently infected with *Strongyloides stercoralis*, the species which causes the vast majority of human strongyloidiasis cases, has recently been corrected upwards to "about 600 million" [2]. Given the difficulties with diagnosis, the true number may be even higher [6–8]. *S. stercoralis* has also been reported to occur in non-human primates (NHPs), dogs and cats, rendering these animals putative sources for zoonotic human *S. stercoralis* infection [6,9–11]. In NHPs and in cats other, more or less host specific species of *Strongyloides* were also described, i.e. *S. fuelleborni* and *S. cebus* in NHPs, and *S. planiceps* and *S. felis* in cats [12]. The species status of *Strongyloides* in dogs has been controversially discussed ever since Brumpt separated the *Strongyloides* in dogs as *S. canis* from the human infective species *S. stercoralis* [13]. Interestingly, Brumpt proposed the existence of this separate species of *Strongyloides* in dogs based on the very same data that had convinced Fülleborn that the *Strongyloides* he found in the dogs belonged to the same species as the ones in humans [14]. While it became clear that dogs can be experimentally infected with at least some isolates of *S. procyonis* (natural host racoon, [15]) and human infective *S. stercoralis* (reviewed in [10]) it remained enigmatic if the species causing most natural *Strongyloides* infections in dogs is different from the one in humans.

Over the last years, the Hyper Variable Regions (HVR) I and IV of the nuclear Small SUbunit ribosomal RNA locus (*SSU*) and the mitochondrial *cox-1* locus have emerged as the standard markers for molecular taxonomy within the genus *Strongyloides* spp. and within the species, *S. stercoralis* [16–19]. A nomenclature system for the different haplotypes has been

proposed and extended [18,20,21]. A few studies, analysing samples from East and southeast Asia [20,22–24], and Iran [25] analysed whole genome data from individual *S. stercoralis* worms in addition to the *SSU* and *cox-1* markers. Jaleta et al. [20] and Nagayasu et al. [26] in southeast Asia and Beknazarova et al. in Australia [27] found that dogs carried at least two types of *Strongyloides*, only one of which appeared to be shared with humans in the same region. In this manuscript, we will refer to them as "human and dog" and "dog only", respectively. Barratt and Sapp [21] compiled all sequence information available from *S. stercoralis* from different hosts and used machine learning approaches to analyse these data in depth. Their findings suggest that *S. stercoralis* is in fact a complex of closely related species with different but overlapping host spectra. From these data it appears most likely that zoonotic *S. stercoralis* infections can happen. Although there are only very few cases of well-documented dog to human transmissions and human to human transmission appears to be the predominant way of acquiring *S. stercoralis*, it is possible that, under certain circumstances, zoonotic transmission is important for the epidemiology of human strongyloidiasis [10]. It is, for example, conceivable that dog derived infective larvae might restart *S. stercoralis* infections in a community after treatment [10].

In addition to *S. stercoralis*, *Strongyloides fuelleborni*, which can be distinguished from *S. stercoralis* morphologically [28] and coprologically (from this species eggs are shed with the faeces and not larvae as in *S. stercoralis* [29]), has been found to be able to infect humans. Two subspecies of *S. fuelleborni* have been described, namely *S. fuelleborni fuelleborni* and *S. fuelleborni kellyi* [29]. While the former is the predominant species of *Strongyloides* in old world non-human primates, *S. fuelleborni kellyi* has been found only in humans in Papua New Guinea [29]. Based on molecular taxonomy [30] we think *S. f. kellyi* should probably be considered a separate species and do not further discuss it in this publication. For the rest of this publication "*S. fuelleborni*" always refers to *S. fuelleborni fuelleborni*. Barratt and Sapp [21] described genetic/genomic differences between *S. fuelleborni* in Africa and *S. fuelleborni* in Asia. The vast majority of human *S. fuelleborni* infections were found in Africa and [21] found genetic indication for a human specialized sub-population within the African clade, suggesting human to human transmission. In Asia, on the other hand, no such genetic hint was found and it appears that human *S. fuelleborni* infections are restricted to individuals with close contact with non-human primates, indicating that most, if not all human *S. fuelleborni* cases in Asia are zoonotic [21 and references therein].

So far, for *S. stercoralis* with molecular taxonomic information, there is a strong sampling bias towards southeast Asia, East Asia and Australia[21]. None of the few dog derived *S. stercoralis* analysed from other places was of the "dog only" type, although this type appears to be very common in southeast Asia [21]. To further extend the geographic range covered by sampling and to see if the "dog only" type extends towards the West of southeast Asia, we collected *S. stercoralis* from two locations in Bangladesh. We knew that *S. stercoralis* is prevalent in Bangladesh ([2] lists an estimated overall prevalence of 17.3%), but we are not aware of any published systematic study on *S. stercoralis* in this country. Contrary to earlier studies in Asia, we noticed a rather high incidence of *S. fuelleborni* in humans. Molecular taxonomically these worms grouped clearly with the Asian clade defined by [21]. Molecular taxonomically, the *S. stercoralis* we found mixed in with the southeast Asian population described earlier [20,22,23,26] and appeared not to form a separate population. However, we found one *S. stercoralis* worm in a human sample that was of a type that had been considered dog-specific and we found two worms in a dog sample that had the nuclear genomes of the "dog only" type but the mitochondrial genome of the "human and dog" type. This suggests that occasional interbreeding between the types does occur and that therefore exchange of genetic properties, such as a drug resistance, between the two types is conceivable.

Methods

Ethics statement

All participants were volunteers and gave formal verbal informed consent. In the case of children, formal verbal consent was obtained from the parent/guardian. The sampling of human-derived material including the procedures to obtain informed consent, was in accordance with the Bangladeshi legal requirements and with the guidelines of the Sylhet Agricultural University. This study was approved by the Ethical Review Committee, Sylhet Agricultural University Research System (SAURES), Bangladesh (SAURES-UGC-2022-04). Interested putative participants were informed orally about the project and, if they chose to participate, were handed collection containers. All participants remained free to return the container or not.

Study area, sample collection and processing

Human faecal samples were collected in December 2022 from two regions in Bangladesh that had been previously identified as high prevalence areas for helminthiasis: Sylhet, and Dhaka. 134 human samples from four different locations in Sylhet; Khadim tea garden, Daldali tea garden, Baluchar and Fotehpur and one dog sample from the premises of Sylhet Agricultural University were analysed. In Dhaka 95 human samples were collected from two locations: Hazaribug and Mohammadpur.

Stool collection jars with a spoon were distributed to the individuals who agreed to participate in the study after explaining how to properly collect the stool sample without soil contamination. The next day the sample jars were collected.

Faecal samples were mixed well with approximately equal volumes of activated charcoal (Roth 5966.1) to facilitate air exchange. Water was added to make the samples well moisturized but not soaking wet. This mixture was incubated at room temperature (R.T.) for 24-48hrs with the lid partially open and re-moisturized on need-to basis. Samples were analysed using modified miniature Baermann apparatuses based on 50ml Falcon tubes as described [31]. In brief, the faeces mixture was wrapped in a 10x10cm cotton gauze, placed in the top part of a 50ml Falcon tube filled with lukewarm water and secured using a toothpick. After 3hrs of incubation at ambient temperature, the sediment was taken out using a Pasteur pipette and observed under a stereo dissecting microscope for the presence of worms. These worms were in part individually and in part bulk preserved in 80% ethanol at the Sylhet Agricultural University as described [32] and brought back to the Max Planck Institute for Biology, Tübingen for molecular/genomic analysis.

Single worm lysis and *cox-1*, *SSU* HVR-I and *SSU* HVR-IV genotyping

Single worm lysis for adults and larvae was performed as described [32]. For infective larvae the lysis was extended to 6 hrs at 65˚C. The lysate was either freshly used for PCR or stored at -20˚C. The *cox-1*, *SSU* HVR-I and *SSU* HVR-IV were PCR amplified using the primers described in [24]. For the *SSU* HVR-I and the *SSU* HVR-IV for all worms the same primer pairs RH5401/RH5402 and 18SP4F/18SPCR, respectively, were used. For *cox-1* the primers designed for *S*. *stercoralis* (but also working for some other species) (ZS6985/ZS6986) were used, unless the worm was already known to be a hookworm, in which case the hookworm optimized primer ZS6989 was used as the reverse primer instead of ZS6986. For the PCR, 10μl of QIAGEN *Taq* PCR master mix (x2) (201443), 0.4μl of 10μM forward and reverse primers, 7.2μl of PCR water and as template 2μl of single worm lysate were added. For *S*. *fuelleborni* the same primers as for *S*. *stercoralis* were used.

For sequencing 1μl of PCR product was mixed with 1μl of the relevant sequencing primer (10μM) listed in [24] and 8μl of water and submitted to Genewiz, Leipzig, Germany. The *S. stercoralis* P203 iL3 HVR-I PCR product and the *S. fuelleborni cox-1* PCR products were gel purified (1% agarose, 1X TAE) using the QIAquick gel extraction kit (Qiagen 28706) prior to sequencing. If the sequencing result was not clean, the PCR products were sequenced again using the alternative sequencing primers listed by [24] or the amplification primers. Sequence quality and the presence of hybrid sequences were manually assessed by looking at the chromatograms using the SnapGene software (from Dotmatrics; available at snapgene.com). The sequences were first compared with published sequences in the National Centre for Biotechnology Information (NCBI) database using the BLAST function (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and then phylogenetically analysed using MEGA11 [33] with the Neighbour-joining method and default settings. The robustness of the trees was assessed with 1000 boot strap repetitions. Position numbers refer to GeneBank entries AF279916 for the *S. stercoralis* and *S. fuelleborni SSU*, LC050212 for the *S. stercoralis* and *S. fuelleborni cox-1* and AJ417719 for hookworm *cox-1* sequences.

We also retrieved *cox-1* sequences from the read data of the whole genome sequenced worms in [20] (see Fig. 4 of this reference) and submitted them to GenBank along with the *cox-1* sequences from this study (accession numbers OR804688-712, OR805174-81, OR810937-54, OR809277-99).

Whole genome sequencing

Illumina paired-end whole genome sequencing with a read length of 150 bp was done as described [25] with slight modifications as follows. In the DNA clean-up step, 11–14μl of lysis were used instead of 10μl. In the pooling and concentration adjustment step the bead clean-up was skipped. The concentrations were calculated for the samples and 2nM of each sample were pooled which resulted in a 1.85nM final concentration in the pool which was then submitted to the MPI for Biology in-house sequencing facility for Illumina NexSeq 2000 sequencing.

Analysis of the whole genome sequences

Whole genome tree. The paired end sequencing resulted in an average of 18325297 read pairs per library (range 8167732–33193716) corresponding to 5.7–21.5 x coverage for the *S. stercoralis* samples. Whole genome sequence data for 12 *S. stercoralis* samples and three *S. fuelleborni* samples (on average 18527739 read pairs per sample) were uploaded to the European Nucleotide Archive under the study accession PRJEB70604.

The read alignment, duplicate removal, variant calling, defining heterozygous sites, creating the genotype using variant positions and constructing the neighbour joining (NJ) tree based on the variant positions were all done as described [25].

Whole mitochondrial (wmit) tree. Whole genome sequencing reads from this study and previous studies were aligned to the *S. stercoralis* wmit reference genome (NC_028624.1 [identical to LC050212]) to generate wmit sequence assemblies. The four wmit sequences from [34] were used directly. Read alignment, binary alignment file generation and duplicate removal were done as mentioned above. BAM files were loaded to IGV_2.16.0 and the consensus sequences were obtained using the 'copy consensus sequence function' in IGV. NJ trees were generated using MEGA 11. The sequence file used as input for MEGA 11 can be found in S2 File.

To generate wmit assemblies from the *S. fuelleborni* in this study we first aligned the reads to two mitochondrial whole genome sequences from [35] (OL505577, arrangement A and OL602833, arrangement B) and visualized the BAM files using IGV. Since the tRNA(Met) gene that is present in arrangement B but absent in arrangement A was absent from all our *S.*

fuelleborni sequences we decided to use OL505577 as reference. A wmit tree for *S. fuelleborni* was generated using sequences from this study and from [35] as described for *S. stercoralis* above.

Heterozygosity analysis. General heterozygosity analysis was done as described [22].

Coverage analysis. Coverage for both autosomal contigs and X-chromosomal contigs was analysed using samtools (0.1.18) depth command and the coverage was plotted against the number of positions using R studio (R 4.3.1, ggplot2 3.4.3).

Results

We analysed 134 human samples (in most cases rather small samples) collected from Sylhet and found worms in 25 of them. In seven samples we found only *Strongyloides*, in five samples *Strongyloides* and hookworms, in 12 samples only hookworms and in one sample we found several worms, all of which, based on their 18S sequence belonged to *Tokorhabditis* spp., which is a genus of free-living nematodes [36]. We think this last case represents a contamination from the ground and this sample is not further discussed. The one dog sample we obtained was positive for *Strongyloides*. From Dhaka, we found *Strongyloides* in only two out of 95 human samples, while in six other samples, based on the 18S sequence, we detected *Caenorhabditis nigoni*, which are free-living nematodes [37]. These worms likely represent ground contamination and are not discussed further.

For 71 hookworms and 99 *Strongyloides* (67 from humans, 32 from the dog) the sequence of at least one out of the *SSU* HVR-I, *SSU* HVR-IV or *cox-1* was successfully determined.

The hookworms found were *Necator americanus*

Initially, 47 worms were confirmed to be hookworms, based on *SSU* sequences. Since the different hookworm species cannot be distinguished based on their *SSU* HVR-I or *SSU* HVR-IV sequences, we determined the *cox-1* sequence using the same primers as used in [24], which was successful for 42 worms. Among these worms, we identified 18 different *cox-1* haplotypes (accession numbers OR810937-54), of which six were identical with existing database entries, while 12 were new (S1 Table). On several occasions, worms with different *cox-1* sequences were found within the same host (S1 Table). All 18 sequences clustered with perfect bootstrap support with the group A [38] which is considered *Necator americanus* (Fig 1). Within the species *N. americanus*, our samples did not cluster together but intermixed with sequences derived from Africa and Asia, arguing against the presence of a Bangladesh specific sub-population. In addition, 24 larvae for which we amplified the *cox-1* sequence using the primers optimized for *S. stercoralis* turned out to be hookworms. Since these sequences were shorter than the ones generated using the hookworm specific primers, they are not included in Fig 1. However, all 24 sequences clearly grouped with *Necator americanus* sequences. Hence, overall, we identified 71 worms as hookworms, of which we confirmed 66 to be *Necator* and not *Ancylostoma*.

High incidence of *S. fuelleborni*

Strikingly, four out of the 12 individuals found to be infected with *Strongyloides* spp. in Sylhet carried *S. fuelleborni* and not *S. stercoralis* (two of them were found to be co-infected with hookworms, in none of them we found *S. stercoralis* along with *S. fuelleborni*).

At the *SSU*, all 16 *S. fuelleborni* genotyped were HVR-I haplotype XIV and HVR-IV haplotype S (cf. [21]). Out of the 16 worms, *cox-1* sequences were obtained for 15. We identified eight different *cox-1* haplotypes (accession numbers OR805174-81), none of which had been reported before (S1 Table). All three persons for whom we obtained the *cox-1* sequence from more than one worm, carried worms with different haplotypes (S1 Table). In a phylogenetic analysis based on the *cox-1* sequences (Fig 2), all eight sequences clearly grouped with *S.*

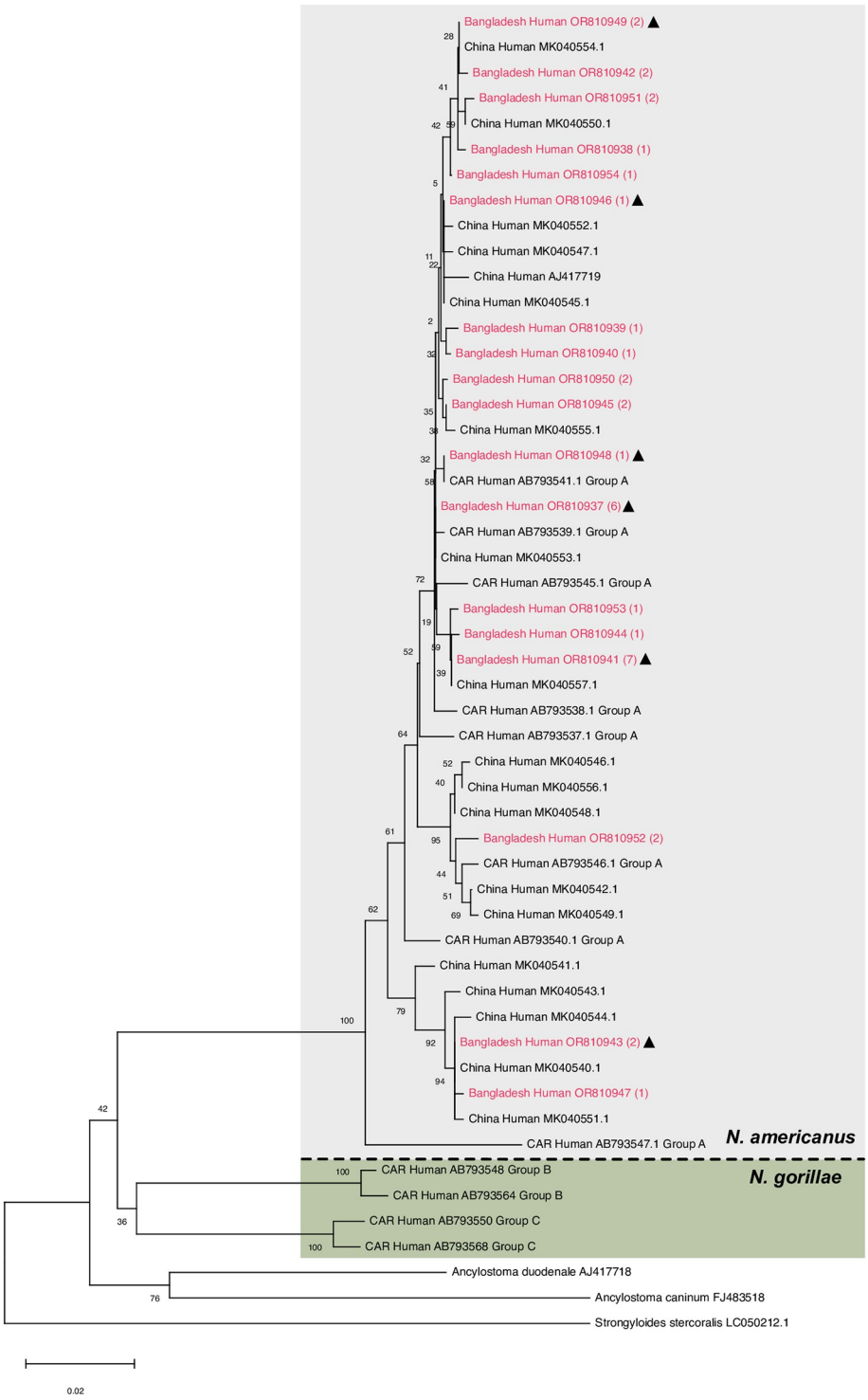

Fig 1. Neighbour joining tree based on partial hookworm *cox-1* sequences (670bp). Sequences found in this study are in red, the number of worms this sequence was found in is in (). Triangles indicate haplotypes that had been previously known. For every sample the country of origin (CAR = Central African Republic), the host and the GenBank accession number are given.

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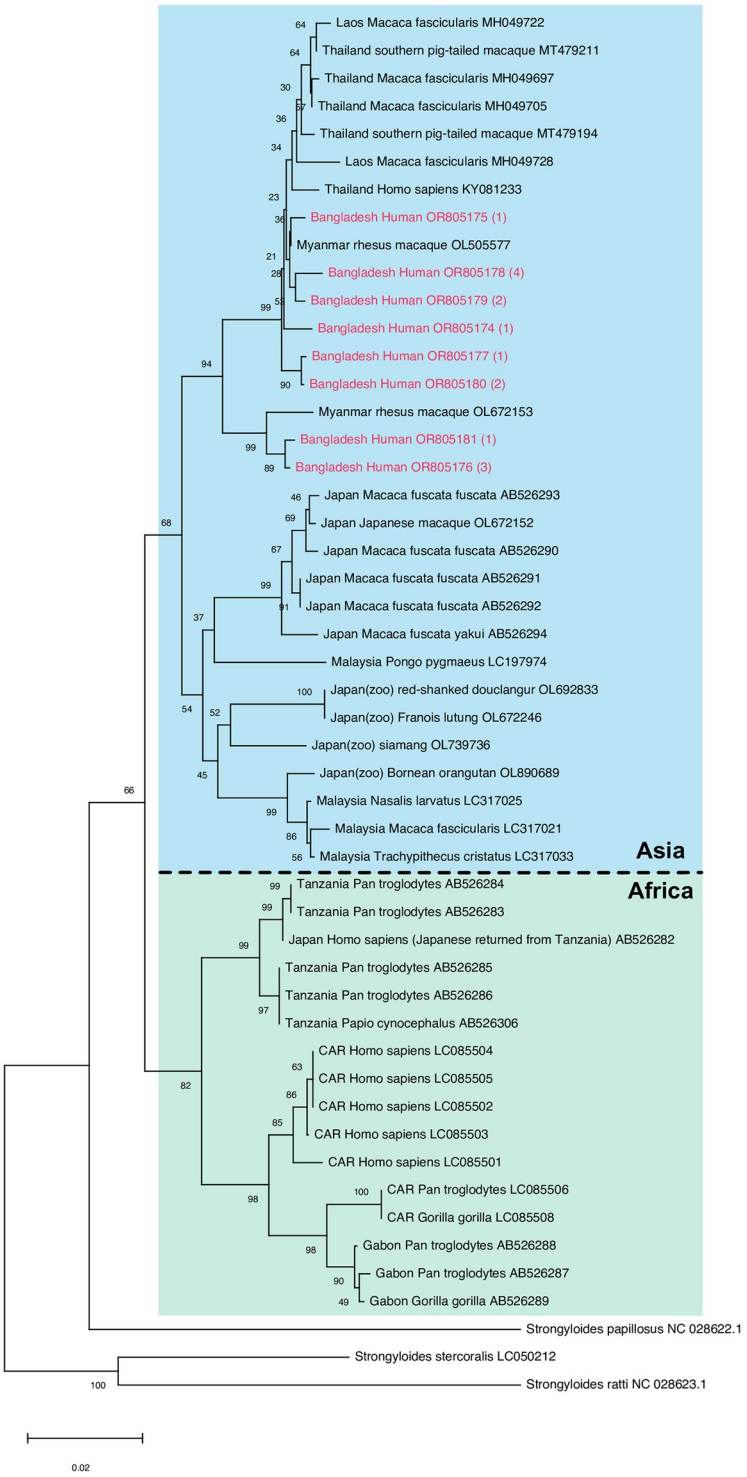

Fig 2. Neighbour joining tree based on partial *S. fuelleborni cox-1* sequences (552bp). Sequences found in this study are in red, the number of worms this sequence was found in is in (). For every sample the country of origin (CAR = Central African Republic), the host and the GenBank accession number are given.

https://doi.org/10.1371/journal.pntd.0012440.g002

fuelleborni from southeast Asia (mainly Thailand, Myanmar and Laos) described as cluster 3 by [21], further supporting the notion that in *S. fuelleborni* (different from *S. stercoralis*) geographic sub-populations exist and an Asian and an African clade exist [21].

From three *S. fuelleborni* derived from three different persons, we performed whole genome Illumina short read sequencing (Table 1) and deposited the read data in the European Nucleotide Archive (accession number PRJEB70604). Since there is no reference nuclear genome for this species available, we make these data publicly available here without further analysis. From these data we extracted the full mitochondrial genomes and compared them with the sequences reported by [35]. The sequences analysed are listed in S1 File. All three worms clustered with the samples containing mitochondrial genome arrangement A and did not contain the tRNA(Met) gene that is absent from arrangement A but present in arrangement B. Arrangement A had been observed in *S. fuelleborni* derived from macaques in Myanmar and Japan and was hypothesized to represent the ancestral state [35] (Fig 3).

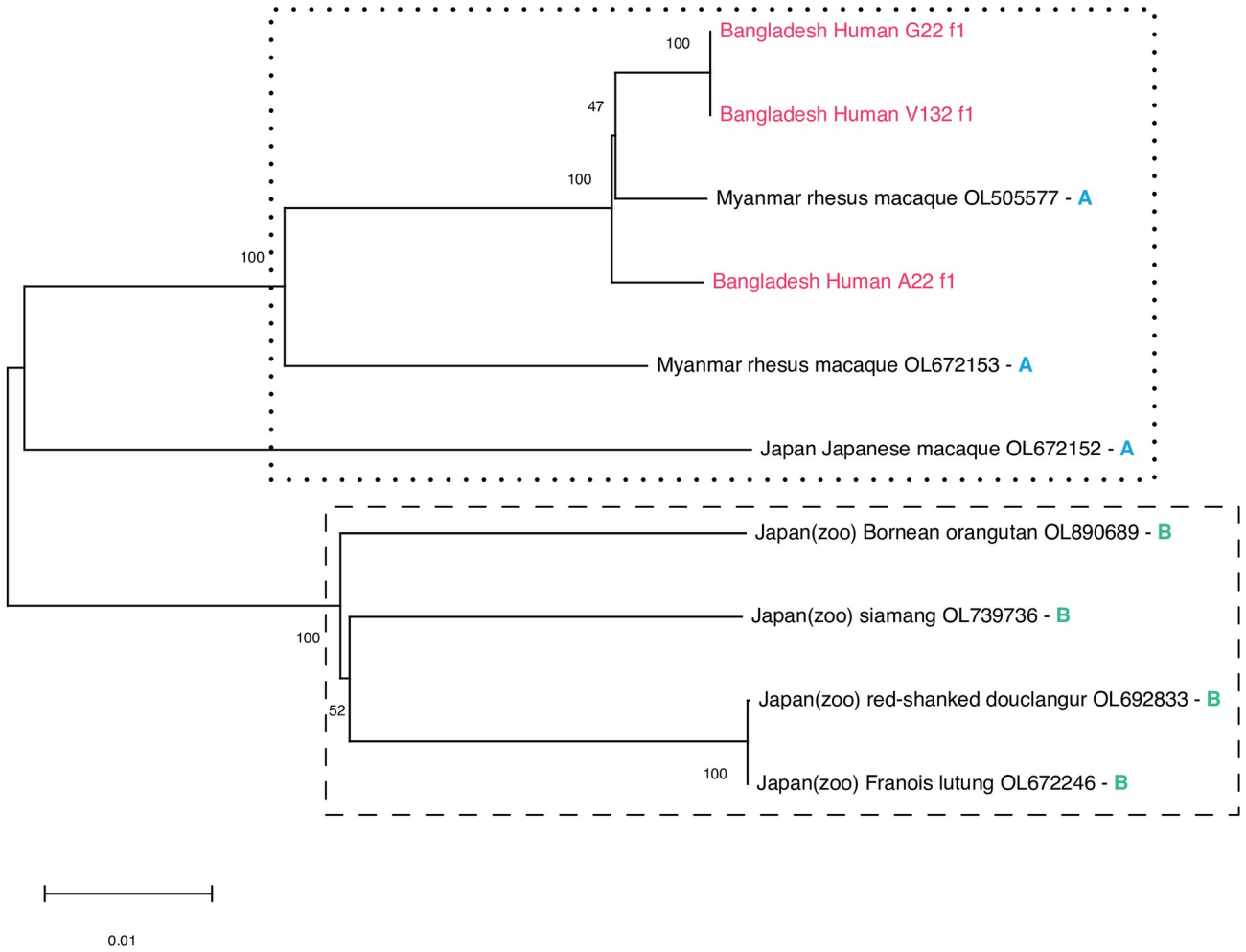

Fig 3. Neighbour joining tree based on full mitochondrial nucleotide sequences. All non-Bangladesh sequences are from Ko et al. (2023). The letters (A or B) refer to the genome arrangement described in Ko et al. (2023) (compared with B, arrangement A lacks a tRNA(Met) gene present adjacent to the *S1* gene). For every sample the country of origin, the host and the GenBank accession number or the worm identifier (for sequences from this study) are given. The sequences from this study were extracted from the data available from the European Nucleotide Archive under the accession number PRJEB70604. A FASTA file with all sequences used is provided as S1 File.

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Table 1. Whole genome sequenced samples.

Sample number	Worm ID	Species	*cox-1* haplotype (type)	*SSU* HVR-I haplotype	*SSU* HVR-IV haplotype	Internal ID in sequencing protocol
1	Human_C19—f2	*S. stercoralis*	OR804711	II	A	1
2	Human_C19—f4	*S. stercoralis*	OR804711	II	A	3
3	Human_F40—f1	*S. stercoralis*	OR804710	II	A	14
4	Human_F10—f1	*S. stercoralis*	OR804710	II	A	17
5	Human_F10—f3	*S. stercoralis*	OR804709	II	A	19
6	Human_J102—f1	*S. stercoralis*	OR804706	I	V	20
7	Dog—f1	*S. stercoralis*	OR804688	I	V	21
8	Dog—f2	*S. stercoralis*	OR804689	I	V	22
9	Dog—f4	*S. stercoralis*	OR804691	I	V	24
10	Dog—f5	*S. stercoralis*	OR804692	I	V	25
11	Dog—f6	*S. stercoralis*	OR804693	I	V	26
12	Dog—m2	*S. stercoralis*	OR804701	I	V	27
13	Human_A22—f1	*S. fuelleborni*	OR805174	XIV	S	29
14	Human_G22—f1	*S. fuelleborni*	OR805178	XIV	S	33
15	Human_V132—f1	*S. fuelleborni*	OR805181	XIV	S	36

https://doi.org/10.1371/journal.pntd.0012440.t001

The *cox-1* haplotypes are referred to by their GenBank accession numbers. The nomenclature for the *SSU* HVRs is taken from [21] with U being a new haplotype found for the first time in this publication (Fig 4A).

S. stercoralis SSU haplotypes

For *SSU* HVR haplotype nomenclature see [21]. At the *SSU* HVR-IV the *S. stercoralis* in our samples had either haplotype A (28 individuals), which was described to be indicative for the "human and dog" type [20,21] or a new haplotype we call V (31 individuals, Fig 4A). All but one (see below) of the carriers of haplotype V were isolated from the dog. At *SSU* HVR-I, 28 worms had haplotype I, 26 worms had haplotype II and one worm each had haplotype III and V. In all cases where we have the sequences for both HVRs, HVR-IV haplotype A co-occurred with HVR-I haplotype II (24 cases) or III (one case) while HVR-IV haplotype V co-occurred with HVR-I haplotype I (28 cases) or V (one case). Notice that in an earlier study in Cambodia [20], HVR-I haplotype I did also co-occur with HVR-IV haplotype A.

S. stercoralis cox-1 haplotypes

In a total of 76 worms, we detected 25 different *cox-1* haplotypes (accession numbers OR804688-712) of which three had been previously reported while 22 were new (S1 Table). Each haplotype was present in between one and 15 different worms with the previously known haplotypes being the first (15 worms), second (11 worms) and fifth (seven worms) most abundant ones (Fig 4). Upon phylogenetic analysis (Fig 4, S1 Fig) the previously described and five of the new haplotypes (representing 48 worms, two of which had been isolated from the dog) grouped with sequences in the "human and dog" clusters according to [20]. 17 haplotypes (representing 28 worms, of which one had been isolated from a human sample [see below]) grouped with one of the "dog only" clusters according to [20].

A "dog only" type *S. stercoralis* in a human host

Strikingly, one of the worms that by *cox-1* sequence fell into the "dog only" cluster had been isolated from a human from Dhaka (worm: Human_J102—f1, asterisk in Fig 4B). This worm

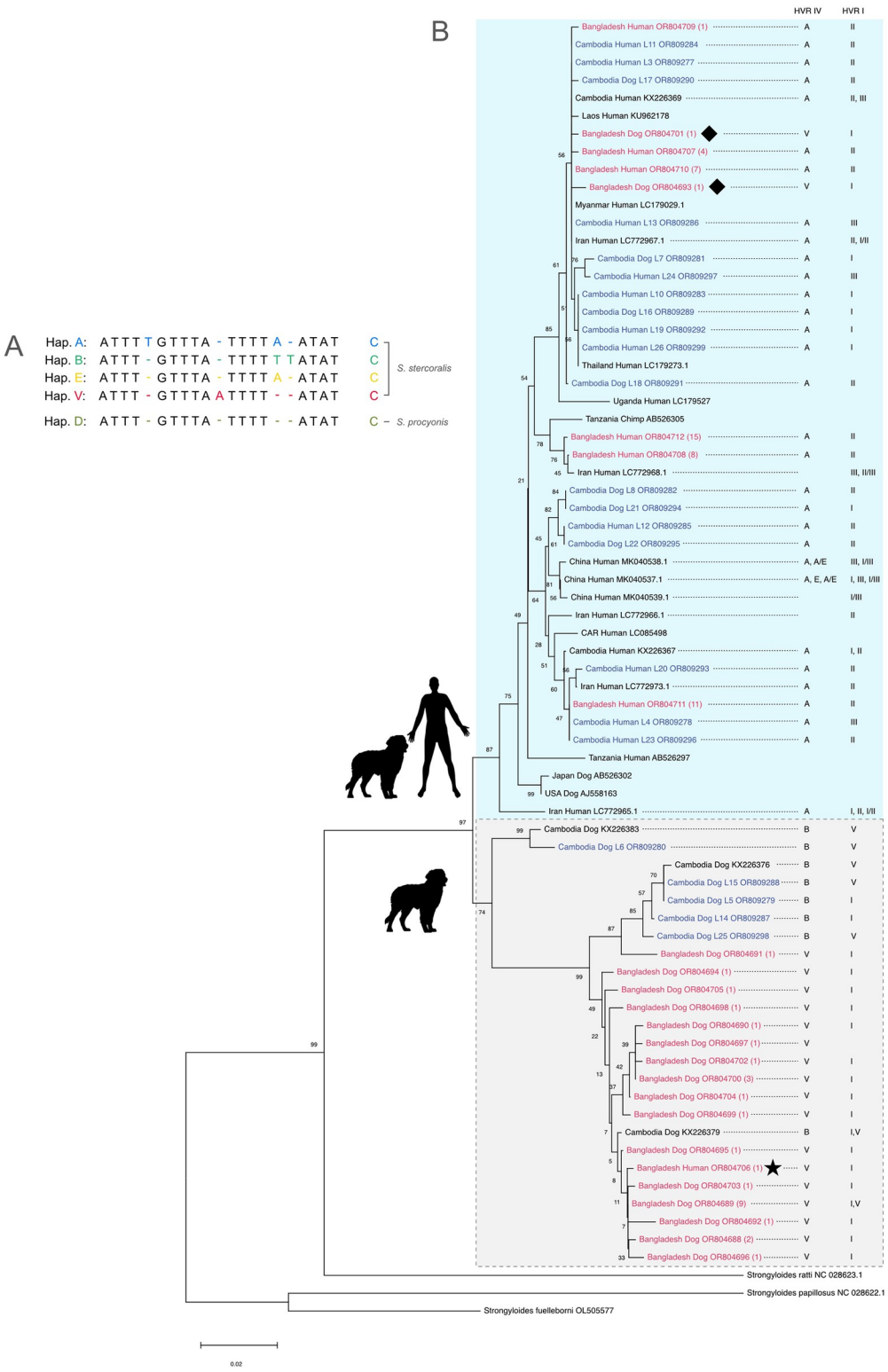

Fig 4. *cox-1*, *SSU* HVR-I and *SSU* HVR-IV haplotypes of our samples compared with selected published sequences. A: sequence of the new *SSU* HVR-IV haplotype V compared with the previously reported haplotypes (nomenclature according to [21]) mentioned in B. Notice that haplotype E was called haplotype C in Zhou et al. (2019). B: neighbour joining tree based on partial *S. stercoralis cox-1* sequences (552bp). Sequences found in this study are in red, the number of worms this sequence was found in is in (). Samples in blue are worms from [20] for which

full genome short read sequences are available. For every sample the country of origin (CAR = Central African Republic, USA = United States of America), the host and the GenBank accession number are given. For worms from Cambodia also the worm individual is given after the host to facilitate cross reference with [20]. For each *cox-1* haplotype the *SSU* HVR-I and *SSU* HVR-IV haplotypes found in individuals with this *cox-1* haplotype are indicated (if known). Samples from Cambodia are from [20], samples from Laos are from [39], samples from Myanmar, Thailand and Uganda are from [26], samples from Iran are from [25], sample from the USA is from [40], samples from Tanzania, and Japan are from [17], samples from CAR are from [41] and samples from China are from [24]. Haplotypes separated by '/' indicates a heterozygous worm. For a *cox-1* tree with more sequences see S1 Fig. Diamonds label the two worms that showed "human and dog" type mitochondrial but "dog only" type nuclear sequences. The asterisk labels the worm in the "dog only" cluster isolated from a human host.

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carried the new *SSU* HVR-IV haplotype V, and HVR-I haplotype I, like all but one (the one with HVR-I haplotype V) of the other worms from our study in this cluster, which all came from the dog. This is the first report of a genomically "dog only" type *S. stercoralis* in a human. However, this was the only worm found in this particular sample and we cannot exclude with absolute certainty that this result is an artefact (see discussion).

First finding of a "human and dog" type mitochondrial genome in combination with a "dog only" type nuclear genome

In all previous studies that we are aware of, where nuclear and mitochondrial sequences from the same worms were determined [20,23,26,42,43], both sequence kinds always fell in the same group as defined by [20,26] ("human and dog" or "dog only"). Here, for the first time, we found worms where the phylogenetic positioning based on the *cox-1* and on the *SSU* sequences was not in agreement. In the dog we found two worms, that based on their *cox-1* sequences fell within the "human and dog" type but had *SSU* haplotypes normally associated with the "dog only" type (diamonds in Fig 4B). This suggests that rare interbreeding between the two types does occur.

The *S. stercoralis* population in humans is genetically close to the one in southeast Asia

Since the conclusions above are based on a rather small number of informative positions, we performed Illumina whole genome sequencing of the three notable worms mentioned above along with nine other individuals isolated from humans and the dog (Table 1). Then we compared their mitochondrial and nuclear genomes. The read data are available from the European Nucleotide Archive (accession number PRJEB70604). The extracted whole mitochondrial genomes are listed in S2 File.

We reconstructed Neighbour Joining cladograms based on the full mitochondrial (Fig 5 and S2 Fig) and nuclear (Fig 6, S3 and S4 Figs) genomes. In all cases the classification as "human and dog" or "dog only" agreed with the one based on only *cox-1* for the mitochondrial genome (cf. Figs 5 and 4B) or only the *SSU* HVR-IV for the nuclear genome (cf. Figs 6B and 4B). Notice that the nuclear genome tree should not be interpreted as a phylogenetic tree because it is a within-species tree with genomes possibly undergoing mixing due to meiotic recombination. Neighbour Joining clustering of the "human and dog" type whole nuclear genome sequences showed that the worms from Bangladesh group with the sequences previously described for southeast Asia, Japan and Iran and away from a possibly asexual population described in southern China and the laboratory reference isolate, which originated from the USA (Fig 6A, for references see figure legend).

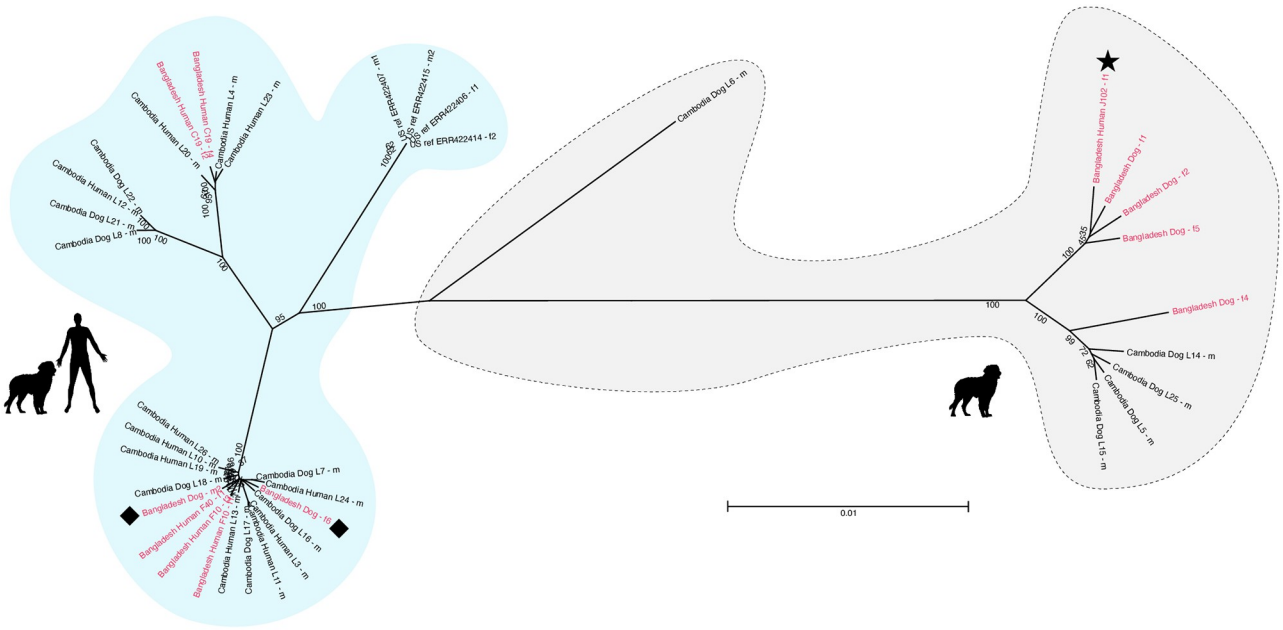

Fig 5. Neighbour joining tree based on full mitochondrial genomes determined in this study (in red) plus the worms mentioned in Fig. 4 of [20] and four worms of the reference isolate [44] for comparison. For a tree with more sequences see S2 Fig. A FASTA file with all sequences used in this figure and in S2 Fig is provided as S2 File. Diamonds label the two worms that showed "human and dog" type mitochondrial but "dog only" type nuclear sequences. The asterisk labels the worm in the "dog only" cluster isolated from a human host.

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Whole genome sequence confirms the unexpected worms

When the sequences of the "dog only" cluster are included in the analysis, there remains little resolution in the "human and dog" branch of the nuclear genome tree (Fig 6B and S4 Fig). This is because, compared with the "dog only" sequences they are very similar to each other and the inclusion of more samples reduced the number of informative sites included in the analysis (only positions covered in all worms in the analysis were considered, see Materials and Methods). However, this analysis confirms the unexpected results based on the *SSU* and the *cox-1* sequences. Worm Human_J102—f1 (isolated from a human host, marked with an asterisk in Figs 5 and 6) grouped with respect to the nuclear and with respect to the mitochondrial genome with the "dog only" cluster, while worms Dog_f6 and Dog_m2 (marked with diamonds in Figs 5 and 6) both show a mitochondrial genome that groups with the "human and dog" cluster but a nuclear genome that belongs to the "dog only" cluster. We conclude from this result that occasional interbreeding of the two types does occur and thereby a "human and dog" type mitochondrial genome introgressed into the "dog only" population. Presumably, a "human and dog" type female and a "dog only" type male interbred and the descendants later bred with "dog only" type partners, thereby rendering the recombining nuclear genome "dog only" type while maintaining the uni-parentally (maternally) inherited mitochondrial genome.

Worms belonging to the "human and dog" type show low heterozygosity

In order to compare our samples from Bangladesh with earlier studies [22–25] we performed a heterozygosity analysis (Fig 7A). The worms of the "human and dog" type from Bangladesh showed very similar heterozygosity like the ones described from southeast Asia [22,23] and a portion of the worms from a recent study in Iran [25]. The heterozygosity was clearly lower

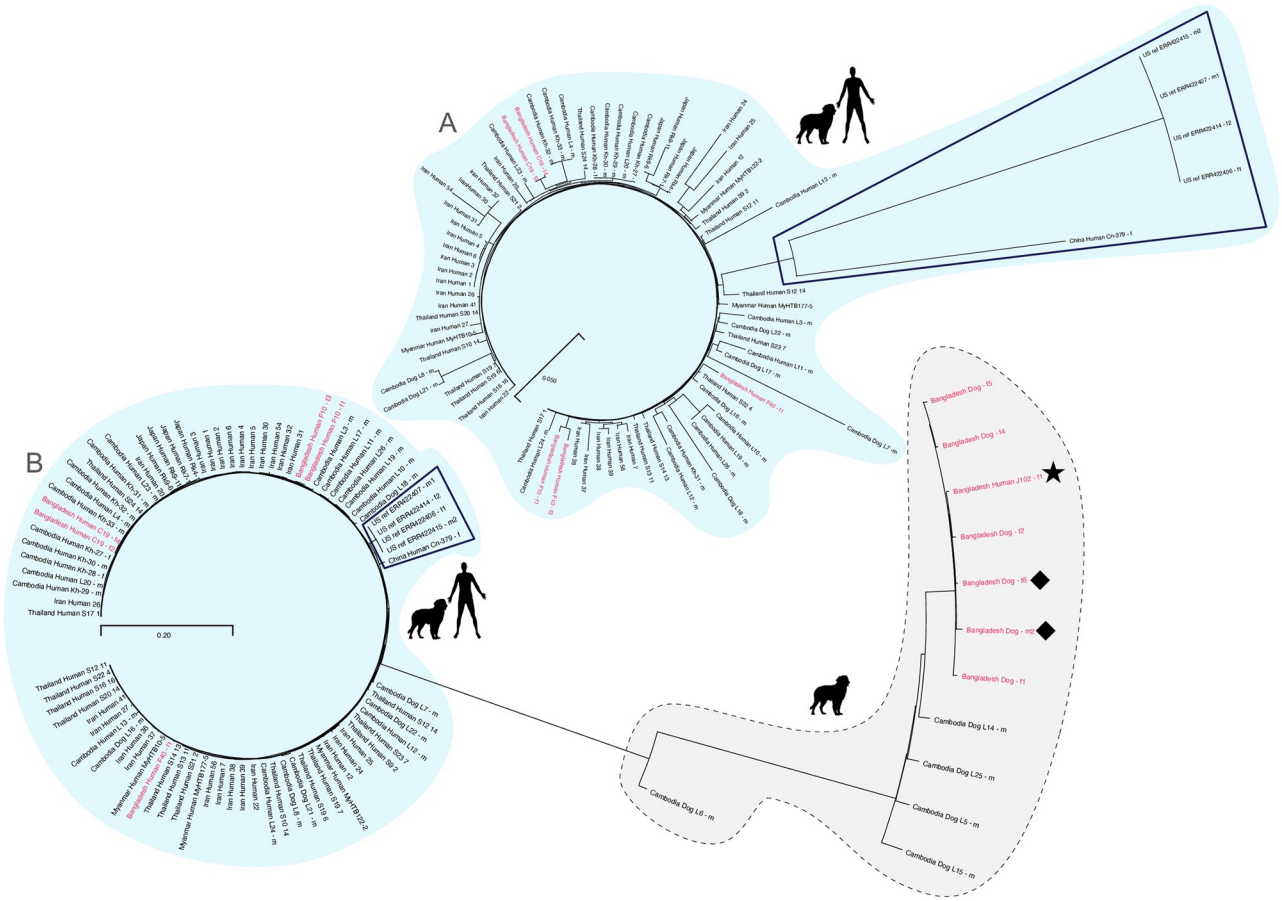

Fig 6. Neighbour joining tree (A without and B with "dog only" type worms) based on full nuclear genomes determined in this study (in red) plus selected worms from earlier studies for comparison. Diamonds label the two worms that showed "human and dog" type mitochondrial but "dog only" type nuclear sequences. The asterisk labels the worm in the "dog only" cluster isolated from a human host. To get an impression on how different the "dog only" type is from the "human and dog" type, compare in A and B the branch lengths of the samples from the USA and China (boxed), which are the "human and dog" type worms with the greatest genomic difference from the southeast Asian human derived *S. stercoralis*. For a different representation, see S3 and S4 Figs. Sequences from Cambodia are from [20], Thailand are from [22], Iran are from [25], Myanmar and Japan are from [23], the USA are from [44] and China is from [24].

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than in the presumably essentially asexual populations in Japan [23] and in southern China [24] and in a portion of the worms from Iran [25].

Worms belonging to the "dog only" type show high apparent heterozygosity that is likely caused in part by structural variations, rather than true heterozygosity

When we attempted to include the "dog only" type worms (including the one isolated from a human host) in the heterozygosity analysis we noticed that they showed very high apparent heterozygosity (Fig 7B). Strikingly, this was also the case for heterozygosity on the X chromosome in the male worm (arrow in Fig 7B). To determine if this was an anomaly of our samples from Bangladesh, we subjected the sequences of the five whole genome sequenced "dog only" type worms from [20] to the same analysis. Except for the worm L6, these sequences showed even higher apparent heterozygosity, including on the X chromosome (circled in Fig 7B, all

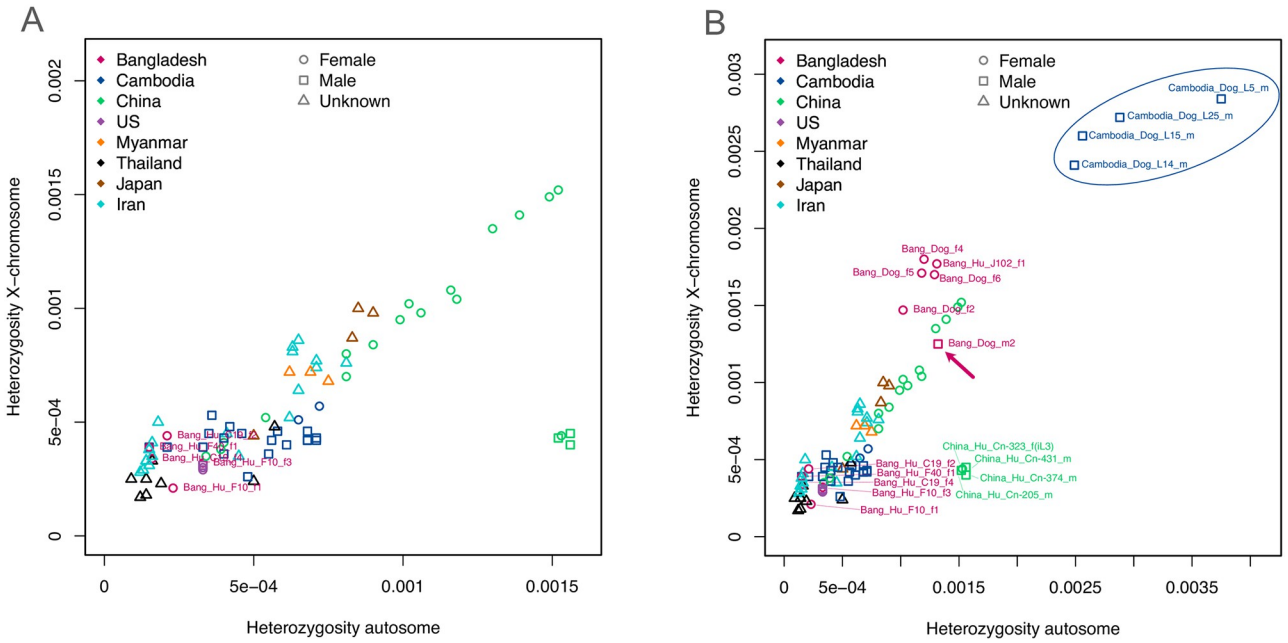

Fig 7. Measured heterozygosity of the whole genome sequenced worms from this and previous studies. The X axis shows the heterozygosity on the autosomes and the Y axis shows the heterozygosity on the X chromosome. A: only "human and dog" type worms. B: the same worms as in A plus the "dog only" type worms from this study and from [20]. Notice the high heterozygosity on the X chromosome in males of the "dog only" type (arrow and circled). The samples from previous studies are from the following references: Thailand [22], Iran [25], USA [44], Japan and Myanmar [23], Cambodia [20] and China [24]. For more detailed explanations, see S3 File.

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"dog only" type whole genome sequenced individuals by [20] were males). Notice that with respect to the mitochondrial and the nuclear genomes, L6 belongs to a separate sub-cluster of the "dog-only" cluster than the other four worms and the worms isolated in this study. It is not clear if worms in this subcluster are more closely related to the other "dog only" worms or to the "human and dog" worms (compare the positions of L6 in Figs 4–6 and S1 and S2 Figs). We think this high apparent heterozygosity is in part a consequence of using a divergent reference genome sequence (the reference sequence for the human infective *S. stercoralis* belongs to the 'human and dog' cluster) for calling the heterozygous positions. The argument for this with Figures is provided in S3 File and briefly summarized here. First, we asked if some of the *S. stercoralis* in Asia, in particular the "dog only" type, might not employ XX/XO sex determination as it is the case in the USA derived *S. stercoralis* reference isolate [44]. We therefore performed read coverage analysis for males and females. Overall, in both types the X chromosome showed lower read coverage in males compared with autosomes and with females, suggesting that males of the "dog only" type do only have one X chromosome. We then analysed the heterozygosity over the length of the chromosomes. Males did indeed show very low heterozygosity over large portions of the X chromosome but there were apparent heterozygosity hot spots. These were visible in both males and in females and also on autosomes. We think these apparent heterozygosity hotspots reflect duplications and X to autosome translocations in the genome of the "dog only" type, compared with the *S. stercoralis* reference genome [44].

Discussion

Among 12 *Strongyloides* positive persons from the Sylhet region we found four who carried *S. fuelleborni* rather than *S. stercoralis*. This was unexpected. In Asia infections of humans with *S.*

fuelleborni are considered very rare and restricted to people with very close interactions with monkeys [45–47]. In the Sylhet region there is a large population of free roaming monkeys. Albeit we have no reason to distrust our study participants, we need to point out that we cannot formally exclude that monkey faeces instead of human stool was returned. We consider it very unlikely that this was done. Nevertheless, in future studies intending to confirm this 'higher than expected' infection of humans with *S. fuelleborni*, an independent confirmation of the host (e.g. through the detection of host specific sequences in the stool), would be desirable in order to dispel all doubts. If confirmed in future studies, our results suggest that *S. fuelleborni* may be more common as a human parasite in at least certain places in Asia than previously thought. Based on the *cox-1* sequences, the *S. fuelleborni* in Bangladesh were most closely related to worms from Thailand, Myanmar and Laos (cluster 3 in [21]) which makes sense due to geographic proximity.

As expected, we also found hookworms. Based on their *cox-1* sequences they were of the species *Necator americanus* (group A in [48]). In agreement with earlier studies [24,48], we found no indication for population separation between Asia and Africa.

Overall, at the level of the nuclear and the mitochondrial genomes, the *S. stercoralis* we found in Bangladesh in humans mixed in with the worms described earlier from southeast Asia. Hence, we have no reason to assume that *S. stercoralis* in humans from Bangladesh and from southeast Asia are genetically distinguishable sub-populations. Together with the recent findings of [25] that *S. stercoralis* in Iran also share much of their genetic diversity with the ones in southeast Asia, these findings support the proposal by [26,34] that *S. stercoralis* has only rather recently established in humans after a host switch of a particular genotype from a canine host (possibly upon domestication of dogs) and then spread in the human population. It should, however, be noticed that based on reviewing published *cox-1* sequences, [19] did detect significant population structure and based on whole genome sequence, a possible asexual population of *S. stercoralis* in southern China and the laboratory reference isolate that originates from the USA, are genomically rather different from the southeast Asian *S. stercoralis* [24].

We found only one *Strongyloides* positive dog and all worms we analysed from this host individual had nuclear genomes that fell into the "dog only" cluster, based on the nuclear *SSU* and (if determined) whole genome sequences (cf. [20,21,26]). *S. stercoralis* of the "dog only" type (based on molecular taxonomy) had so far been described only in southeast Asia [20,26] and Australia [27] such that our findings extend the range, in which this type is known to occur, further West. The "dog only" type worms in this study showed a new *SSU* HVR-IV haplotype (now called haplotype V) that differs by one nucleotide from haplotype D (c.f. [21]). The fact that based on whole genome neighbour joining clustering all our dog derived worms grouped together with perfect bootstrap support should not be overinterpreted given that these worms were all derived from the same host individual and therefore might have been closely related. It is, however, noteworthy that the one worm that was isolated from a human but appeared genomically to belong to the "dog only" type also grouped with the dog derived worms although it had been isolated from Dhaka while the dog had been sampled in Sylhet. Again, given an N of one, this should not be overinterpreted.

In this study we made two unexpected observations. First, one of the human derived worms belonged genomically to the type that was so far considered to occur only in dogs. We must point out that there is a certain chance that this finding is an artefact. We cannot fully exclude that the human sample was contaminated with dog faeces or that dog faeces instead of human stool was returned. However, even if this result is real (and we think with high likelihood it is) this one "dog only" type worm in a human does not invalidate the general conclusion about species specificity by [20,21,26]. Occasional zoonotic infections of humans with

animal parasitic nematodes have been observed before, for example with filarial nematodes [49–53] or, even rather frequently, with animal parasitic hookworms [54,55 and references therein]. It is therefore not really astonishing that with increasing sampling such a case emerged also for *Strongyloides*. Second and more importantly, we found two worms in the dog with nuclear genomes of the "dog only" type but mitochondrial genomes of the "human and dog" type. This was rather astonishing because, so far, in all cases where nuclear and mitochondrial sequences from the same worms had been determined [20,23,26,42,43] both sequence kinds always fell in the same cluster as defined by [20,26] ("human and dog" or "dog only"). This finding suggests that at least occasional interbreeding of the two types does occur. A rare productive mating between a "human and dog" type female and a "dog only" type male followed by breeding with "dog only" type partners may have led to the introgression of the "human and dog" mitochondrial haplotype into the "dog only" population.

We found a very high apparent heterozygosity in worms of the "dog only" type. We think that this is in part an artefact caused by using the *S. stercoralis* reference genome, which is derived from a human infective isolate [44]. Compared with the reference, the "dog only" type, which, in our opinion, is likely to be a different species, might have a number of duplications with slightly deviating sequences. The positions that differ between the copies will be falsely considered heterozygous positions when the sequencing reads are aligned to the reference sequence without the duplication. Further, there might be translocations that are X chromosomal in the reference but autosomal in the "dog only" type. We think these findings illustrate that the two types are genomically rather different and that the *S. stercoralis* reference sequence is not suitable as a reference for certain genomic analyses of at least some of the "dog only" type *S. stercoralis*.

Supporting information

S1 File. FASTA file of the *S. fuelleborni* whole mitochondrial sequences used in Fig 3.
(TXT)

S2 File. FASTA file of the *S. stercoralis* whole mitochondrial sequences used in S2 Fig (contains all sequences used in Fig 5).
(TXT)

S3 File. Full argument for result section "Worms belonging to the "dog only" type show high apparent heterozygosity that is likely caused in part by structural variations, rather than true heterozygosity" (text and three figures).
(PDF)

S1 Table. Excel table showing the different *cox-1* haplotypes for the *S. stercoralis*, *S. fuelleborni* and *N. americanus* found in this study in different tabs. The last tab shows the sequences extracted from the *S. stercoralis* whole genome sequencing read data from [20].
(XLSX)

S1 Fig. NJ tree based on *cox-1* with more sequences compared with Fig 4. Diamonds label the two worms that showed "human and dog" type mitochondrial but "dog only" type nuclear sequences. The asterisk labels the worm in the "dog only" cluster isolated from a human host. Sequences found in this study are in red, the number of worms this sequence was found in is in (). Samples in blue are worms from [20] for which full genome short read sequences are available. For every sample the country of origin (CAR = Central African Republic, USA = United States of America), the host and the GenBank accession number are given. For worms from Cambodia also the worm individual is given after the host to facilitate cross

reference with [20]. Samples from Cambodia are from [20], samples from Laos are from [39], samples from Myanmar, Thailand and Uganda are from [26], samples from Iran are from [25], sample from the USA is from [40], samples from Tanzania, and Japan are from [17], samples from CAR are from [41] and samples from China are from [24].
(PDF)

S2 Fig. NJ tree based on whole mitochondrial genome sequences with more sequences compared with Fig 5. Diamonds label the two worms that showed "human and dog" type mitochondrial but "dog only" type nuclear sequences. The asterisk labels the worm in the "dog only" cluster isolated from a human host. The sequences from this study are in red. The other sequences were extracted from the whole genome sequencing read data of the following references: Cambodia [20], Thailand [22], Iran [25], Myanmar and Japan [23], USA [44] and China [24]. A FASTA file with all sequences used is provided as S2 File.
(PDF)

S3 Fig. Different representation of the cladogram based on whole genome sequences, without the "dog only" cluster, compared with Fig 6A. The sequences from this study are in red. The other sequences were extracted from the whole genome sequencing read data of the following references: Cambodia [20], Thailand [22], Iran [25], Myanmar and Japan [23], USA [44] and China [24].
(PDF)

S4 Fig. Different representation of the cladograms based on whole genome sequences, including the "dog only" cluster, compared with Fig 6B. Diamonds label the two worms that showed "human and dog" type mitochondrial but "dog only" type nuclear sequences. The asterisk labels the worm in the "dog only" cluster isolated from a human host. The sequences from this study are in red. The other sequences were extracted from the whole genome sequencing read data of the following references: Cambodia [20], Thailand [22], Iran [25], Myanmar and Japan [23], USA [44] and China [24].
(PDF)

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Author Contributions

Conceptualization: Veroni de Ree, Tilak Chandra Nath, Priyanka Barua, Dongmin Lee, Christian Rödelsperger, Adrian Streit.

Data curation: Veroni de Ree, Christian Rödelsperger.

Formal analysis: Veroni de Ree, Christian Rödelsperger, Adrian Streit.

Funding acquisition: Adrian Streit.

Investigation: Veroni de Ree, Tilak Chandra Nath, Priyanka Barua, Dorothee Harbecke, Dongmin Lee, Adrian Streit.

Methodology: Veroni de Ree, Dorothee Harbecke, Christian Rödelsperger, Adrian Streit.

Project administration: Tilak Chandra Nath, Priyanka Barua, Dongmin Lee, Adrian Streit.

Resources: Tilak Chandra Nath, Adrian Streit.

Software: Christian Rödelsperger.

Supervision: Tilak Chandra Nath, Priyanka Barua, Dongmin Lee, Adrian Streit.

Visualization: Veroni de Ree, Adrian Streit.

Writing – original draft: Veroni de Ree, Adrian Streit.

Writing – review & editing: Veroni de Ree, Tilak Chandra Nath, Priyanka Barua, Christian Rödelsperger, Adrian Streit.

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Poly(UG)-tailed RNAs are involved in the control of thousands of genes predominantly in the germline in *Pristionchus pacificus*

Veroni de Ree, Dorothee Harbecke, Hanh Witte, Christian Rödelsperger, Catia Igreja and Adrian Streit*

Department of Integrative Evolutionary Biology, Max Planck Institute for Biology Tübingen, Max-Planck-Ring 9, 72076 Tübingen, Germany

*Corresponding author:

adrian.streit@tuebingen.mpg.de

Orcid AS: 0000-0002-7838-621

Orcid V dR: 0009-0001-9026-4514

Abstract

In the nematode *Caenorhabditis elegans*, the terminal transferase RDE-3 adds a poly(UG)-tail to the free 3' end of RNA molecules generated by the action of primary siRNAs or piRNAs. The tailed RNA serves as a template for RNA-dependent RNA polymerases (RdRp) to generate secondary siRNAs, thereby reinforcing the RNAi effect. In this iterative process, progressively shorter tailed RNAs are formed. In *C. elegans*, injection of poly(UG)-tailed single-stranded RNA (ssRNA) leads to RNAi-mediated gene silencing, thereby bypassing the need for the processing of double-stranded RNA into siRNAs. We wondered if poly(UG)-tailed ssRNAs could also be used for experimental gene knockdown in nematodes where long dsRNA-mediated RNAi does not work reliably, such as the satellite model organism *Pristionchus pacificus* or parasitic nematodes of the genus *Strongyloides*.

Here we show that injection of poly(UG)-tailed RNA leads to gene knock down in *P. pacificus* and that the injected RNA, as well as the corresponding endogenous RNA, serve as substrate for the formation of new poly(UG)-tailed RNAs. Different from *C. elegans*, in *P. pacificus,* the knockdown effect depends on the redundant activity of the three *rde-1* paralogs present in this species. We detected endogenously occurring poly(UG)-tailed RNAs derived from thousands of genes, more than half of which belong to germline-specific co-expression clusters. Mutations in *Ppa-rde-3*, lead to sterility. In contrast, in *Strongyloides* spp., we found poly(UG)-tailed RNAs to be much less abundant, if not absent. Our results show that poly(UG)-tailed RNAs are not restricted to *C. elegans* and suggest that they play an important function in the germ line in *P. pacificus*.

Keywords: RNAi, poly(UG) tailed RNA, *Pristionchus pacificus*, *Strongyloides* spp. *rde-1*, *rde-3*.

1.0 Introduction

Gene silencing in response to double stranded RNA (dsRNA) is believed to be a natural mechanism to control the activity of transposable elements that can be exploited for the experimental knock down of genes in various organisms (RNA interference [RNAi]) (Svoboda, 2020). Such work was pioneered in the model nematode *Caenorhabditis elegans* (Fire et al., 1998) and adapted to other systems (Svoboda, 2020), of which only nematodes are discussed here. In *C. elegans,* the experimental silencing is achieved by applying long (several hundred to a few thousand bp) double stranded RNA (dsRNA) by micro injection, soaking the worms in dsRNA solution or feeding the worms with dsRNA expressing bacteria (Ahringer, 2006; Conte et al., 2015). The long dsRNA is processed by Dicer and other factors into primary small interfering RNAs (siRNAs) (Seroussi et al., 2022). The siRNAs form an active complex together with Argonaute proteins, and recognize their target through base pairing (Liu et al., 2023; Seroussi et al., 2022; Seroussi et al., 2023). While very successful in *C. elegans* and most plant nematode parasites (Maule et al., 2011), many animal parasitic nematodes remained largely refractory to the approach of treatment with long dsRNA (Maule et al., 2011; Viney and Thompson, 2008). In some cases, application of *in vitro* synthesized siRNAs was successful (Dulovic and Streit, 2019; Misra et al., 2017).

A possibly nematode specific amplification mechanism is known to lead to the formation of secondary siRNAs in *C. elegans* (Pak and Fire, 2007) and other nematodes (Holz and Streit, 2017; Sarkies et al., 2015; Wang et al., 2011). These molecules are around 22 nt long, have a G at their 5'end (22G RNAs), and interact with members of the family of Worm specific Argonautes (WAGOs) (Seroussi et al., 2023). 22G RNAs are formed by RNA dependent RNA polymerases, without 5'

processing. Hence, they have a 5' tri-phosphate that distinguishes them from other small RNAs like primary siRNAs, miRNAs or piRNAs (Pak and Fire, 2007). (Shukla et al., 2020), in a seminal paper, showed that the terminal transferase RDE-3 (also known as MUT-2) adds poly(UG) tails to target RNAs cut by primary or secondary siRNA complexes. These tailed RNAs serve as templates for the synthesis of new secondary siRNAs by RNA dependent RNA polymerases (RdRPs). These authors also showed that injection of poly(UG) tailed RNAs is sufficient to induce gene silencing. This silencing effect is independent of *rde-1*, which encodes an argonaute family protein and is a key component of the primary RNAi mechanism (Tabara et al., 1999).

Despite the success in *C. elegans*, RNAi is not routinely used in other nematode species. In the free-living model nematode *Pristionchus pacificus* RNAi has been very limited and restricted to particular genes (Aurilio and Srinivasan, 2015), although a protocol using lipofectamine in the injection mix has been proposed to improve the efficacy (Adams et al., 2019). Animal parasitic nematodes of the genus *Strongyloides* appear completely refractory to RNAi through long dsRNAs (Viney and Thompson, 2008) but moderate gene knock down by applying siRNAs has been reported (Dulovic and Streit, 2019). For both these taxa the presence of naturally occurring 5'-tri-phosphorylated small RNAs, putatively secondary siRNAs, has been shown (Holz and Streit, 2017). 22-23G secondary siRNAs are present in *P. pacificus*, while in *Strongyloides* spp. these small RNAs are longer (27 nucleotides) and initiate with G or A nucleotides (27GA RNAs).

In this study, we show that poly(UG) tailed RNAs can be used for experimental gene knock down in *P. pacificus* and that such RNAs are naturally formed from thousands of genes, many of which are expressed in the germ line.

Results

Injection of poly(UG)-tailed RNAs leads to loss of function phenotypes in *P. pacificus*

P. pacificus has an extensively studied mouth morphology polyphenism (Sommer, 2020). In response to environmental conditions, these worms form either a wide mouth with two teeth that allows them to kill other nematodes (eurostomatous, Eu) or adopt a narrower mouth with only one tooth characteristic of the strictly bacterivorous animals (stenostomatous, St). Under standard laboratory conditions on NGM plates, almost 100% of the hermaphrodites of the strain PS312 develop into Eu morphs. At the molecular level, expression of *eud-1* is essential for the formation of the Eu animals (Sommer, 2020). In a pilot experiment, we injected poly(UG)-tailed RNA targeting the gene *eud-1* (the RNA contained 464bp fragment of the *eud-1* transcript) at different concentrations, along with a plasmid encoding RFP under the control of the *eft-3* promoter, into the gonads of PS312 hermaphrodites. For the entire progeny of injected animals that produced any RFP-expressing progeny, we determined the mouth morphology (Suppl Fig. 1). In the non-injected controls, all progeny were Eu. At low poly(UG)-tailed RNA concentrations (0.17 and 0.5 pmol/μl) majority of mothers produced progeny that were only Eu (0% St). At 1.5 and 4.5 pmol/μl concentrations of poly(UG)-tailed RNA, however, the majority of injected mothers produced various numbers (4-40%) of St progeny, indicating that reduction of *eud-1* expression occurred at different levels in the new generation of worms. Since the variation of the percentage of St progeny from RFP positive injected mothers was smaller at 4.5 pmol/μl, we decided to continue our experiments with a concentration of 4.5 pmol/μl of RNA in the injection mix. This is considerably higher than the 0.5 pmol/μl used by (Shukla et al., 2020) for *C. elegans*.

In addition to *eud-1* we targeted *dpy-1,* another gene with a visually recognizable mutant phenotype (Dpy, short and fat body shape) (Witte et al., 2015). We injected poly(UG)-tailed, poly(AC)-tailed and non-tailed RNAs targeting the two genes into PS312 hermaphrodites and scored for the St and the Dpy phenotypes (Fig. 1). In both cases, we observed gene silencing as multiple St and Dpy animals were registered in the progeny of the injected animals. Importantly, in the progeny of *eud-1* injected animals we did not observe any Dpy animals and in the progeny of *dpy-1* injected animals no St worms were present, demonstrating the sequence specificity of the knock down. Strikingly, and different from the finding of (Shukla et al., 2020), RNAs without tail and RNAs with a poly(AC)-tail also induced gene silencing, although with clearly lower penetrance.

Next, we targeted the RFP transgene in the strain RS3832, which carries a *daf-1::Turbo-rfp* transgene in the PS312 genetic background (Fig. 2). Injection of poly(UG)-tailed RNA led to worms with strongly reduced red fluorescence in the progeny of injected hermaphrodites with moderate to high penetrance. Again, also poly(AC)-tailed and non-tailed RNAs induced RFP silencing, with comparable penetrance to poly(UG)-tailed RNA in the case of poly(AC)-tailed RNA but with clearly lower penetrance in the case of non-tailed RNA. Thus, for three different genes, we have demonstrated that poly(UG)-tailed RNAs can be used to specifically knock down individual genes in *P. pacificus*. Since the penetrance of gene silencing was the highest for the RFP transgene, for the following experiments, we concentrated on knocking down the RFP in RS3832.

Gene silencing is dependent on *Ppa-rde-1*

In contrast to the findings of (Shukla et al., 2020) in *C. elegans*, in our experiments, injection of poly(AC)-tailed and non-tailed RNAs also induced gene silencing. To exclude that the silencing effects were caused by unavoidable small amounts of double-stranded RNA produced during the synthesis of the injected RNAs, we followed the strategy of (Shukla et al., 2020) to use *rde-1* mutants. In *C. elegans*, these mutant worms are resistant to RNAi by injection of long double stranded RNA due to deficiency in an earlier step of the pathway, but still susceptible to gene silencing by injection of poly(UG) tailed RNA (Shukla et al., 2020).

In *P. pacificus* we identified three *rde-1* orthologs (Fig. 3). One (protein sequence accession number KAF8384843) had been annotated as *Ppa*-RDE-1 in the databases (encoded by gene model PPA37777 on chromosome I, Fig. 3 and http://www.pristionchus.org - Genome Browser) and is the best reciprocal BLASTP hit with *Cel*-RDE-1. We will refer to this gene as *Ppa-rde-1.1*. The two other paralogs, protein sequence accession numbers KAF8358727 and KAF8358512 are encoded by two genes (gene models PPA00739 and PPA36770) located adjacent to each other in head-to-head arrangement on chromosome III. We will refer to these two genes as *Ppa-rde-1.2* (encoding KAF8358727) and *Ppa-rde-1.3* (encoding KAF8358512) (for the full argument of orthology assignment see Materials and Methods and Fig. 3). We generated CRISPR induced mutations in the *rde-1* paralogs in the RS3832 background (Tables 1 and 2, Suppl. Table 1, see Materials and Methods). As described for *C. elegans rde-1* mutations, (Grishok et al., 2005; Kim et al., 2005) we noticed an increase in the expression of the transgene in triple mutants, suggesting that the *rde-1* orthologs are involved in limiting the expression from multi-copy transgenes as in *C. elegans*. Other than this, these mutants did not show any obvious phenotype. All single and double mutants we tested still showed strongly reduced

RFP upon poly(UG)-tailed RNA (Fig. 4, panel b-d) and the effect of non-tailed RNA was not abolished (Fig. 4, panel e and f). However, in the triple mutant, the poly(UG)-tailed RNA had no silencing effect (Fig. 4, panel a), suggesting that either, different from *C. elegans*, *rde-1* function is required also for the poly(UG)-tailed RNA dependent RNAi amplification mechanism or that the silencing we observed was due to a different mechanism.

New tailed RNAs are formed from injected and endogenous RNAs in *P. pacificus*

If the same process as in *C. elegans* is at work, one would expect to see newly formed poly(UG)-tailed RNAs upon injection. We isolated RNA from the injected worms 24 hours after injection and from the progeny of injected worms when they were second and third juvenile stage (notice that *P. pacificus* hatches from the egg shell only as a J2). Following the example of (Shukla et al., 2020), we reverse transcribed the RNA with a primer recognizing the poly(UG)-tail and containing two adaptor sequences. Then, we performed a nested PCR reaction using primers recognizing the 5' end of the injected RNA and the adaptor sequences (for details see Materials and Methods). In order to avoid that the experiment was dominated by the injected RNA and to increase the chances of identifying poly(UG)-tailed RNAs of different lengths, we separated the PCR products on an agarose gel and isolated and cloned the DNA from different fractions. This rendered this experiment not really quantifiable but merely qualitative. We then sequenced individual clones and found clones with 9 to 26 UG repeats. Notice that the tail length we observed is not indicative for the length of the tail on the RNA molecule the clone was derived from but reflects the place where the reverse transcription primer annealed. For our interpretation we only considered clones with 10 or more UG repeats (Fig. 5A), because the primer used for reverse

transcription contained 9 AC repeats and hence the last 9 repeats in the PCR product are derived from the primer. We also manually double checked that there were no DNA encoded UG repeats at the position of the supposed poly(UG) addition site.
In the sample 24 hours after the injection (Injected P0 in Fig. 5A), as expected, the strongest signal was derived from the injected RNA since 20 out of 35 clones with 10 or more UG repeats contained the full sequence of the injected RNA. The other 15 clones defined ten different poly(UG) addition sites along the RFP sequence. Five of them were represented by more than one clone. These clones had different numbers of UG repeats, indicating that they were independent. In the progeny of the injected worms, we did not detect the injected RNA anymore, but the 21 clones with 10 or more UG repeats defined 14 different poly(UG) addition sites along the RFP sequence of which four were represented by at least two independent clones. Strikingly, two of these addition sites were upstream of the region covered by the injected RNA (both contained exons 1-3 and a portion of exon 4 of the RFP mRNA, one of them extending into the 3'UTR), indicating that poly(UG) tailed endogenous RFP mRNAs occur naturally. Consistent with the notion that tailed RNAs are naturally produced in *P. pacificus*, we also found six clones derived from the progeny of un-injected P0s with 10 or more UG repeats. These clones defined six different poly(UG) addition sites along the RFP sequence. The presence of poly(UG)-tailed *rfp* RNAs in un-injected worms was also confirmed in adult worms (Fig. 6). In order to determine if the injected RNA was used as a substrate for the production of shorter tailed RNAs, we repeated the experiments with an RNA that, compared with the endogenous one, contained two point mutations at the 5' end and used primers that either detect preferentially the injected RNA or preferentially the endogenous RNA (see Materials and Methods). We detected tailed products that were shorter than the

injected RNA with the point mutations and with the endogenous sequence (Fig. 5B), suggesting, that both, the injected and the endogenous RNA served as substrate for the formation of shorter tailed RNAs.

UG tail addition depends on *Ppa-rde-3*

In *C. elegans*, the polymerase that adds the poly(UG)-tails is encoded by *rde-3*. *P. pacificus* has a one-to-one ortholog of *rde-3* (Fig. 3B, for the full argument for orthology assignment, see Materials and Methods). In order to test if RDE-3 is responsible for the poly(UG) addition in *P. pacificus*, we generated CRISPR induced mutations in this gene in the RS3832 background (Tables 1, 2). All seven *rde-3* alleles isolated are maternal effect sterile (Mes). While most homozygous *rde-3* maternal and zygotic mutant worms are sterile, some mutants lay very few eggs, but the embryos arrest at various stages before hatching (we only ever observed one larva that hatched but did not develop). In order to exclude that the Mes phenotype was aggravated by the stress imposed by the presence of a multi-copy transgene, we backcrossed QA444 (*Ppa-rde-3(yt61)*/+; *tuEx333* [*daf-1p::TurboRFP*]) against PS312 and selected RFP negative worms resulting in QA453. Again, *rde-3(yt61)* showed the Mes phenotype with the very few eggs laid by *rde-3(yt61$^{m/z}$)* being inviable.

We isolated 214 QA444 *rde-3(yt61)* homozygous maternally and zygotically mutant worms (the sterile progeny of maternally rescued homozygous mutants) and tested them for the presence of poly(UG)-tailed *rfp* RNAs (without injection) following the experimental procedure described above. We found only two clones with more than 10 UG repeats (Fig. 6). These two clones were identical (same UG addition site, 17.5 UG repeats) and hence presumably derived from the same original PCR product. In

contrast, from an equal number of progeny of QA444 *rde-3(wt)* worms we found 25 clones representing at least 20 different PCR products and 15 different UG addition sites (Fig. 6). Although our assay is not really quantitative, this indicates that RNA tailing is strongly reduced in the absence of RDE-3. The remaining tails may be the product of a small amount of persisting maternal *rde-3* function (protein that remains in the mothers and is passed to the eggs).

Poly(UG)-tailed RNAs are naturally produced from many endogenous mRNAs

In order to assess whether poly(UG)-tailed RNAs can be naturally generated from mRNAs other than those derived from multi-copy transgenes, we performed RNA 3'-end sequencing. Ribosome and mRNA-depleted RNA from the wt strain PS312 was reverse transcribed with poly(UG) recognizing primers (for details see Materials and Methods). The resulting requesting Illumina data are deposited in the European Nucleotide Archive (Accession number PRJEB9683). We screened for reads that, in addition to a portion that aligned with the *P. pacificus* genome sequence, contained a stretch of (UG) repeats. Since the primer used for the reverse transcription contained 9 UG repeats, we only considered reads that contained 10 or more UG repeats. Out of 8,386,810 total reads that mapped to the genome, 200,087 (2.4%) fulfilled this criterion (Suppl. Tab. 2). In 21,270 (out of a total of 28,896) gene models, we did not find any such reads (73.6%). However, 26.4% of the *P. pacificus* genes produced poly(UG)-tailed RNAs in various amounts. In detail, read number of poly(UG)-tailed RNAs per gene were grouped arbitrarily in very low (1-5 reads; 2,520 genes), low (6-50 reads; 4,356 genes), median (51-100 reads; 466 genes), high (101-500 reads; 266 genes) and very high (501-16,008 reads; 18 genes). At the very top, for instance, were

11 genes with 501 to 1,000 reads, 5 with 1,001 to 2,000 reads and finally one each with 3,331 and with 16,008 reads.

We then filtered out duplicate reads to get an overview of the number of unique reads per gene, which represent independent detection events (reads derived from independent reverse transcription events) (Suppl. Tab. 3). In 5,129 (17.7%) gene models, we found three or more unique reads, of which 1,897 (6.6% of the total) had 10 or more unique reads. 79 models had 100 or more unique reads, among them the top three with 516, 676 and 834. Interestingly, the majority (58.8%) of the 5,129 genes with three or more unique reads fall into one of the five gametogenesis related co-expression modules described by (Athanasouli et al., 2023) and amounted to a considerable fraction of the corresponding module. Specifically, 1,758 genes belong to module 1 (oogenesis 1) and represent 54.9% of the 3,202 genes in the module; 542 genes belong to module 5 (oogenesis 2) and represent 49.3% of the 1,099 genes in the module; 452 genes belong to module 2 (spermatogenesis) and represent 22.7% of the 1,995 genes in the module); 227 genes belong to module 7 (oogenesis 3) and represent 48.1% of the 472 genes in the module; and 36 genes belong to module 23 (oogenesis 4) and represent 50.0% of the 72 genes in the module. We then visually inspected the randomly selected gene models to determine how the reads are distributed over the gene models. Consistent with the hypothesis that a mechanism as described by (Shukla et al., 2020) for *C. elegans* is also at work in *P. pacificus*, the reads aligned to multiple places along the CDS of the gene models.

We wondered if poly(UG)-tailed RNAs exist also in *Strongyloides* spp., a genus of parasitic nematodes in a different clade (clade IV, Blaxter et al. 1998) than *C. elegans* and *P. pacificus* (both in clade V). Since we had found that in *P. pacificus* the injected

poly(UG)-tailed RNA served as substrate for the formation of new poly(UG)-tailed RNAs, we injected the same poly(UG)-tailed *rfp* RNA into the gonads of *Strongyloides stercoralis* free-living females and isolated RNA from all injected animals (pooled) 24 h after injection. A co-injection marker (designed for *S. stercoralis*) was included in the injection mix for consistency with the other experiments, but it was not considered in this experiment. In four independent experiments (injecting 44, 29, 28 and 33 animals) with tailed RNAs from two independent in vitro transcription reactions, we detected the injected RNA, but failed to detect RNAs with other poly(UG) addition sites. Next, we performed RNA 3' end sequencing with RNA extracted from *Strongyloides ratti* and *S. stercoralis*.

In *S. ratti,* we also detected tailed RNAs with 10 or more UG repeats that mapped to multiple, but many fewer genes, compared with *P. pacificus*. We found reads mapping to 7.6% (995) genes of *S. ratti*. As seen in *P. pacificus*, the number of tailed reads per gene also varied. The majority of the tailed RNAs had a very low (1-5 reads; 783 genes), low (6-50 reads; 190 genes), median (51-100 reads; 8 genes) and high (101-1,000 reads; 9 genes) number of reads, but the five top genes had over 1,000 copies of tailed RNA (Suppl. Tab. 4).

In only 212 (1.6%) gene models, we found three or more unique reads, of which 53 had 10 or more unique reads (Suppl. Tab. 5). There were only six gene models with 100 or more reads, with the top three having 169, 639 and 932 reads. Upon visual inspection, we noticed that five of the six gene models with more than 100 unique reads were mitochondrial genes, including the two genes with the highest number of unique reads.

We have also found reads with 10 or more (UG) repeats that mapped to the genome of *S. stercoralis*. However, we only observed tailed RNAs derived from 3.2% (557) of

the coding sequences, and for 470 of these the number of reads per gene was a maximum of 5. In only 87 (0.5%) genes, we found more than 5 reads, of which 84 had less than 100 reads. The top six genes produced 53, 67, 82, 100, 273 and 826 copies of the tailed RNA, respectively (Suppl. Tab. 6). In addition, the number of unique reads per gene was low, with 106 genes (0.6%) varying between 3 and 50 unique reads (Suppl. Tab. 7).

The following observations make us believe that most, if not all, of the reads in *Strongyloides* spp. correspond to artefacts. We noticed that the vast majority of reads that aligned with the genome had a large number of mismatches and were therefore not really derived from the sequence they aligned to. Further, if there were multiple unique reads in a particular gene, they tended to map to the same position within the gene. Compared with *P. pacificus*, both, the fraction of reads with 10 or more UG repeats and the number of genes hit by these reads are much lower in *Strongyloides* spp. Our data are not suitable to decide if all these reads are artefacts and poly(UG)-tailed RNAs do not exist in *Strongyloides* spp. or if poly(UG)-tailed RNAs are just less common in this taxon. However, we think that these data can be seen as a maximum estimate of the methodologically introduced background. This supports the notion that most of the poly(UG)-tailed RNAs observed in *P. pacificus* were real.

Discussion

In *C. elegans*, poly(UG)-tailed RNAs have been shown to be formed in response to the activity of primary siRNAs and piRNAs through the activity of the terminal transferase RDE-3, also known as MUT-2 (Shukla et al., 2020). These RNAs are part of a presumably nematode specific amplification step for the action of siRNAs and

piRNAs (Pak and Fire, 2007; Shukla et al., 2020). Injection of poly(UG)-tailed RNAs can be used to experimentally knock down genes (Shukla et al., 2020). Here, we show that a similar mechanism exists in *P. pacificus*. We showed gene knockdown for two endogenous genes and a multi-copy transgene. Different from (Shukla et al., 2020) in *C. elegans*, we observed that injection of non-tailed RNAs or RNAs with a poly(AC)-tail also induced gene silencing. (Shukla et al., 2020) had done their experiments in *rde-1* mutant worms, which are resistant to RNAi initiated by long double-stranded RNAs, which may arise in small quantities in the in *vitro transcription* reaction used to generate the RNAs to be injected. (Shukla et al., 2020) found the silencing effect of poly(UG)-tailed RNAs not to be affected by mutations in *rde-1*. We generated mutations in all three *rde-1* paralogs present in *P. pacificus,* and in our hands, the silencing effect of poly(UG)-tailed RNAs was abolished if all three paralogs were mutant. This may reflect a biological difference between *C. elegans* and *P. pacificus,* but we can also not completely exclude that the silencing we observed is caused, at least in part, by a mechanism different from the one described by (Shukla et al., 2020). However, the fact that we found the injected and the endogenous RNA to be a substrate for the addition of new poly(UG)-tails strongly argues that a similar mechanism is in place in *P. pacificus*. Further, we detected endogenously present poly(UG)-tailed RNAs in thousands of genes (if a cut off of at least three unique reads detected in a gene in our experiment is applied, 5129 genes, which is 17.7% of all predicted genes). Interestingly, more than half (58.8%) of these genes belong to germline-specific co-expression clusters (Athanasouli et al., 2023) and they represent about half of all genes in the four oogenesis clusters and almost a quarter of the genes in the spermatogenesis cluster. There is, however, also a moderate correlation of the number of poly(UG)-tailed RNAs detected and the expression level of genes

(germline genes tend to be fairly highly expressed). The notion that poly(UG)-tailed RNAs play an important role in the germline is also supported by the maternal effect sterile phenotype we observed in worms mutant for *Ppa-rde-3*. In *C. elegans*, RDE-3 has been shown to add poly(UG)-tails to RNAs of germline and soma expressed genes.

We failed to find convincing evidence for the existence of a poly(UG)-tailed RNA dependent silencing pathway in *S. stercoralis* or *S. ratti*. However, it should not be firmly concluded that such a pathway does not exist in *Strongyloides* spp. solely based on our negative results. It could be that *Strongyloides* spp. uses a different tail for the same purpose (to elucidate this, a non-tail-biased RNA end seq. could be of help). However, we think that it can be concluded that, if the pathway exists with poly(UG) tails, it is not as widely used in *Strongyloides* spp. as it is in *P. pacificus*. In our RNA end seq experiment that was designed to enrich for poly(UG)-tailed RNAs the proportion of reads with 10 or more UG repeats (and with this more repeats than introduced through the primer used) was about 50 times higher in *P. pacificus* than in the two *Strongyloides* samples and the fraction of genes in which we found three or more unique reads was about 10 times higher in *P. pacificus* compared with *Strongyloides*. While it is not possible to conclude that the pathway is absent from *Strongyloides* spp., the results of the two *Strongyloides* experiments can be taken as maximum estimates for the background detection of poly(UG)-tailed RNAs, which supports that the majority of the reads in the *P. pacificus* experiment were real.

Limitations: Since the RNAi mechanism in *P. pacificus* is poorly characterized we have no mutants of which it is known that they are insensitive to silencing through the primary RNAi pathway that is triggered by long double-stranded RNAs. Although

RNAi by the injection of such RNAs is believed to work only in exceptional cases, we cannot exclude that some of the effects we saw were caused by small quantities of double-stranded RNAs that arose in the *in vitro* transcription reaction.

We would like to point out very clearly that our PCR and cloning-based experiments used to detect poly(UG)-tailed RNAs are purely qualitative (demonstrate the existence and identify addition sites) but not quantitative. Although in principle more quantitative, also in the RNA end sequencing experiment, the quantitative information is limited, due to the extensive pre-treatment of the RNA (rRNA and poly(A) RNA depletion) and the PCR amplification step during the library construction.

Conclusions

Here, we show that in *P. pacificus,* a gene regulatory mechanism that involves poly(UG)-tailed RNAs whose formation is dependent on *Ppa-rde-3* is in place. It is of particular importance in the germ line. Poly(UG)-tailed RNAs can be used to experimentally downregulate genes in *P. pacificus*. Different from *C. elegans*, the knockdown requires the redundant activity of the three *rde-1* homologs. In *P. pacificus*, knockdown through injection of poly(UG)-tailed RNAs works more reliably than RNAi based on the injection of double-stranded RNA, but not as efficiently as RNAi works in *C. elegans*. If the clade IV nematodes *Strongyloides* spp. have a similar mechanism, it is much less widely used than in the clade V nematodes *P. pacificus* and *C. elegans*.

Materials and Methods

Worm Strains and Cultures

The genotypes of all *P. pacificus* strains constructed and/or used are given in Table 2. RS3832 is not yet published and was a gift from Wensui Lo (at the time at our institute, now at Northwest A&F University, Yangling, China). The strain carries a multi-copy *daf-1::Turbo-rfp* transgene in the PS312 genetic background and was constructed by Wen-Sui Lo when she was in Ralf Sommer's lab at our institute. Requests for this strain should be addressed to Wen-Sui Lo or Ralf Sommer. Although the transgene was originally established as an extrachromosomal array, it is transmitted to 100% of the progeny and, in crosses, behaves like a Mendelian locus. Hence, we suspect that the array did integrate into a chromosome. *P. pacificus* was maintained on NGM agar plates at 20°C as described for *C. elegans* by (Stiernagle, 2006).

S. stercoralis PV001 (Hunt et al., 2016) was a gift from James Lok (University of Pennsylvania) and had been maintained in Mongolian Gerbils (*Meriones unguiculatus*) in the laboratory since April 2021 as described by (Lok, 2007) and (Nolan et al., 1993) with occasional reversion to frozen stock. *S. ratti* ED321 was a gift from Mark Viney (at the time University of Bristol, now University of Liverpool) and had been maintained in the lab since 2010 in female Wistar rats as described by (Viney et al., 1992) with occasional reversion to frozen stock. All relevant animal welfare regulations (the German "Tierschutzgesetz" and the "Verordnung zum Schutz von zu Versuchszwecken oder zu anderen wissenschaftlichen Zwecken verwendeten Tieren) were followed. Permits with respect to animal experimentation (EB 04/20 A and EB 02/23 V), infection protection (AZ: 25-28/5420.11-3.9 / Streit, Adrian) and animal health (AZ: 3STV/9114.51 / MPI Streit) were granted by the "Regierungspräsidium Tübingen". Animal and laboratory facilities are subject to regular inspection by the authorities.

PCR and sequencing

All PCR primers were purchased from Eurofins Genomics unless otherwise specified. All PCR reactions were done using the Qiagen Taq master mix (201445) or DreamTaq PCR Master Mix (K1081). All primer sequences are listed in Table 3. All PCR products to be sequenced were submitted to Genewiz from Azenta Life Sciences. 0.5-2μl of the PCR product mixed with 1μl of the sequencing primer (stock concentration 10 mM) were sent in a 10μl.

Construction of the template DNA for *in vitro* transcription

Total RNA (1-2μg) extracted from mixed stage RS3832 using TRIzol (life technologies, 15596026) and following the manufacturer's instructions was used to synthesize cDNA using superscript II (Invitrogen, 18064-014) with oligo(dT) - (QT796) primers in a 20μl reaction. RNA was heated with 1μl of 10mM dNTPs, 1μl of reverse transcription (RT) primer (oligo(dT) - QT796) and H_2O to 65 °C for 5 min and immediately chilled on ice. Then the 4μl of 5x first strand buffer, 2μl of 0.1M DTT and 1μl of H_2O were added to the reaction and incubated at 42°C for 2mins. Finally, 1μl of Superscript II (or H_2O for the negative control) was added and incubated for 2hrs at 42°C followed by 15min at 70°C.

1μl of the cDNA was used as template to amplify the target portion of the gene with gene-specific primers in a 10μl reaction:eud-1 (F-7291 & R-7294), dpy-1 (F-7275 & R-7276), rfp (F-7307 & R-7311). The PCR products were sequenced from both sides using the amplification primers. The PCR products were diluted 1:100 and 0.5μl was used as template for a second PCR with primers containing overhang sequences to add the T7 promoter in the 5' end and the desired tail on the 3' end (see primer list: 7297-eud-1 F_T7, 7283-dpy-1 F_T7, 7312-rfp F_T7, 7299-eud-1 R_pUG, 7300-eud-

1 R_pCA, 7284-dpy-1 R_UG, 7285-dpy-1 R_CA, 7318-rfp R_pUG, 7317-rfp R_pAC and for the no tail PCR the corresponding T7 primer and the non-overhang R primer was used). These products were gel purified on 1% agarose, 1x TAE gels and the DNA was extracted from the gel using the QIAquick Gel Extraction Kit (28706) following the manufacturer's instructions. The purified PCR products were sequenced from both sides using the amplification primers. As an alternative template for the generation of *rfp* targeting RNAs and to avoid problems with a non-productive alternative splice form of the *rfp*-RNA we found to be present in the worms, the 1st PCR of the *rfp* fragment (without overhangs) was cloned using the TOPO TA cloning kit (Invitrogen 450071 & 450641) and one shot competent cells (Invitrogen C404010) resulting in plasmid pVdR1. The correct sequence was confirmed by sequencing from both sides using the M13 primers.

Generation of SNP marked RNAs

In order to obtain *rfp* RNA marked with two point mutations, instead of F-7307 we used a forward primer (7344) carrying the SNP and the T7 promoter to amplify the template for *in vitro* transcription. In these RNAs the SNPs are close to the 5' end such that even very short molecules could be detected. However, for RNA detection, only one primer site was available at the 5' end, such that the "semi-nested" protocol was used for detection (see below). In order to also have the opportunity for detection with fully nested PCR, we also generated a construct with SNPs a bit further downstream. First, we used primer F-7375, carrying the SNPs for a PCR with R-7311 for 10 cycles. From this PCR reaction, 0.5 μl was used for 20 cycles of PCR with the primer F-7376 (whose 3' part overlaps with the 5' part of 7375) and R-7311. This PCR product was cloned as before, resulting in plasmid pVdR2. Then this plasmid was

used as the template for the PCR with the overhang primers that introduce the T7 promoter and the tails as described above.

In vitro transcription of test RNAs

In vitro transcription of the constructs described above was performed using the Invitrogen, MEGAscript™ T7 Transcription Kit (AM1333) according to the manufacturer's instructions with the following modifications. The transcription reaction volume was scaled up to 40μl and the input DNA was increased to 1.5μg. The incubation step was increased to 37°C overnight for a better yield. Turbo DNase treatment at 37°C was done for 15-40 min.

Following the Turbo DNase treatment, the reaction was extracted once with Phenol: Chloroform:Isoamyl Alcohol 25 : 24 : 1 (PanReac, AppliChem, A0889,0100) and the RNA was precipitated with one volume of isopropanol (ROTH, 7343.1).

The RNA pellet was washed twice with ice-cold 80% Ethanol, air dried and resuspended in 15μl of H_2O. The RNA concentration was determined by nanodrop analysis of 1 μl of a 1:10 dilution, and 200 ng of the RNA were analyzed on a 10% polyacrylamide, 1x TBE, 8M urea gel.

Injection procedures

The injection mix consisted of the RNA to be tested and a DNA plasmid encoding a fluorescent protein as a co-injection marker in TE. In the pilot experiment (Suppl. Fig. 1), RNA concentrations of 0.17pmol/μl, 0.5pmol/μl, 1.5pmol/μl and 4.5pmol/μl were tested for *eud-1*. All later experiments were done using 4.5pmol/μl.

For *P. pacificus*, the co-injection markers were plasmids encoding RFP (for experiments targeting *eud-1* or *dpy-1*) or GFP (for experiments targeting *rfp*) under

the control of a *Ppa-eft-3* promoter. Requests for detailed maps of these plasmids or the plasmids themselves should be addressed to Hanh Witte or Ralf Sommer. The co-injection markers were injected at 30ng/μl. For *S. stercoralis,* the co-injection marker was 50ng/μl of the commercially available plasmid pAJ50 (Addgen), which encodes RFPmars under the *Sst-act-2* promoter.

The injection mix was micro-injected into the gonads of the young adult worms (P0s). All the P0s were singled out individually on NGM plates. In the case of *S. stercoralis*, 3-5 males were added to each female. The injected P0s were allowed to lay eggs for 24 hrs. Then the adult worms were removed from the plates and the F1 progeny were allowed to develop into the developmental stage desired for the respective experiment.

Detection of tailed *rfp* RNAs

The worms specified for the particular experiment in the result section were flash frozen in as little H_2O as possible. Total RNA was extracted using TRIzol (life technologies, 15596026), DNase I treated at RT for 25mins and cleaned and concentrated using Zymogen Clean & Concentrator (R1013) according to the manufacturer's instructions.

The RNA was reverse transcribed using a tail recognizing primer 7755 with 9UG repeats and 2 adaptors (Ad1 and Ad2) as described above.

For the following PCR reaction, one of three forward primers was used: primer 7307 (for the nested protocol, this primer recognizes the injected and the endogenous RNA equally well), 7363 (for the semi-nested protocol, this primer recognizes the 5'end of the injected RNA including the 10 first nucleotides that are derived from the transcription template and are not present in the endogenous RNA and therefore biases towards the detection towards the injected RNA) or 7364 (for the semi-nested

protocol, this primer extends 10 nucleotides further upstream than the injected RNA and therefore biases towards the detection towards the endogenous RNA). A first PCR was done using the desired forward primer and the Ad1 7757 reverse primer. The product was diluted 1:1000, and 1μl was used as template in the second PCR with the Ad2 reverse primer 7758 and either the nested (7319, nested protocol) or the same forward primer as in the first round of PCR (semi-nested protocol). The resulting PCR products were separated on a 1% agarose, 1x TAE gel. The size range between 1000 bp and 100bp of the lane was divided into multiple pieces. The DNA was extracted using the QIAquick Gel Extraction Kit (cat no. 28706) according to the manufacturer's instructions and cloned using the Invitrogen TOPO TA cloning kit (450071 & 450641). All (if there were few) or 20 colonies per fraction were tested by colony PCR using the standard M13 primers and analyzed on a 1% agarose, 1x TAE gel. Clones that appeared to be derived from primer dimers were discarded. The remaining clones were sequenced using the standard M13 primers.

4.7. Sequence analysis

Sanger sequences were manually checked using the SnapGene software (SnapGene® software (from Dotmatics; available at snapgene.com) in order to determine the position to which the poly(UG)-tail was added. Only clones with 10 or more TG repeats were considered (9 repeats are in the primer used for reverse transcription). If the position of the UG tail was a TG rich site, the sequence was disregarded. For Illumina sequences, adapter sequences were removed by the cutadapt tool (version 4.4 with options -a TGGAATTCTCGGGTGC -A GATCGTCGGACTGTAG -m 20:20). Trimmed read pairs with minimum length of 20 nucleotides were then aligned against the *P. pacificus* genome (version El Paco) by the STAR alignment

tool (version 1.7.10b with option --alignIntronMax 10000) (Dobin et al. 2013; Rödelsperger et al. 2017). Duplicate reads were removed by the samtools rmdup program and the binary alignment file (.bam) was converted into text format (.sam) using the samtools view command (version 0.1.18) (Li et al. 2009). Based on the cigar string and the nucleotide sequence in the .sam file, we extracted reads containing a portion that aligned to the genome and also contained 10 or more unaligned TG repeats. Quantification of UG-containing reads against the *P. pacificus* gene annotations (version El Paco gene annotation version 3) was performed using the featureCounts program as implemented in the R subreads package (version 4.0.0) (Liao, Smyth, and Shi 2014; Athanasouli et al. 2020). For further manual inspection, the extracted reads were converted into .bam format using the samtools view command and then visualized in the genome browser.

Microscopy

Phenotyping for all 3 genes was done when the worms were in the young adult stage. The morphological phenotypes (mouth form and Dpy) were scored while the worms were on NGM plates, using Zeiss Discovery V20 and Olympus SZX10 high-power dissecting scopes with illumination from underneath. RFP and GFP fluorescence were assessed while the worms were on NGM plates, using a Leica M205 FCA fluorescence dissecting scope. For higher magnification images, a Zeiss Imager Z1 fitted with an Axiocam 506 mono camera was used.

Identification of *P. pacificus* RDE-1 homologs

All BLAST analyses were performed at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and repeated on March 24th 2025. The

protein sequence of *C. elegans* RDE-1 was retrieved from the databases (accession number NP_741611.1). BLASTP analysis against *C. elegans* and *P. pacificus* protein sequences revealed NP_741611.1 and KAF8384843.1 as best reciprocal BLASTP hits. KAF8384843.1 had been annotated as *P. pacificus* RDE-1 in the database entry. Both, NP_741611.1 and KAF8384843.1, when used as bait for BLASTP searches against *P. pacificus*, returned the same six top hits with a large increase of the e-values between the third and the fourth hit (accession numbers KAF8384843.1 (annotated as *Ppa*-RDE-1), AF8358727.1 (PRIPAC_93722), KAF8358512.1 (PRIPAC_93507), KAF8381868.1 (annotated as *Ppa*-ALG-1), KAF8373425.1 (PRIPAC_79854), KAF8368124.1 (annotated as *Ppa*-ALG-4).

When used as bait for BLASTP searches against *C. elegans*, they returned the same five top hits with a large increase in the e-values between the first and the second hit (accession numbers NP_741611.1 (*Cel*-RDE-1), NP_001367206.1 (*Cel*-ALG-1), NP_493837.1 (*Cel*-ALG-2), NP_502218.1 (*Cel*-ALG-3), NP_499191.1 (*Cel*-ALG-4). From these 11 sequences, a Neighbour Joining (NJ) tree (Fig. 3) was reconstructed as described below. A Maximum likelihood tree was also computed and showed the same topology as the NJ tree.

Identification of *P. pacificus* RDE-3

All BLAST analyses were performed at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and repeated on March 24th 2025. The protein sequence of *C. elegans* RDE-3 was retrieved from the databases (accession number NP_491834.1). BLASTP analysis against *C. elegans* and *P. pacificus* protein sequences revealed NP_491834.1 and KAF8387490.1 as best reciprocal BLASTP hits. KAF8387490.1 had been annotated as *P. pacificus* MUT-2 (a.k.a. RDE-3, see

introduction) in the database entry. Both NP_491834.1 and KAF8387490.1, when used as bait for BLASTP searches against *P. pacificus*, returned the same three top hits with a large increase in the e-values between the first and the second hit (accession numbers KAF8387490.1 (annotated as *Ppa*-MUT-2), KAF8385952.1 (PRIPAC_75094), KAF8366393.1 (annotated as *Ppa*-GLD-2). When used as bait for BLAST searches against *C. elegans*, they returned the same three top hits with a large increase in the e-values between the first and the second hit (accession numbers NP_491834.1 (*Cel*-RDE-3), NP_001021434.1 (*Cel*-GLDR-2), 5JNB_A (*Cel*-GLD-2)). From these 6 sequences, a Neighbour Joining (NJ) tree (Fig. 4) was reconstructed as described below. A Maximum likelihood tree was also computed and showed the same topology as the NJ tree.

Phylogenetic analysis and tree reconstruction

The sequences were aligned with Muscle in MEGA12 (Kumar et al., 2024; Stecher et al., 2020) using default settings. The following information was retrieved from the captions to the trees provided by MEGA. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal trees with the sum of branch length = 4.170 (tree in Fig. 3A) and 3.216 (tree in Fig. 3B) are shown in Fig. 3. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein, 1985). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The pairwise deletion option was applied to all ambiguous positions for each

sequence pair resulting in a final data set comprising 1454 positions for the tree in Fig. 3A and 995 positions for the tree in Fig. 3B. Evolutionary analyses were conducted in MEGA12 (Kumar et al., 2024; Stecher et al., 2020) utilizing up to 7 parallel computing threads.

CRISPR knockout mutants of *rde-1* and *rde-3*

Knockout mutants were generated via CRISPR/Cas9 mutagenesis following (Witte et al., 2015), with modifications described in "Co-Crispr injection protocol by Hanh Witte (version 2019-10-23)" in the "protocols" section of Pristioncus.org (http://www.pristionchus.org/download/protocol_cocrispr_2019_10.pdf). The co-injection marker used was the same *Ppa-eft-3::rfp* construct as used for the RNA injection experiments. The sequences targeted are listed in Table 1.

Detection of poly(UG)-tailed RNA detection in *P. pacificus*, *S. ratti* and *S. stercoralis* via [poly(UG) biased] RNA sequencing

Total RNA was extracted from mixed-stage cultures of *P. pacificus* (PS312) and free-living *S. ratti* (ED321) and *S. stercoralis* (PV001) animals as described above. The RNA samples were depleted from ribosomal RNA and poly(A) RNA using the *P. pacificus* (Ribo-Seq) riboPOOL (siTOOLs BIOTECH, dp-K006-000082) kit (for *P. pacificus*), the *S. ratti* (Ribo-Seq) riboPOOL (siTOOLs BIOTECH, dp-K006-000106) (for *S. ratti* and *S. stercoralis*), and the Poly A riboPOOL (siTOOLs BIOTECH, dp-K012-34) (all three samples) according to the manufacturer-provided protocols as follows. The rRNA depletion RP and poly A RNA depletion reagents were mixed 1:10 and 1μl of this mix was used in the hybridization step with total RNA (14μl of 2μg) and hybridizing buffer (RNase inhibitor was skipped). The mixture was

incubated at 68°C for 10mins and allowed to cool down slowly to 37°C. 80μl of prepared beads were mixed with the hybridized riboPOOL and total RNA and incubated at 37°C for 15mins, followed by an 50°C incubation for 5 minutes. The tube with the mix was placed on a magnetic rack for two minutes to pellet the beads and the supernatant with the rRNA and poly A-depleted RNA was kept. The last step was repeated with the supernatant to get rid of remaining tract amounts of beads. The RNA was purified using the clean-up beads purification provided with the kit as per the manufacturer's instructions.

The rRNA and poly A depleted RNAs were analyzed on an Agilent Bioanalyser 2100 along with an aliquot of the total RNA to assess the depletion quality. The *S. stercoralis* RNA sample contained some remaining ribosomal RNA compared with the other two. This might be because no rRNA species-specific kit is currently available for *S. stercoralis* and the one designed for *S. ratti* was not fully effective.

The RNAs were precipitated overnight using x3 RNA volumes of ice cold 100% EtOH, 10% of RNA volume of 3M NaOAc and 1μl of Glycol Blue. The pellets were washed twice with 70% EtOH, air dried and resuspended in 5μl of nuclease-free water.

To remove the 5' cap and generate 5' mono-phosphate RNAs, the RNAs were treated with CapClip Pyrophosphatase (CCP, Biozym) as follows: 2μl of x10 CCP buffer, 2.5μl (1 U/μl) of CCP enzyme (freshly diluted in water from the 5 U/μl stock), 10.5μl water and the 5μl of RNA from the previous step were mixed and incubated for 1hr at 37°C followed by a cleaning and concentration step using the Zymo Research RNA clean and concentrator-5 kit (R1013). The RNA was eluted in 6.5μl of water.

Ligation of the 5' adaptor was achieved by incubating 1μl of 5μM RA5 adaptor (see the primer list) with the RNA at 70°C for 2min and then transferred immediately to

ice. Then 0.8μl of x10 RNA ligase buffer (NEB, B0216S), 0.8μl of T4 RNA ligase 1 (NEB, M0204S) and 0.8μl of 10mM ATP were added and the mix was incubated at 37°C for 2hrs.

The 5' adaptor ligated RNA was reverse transcribed using a primer recognizing the poly(UG)-tail (7485): 1μl of the 4μM 7485 primer was mixed with 8μl of the RNA, incubated at 70°C for 2mins and immediately put on ice. Then 4μl of 5x 1st strand buffer, 4μl of 10mM dNTP, 1μl of 0.1M DTT, 1μl of superscript III (Invitrogen, 18080-044) and 1μl of water were added and incubated at 50°C for 1hr followed by heat inactivation at 70°C for 15mins.

Then the libraries were prepared using the Illumina TrueSeq kit (15016912). Based on a small-scale trial PCR, we decided to use 21, 25 and 24 amplification cycles for *S. stercoralis*, *S ratti* and *P. pacificus,* respectively. For each sample, three 50μl reactions were set up in parallel. The 3 reactions were pooled, 2μl of the *Strongyloides* and 3μl of the *P. pacificus* were run in a 1% agarose gel to check the quality. The pooled reactions were precipitated overnight using three volumes of 100% ice-cold EtOH. After the centrifugation, the pellets were washed twice with 70% EtOH, air dried and was resuspended in 25μl of water and 1μl was diluted 1:10 and analyzed on Qubit (Thermo Fisher) and an Agilent Bioanalyser 2100.

The libraries were fractionated/size selected using the BluePippin system (1.5% gels with internal marker) to obtain 2 libraries of the same sample with two size ranges: 300-600bp and 600-1500bp. 2.5nM of the cDNA library from each of the 3 species were pooled to result in 2 pools of the 2 library sizes. The libraries were submitted for Illumina sequencing on an Illumina Nexseq 2000 instrument.

Figure Legends

Fig. 1: Targeting *eud-1* and *dpy-1*.

A and B illustrate the expected phenotypes. A: mouth openings of a eurostomatous (Eu, wide mouth with two teeth) and a stenostomatous (St, narrow mouth with only one tooth) adult *P. pacificus* hermaphrodite. The images are reproduced from (Dardiry et al., 2023) under the CC BY 4.0 license. B: a wild type (Wt) and a dumpy (Dpy, genotype *dpy-1(sy304)*) adult *P. pacificus* hermaphrodite. The indicated RNA was injected into PS312 *P. pacificus* young adult hermaphrodites along with a plasmid encoding RFP under the control of the *eft-3* promoter as a marker for successful injection (co-injection marker). The entire progeny of injected animals that produced any RFP positive progeny was scored. On the Y-axis the % of phenotypically mutant (stenostomatous (St) in C and Dpy in D) progeny of the injected worms is indicated. Every dot represents the brood of one injected animal. The means plus/minus one standard deviation are indicated.

Fig. 2: Targeting the multi copy *daf-1::rfp* transgene in RS3832.

A: Illustration of the range of the phenotype observed. In the top and the bottom panels, one worm that is the progeny of a poly(UG)-RNA injected mother is shown above a control worm. The central panels show the red channel, the left panels show merged fluorescent and DIC images and the right panels show false colored images of the red channel, indicating signal intensity. The worms scored as "reduced RFP expression" were in the range from the one at the top to the one at the bottom panel. Two cells whose signal never disappeared completely are circled. B: The indicated RNA was injected into RS3832 *P. pacificus* young adult hermaphrodites along with a plasmid encoding GFP under the control of the *eft-3* promoter as a marker for successful injection (co-injection marker). The entire progeny of injected animals that

produced any GFP-positive progeny was scored when they were young adults. On the Y-axis, the % of worms with reduced RFP for each brood is given. Each dot represents the brood of one injected animal. The means plus/minus one standard deviation are indicated. Notice that the experiment was reproduced later multiple times with independently prepared poly(UG)-tailed and non-tailed RNAs in the context of the experiments shown in Fig. 4.

Fig. 3: Orthology relationship of *C. elegans* and *P. pacificus* RDE-1 (A) and RDE-3 (B).

Neighbor joining trees of *P. pacificus* and *C. elegans* RDE-1 (A) and RDE-3 (B) related genes. Bootstrap values based on 1000 replicates are shown next to the branches. The trees are drawn to scale in units of the number of amino acid substitutions per site. For details on the selection of genes included and for tree reconstruction, see Materials and Methods. Maximum Likelihood trees were also calculated and showed the same topology. GeneBank accession numbers followed by the gene names are given. All gene names other than RDE-1 and RDE-3 are as annotated in WormBase (for *C. elegans*) or Pristionchus.org (for *P. pacificus*) on March 24th 2025. *Ppa-rde-3* was annotated as *Ppa-mut-2* (in *C. elegans*, *mut-2* is a synonym for *rde-3*).

Fig. 4: Silencing is dependent on RDE-1

Young hermaphrodites of the indicated strains were injected with poly(UG)-tailed RNA (a-d) or non-tailed RNA (e,f) along with a plasmid encoding GFP under the control of the *eft-3* promoter as a marker for successful injection (co-injection marker). The entire progeny of injected animals that produced any GFP-positive progeny was

scored when they were young adults. On the Y-axis, the % of worms with reduced RFP for each brood is given. Each dot represents the brood of one animal in an experiment with poly(UG)-tailed RNA; each triangle represents the brood of one animal in an experiment with non-tailed RNA. The means plus/minus one standard deviation are indicated. Each panel was generated in a different injection session. For a (triple mutant), two injection sessions were done. Control experiments were done in every injection session. The RNAs were synthesized in different/multiple *in vitro* reactions from the ones used in Fig. 2.

Fig. 5: New poly(UG)-tailed RNAs are formed using endogenous and the injected RNA as substrate.

A: poly(UG)-tailed RNA was injected into 67 RS3832 *P. pacificus* young adult hermaphrodites. 24h after the injected worms had laid eggs, the worms were collected, pooled and RNA was extracted. The progeny of the injected worms was allowed to hatch and become 2nd and 3rd-stage larvae. Of those, all 608 larvae with clearly reduced RFP expression were picked, pooled and used for RNA extraction. An equal number of progenies from un-injected worms were treated in the same way. The RNAs were reverse transcribed with a poly(UG) recognizing primer, amplified according to the "nested PCR" protocol and cloned, as described in Materials and Methods. Clones representing independent reverse transcription events are shown. Only the part of the clone that is derived from the RNA and not from the primers is shown. Notice the two clones from the progeny that extend further 3' than the injected RNA (see text). B: poly(UG)-tailed RNA marked with two-point mutations was injected into 46 RS3832 *P. pacificus* young adult hermaphrodites. After the injected worms had laid eggs for 24 h, they were pooled and RNA was extracted and analyzed

as in A, except that the semi-nested PCR amplification was done using the primers 7363 (biases towards the detection towards the injected RNA) or 7364 (biases towards the detection towards the endogenous RNA) for half of the sample each. The knockdown effect was confirmed in the progeny of these worms. Co-injection marker was included in the injection mix for consistency with the other experiments, but it was not considered in this experiment.

Fig. 6: poly(UG) addition is dependent on RDE-3.

QA444 segregates *rde-3(yt61)* homozygous, heterozygous and Wt worms. RNA was isolated from 214 adult progenies of *rde-3(yt61)* homozygous mothers and from 214 adult progenies of Wt mothers RNA, and cDNA derived from poly(UG)-tailed RNA was cloned as described in Materials and methods. Clones showing 10 or more UG repeats are shown.

Suppl. Fig. 1 Pilot experiment: poly(UG) tailed RNA targeting *eud-1* at the indicated concentrations was injected into PS312 *P. pacificus* young adult hermaphrodites along with a plasmid encoding RFP under the control of the *eft-3* promoter as a marker for successful injection. The entire progeny of injected animals that produced any RFP positive progeny was scored. Since the injections had to be done sequentially, at every time worms were picked for injection, control worms were also picked from the same culture. Each dot represents the progeny of one mother. %ST: % stenostomatous individuals.

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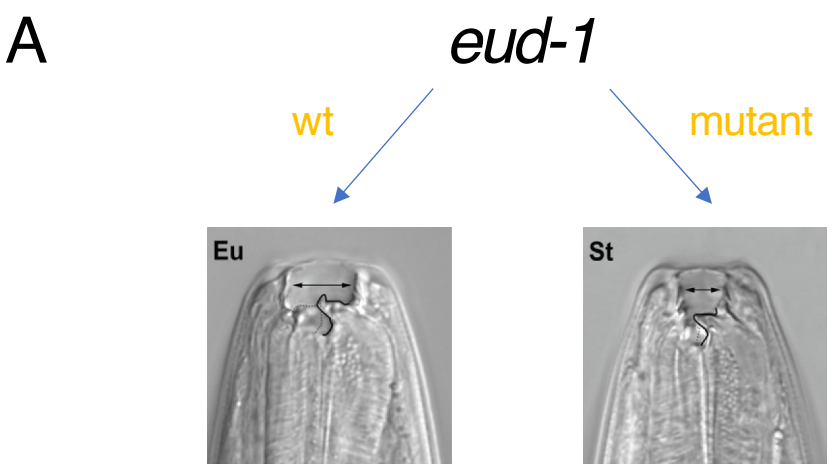
Figure 1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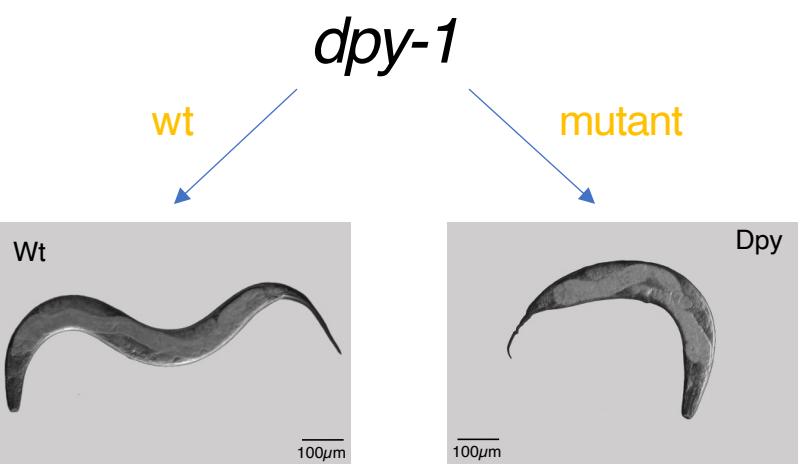
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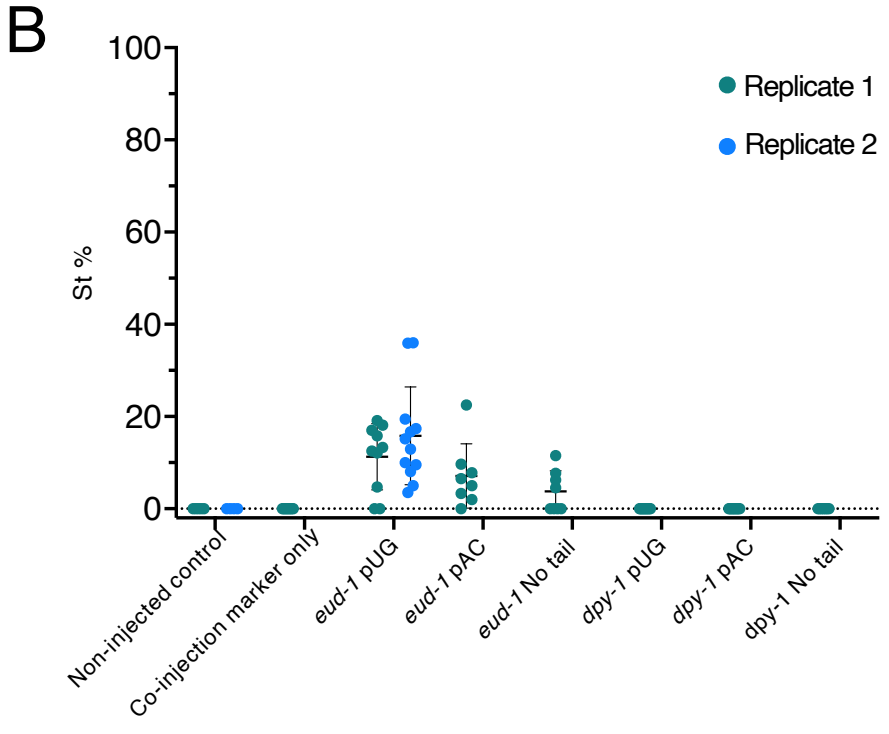
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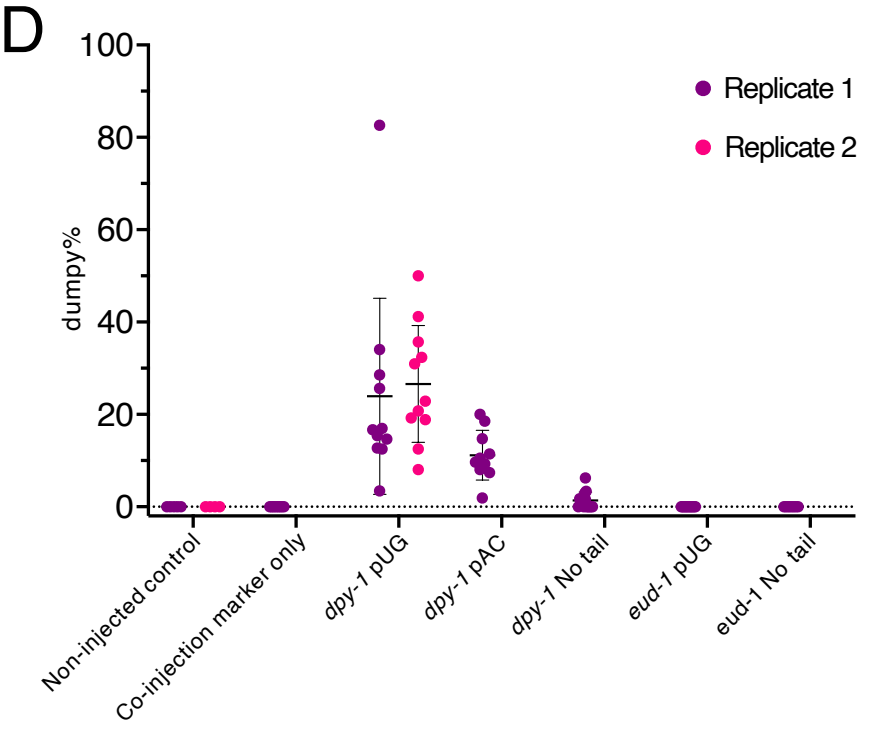
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Figure 2

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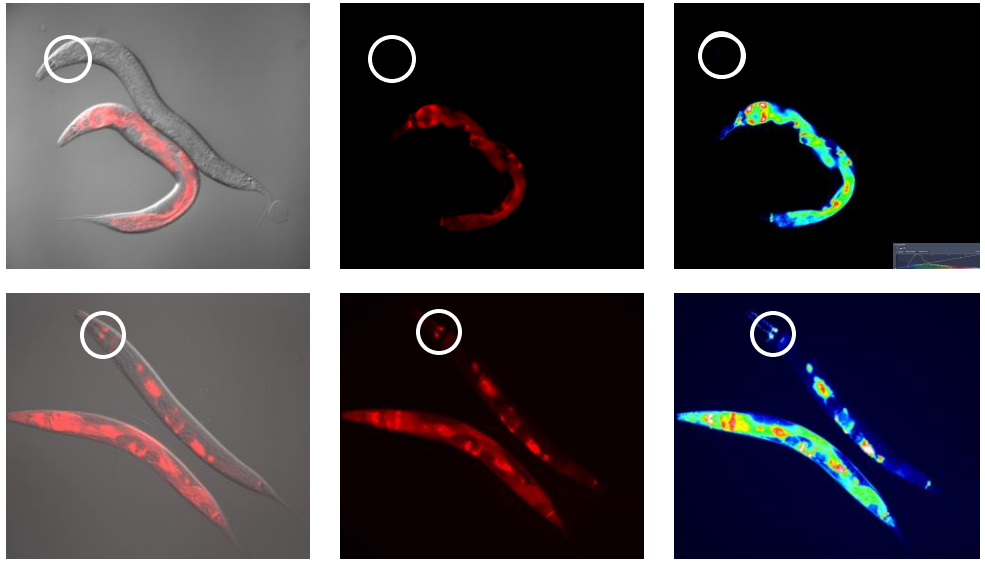

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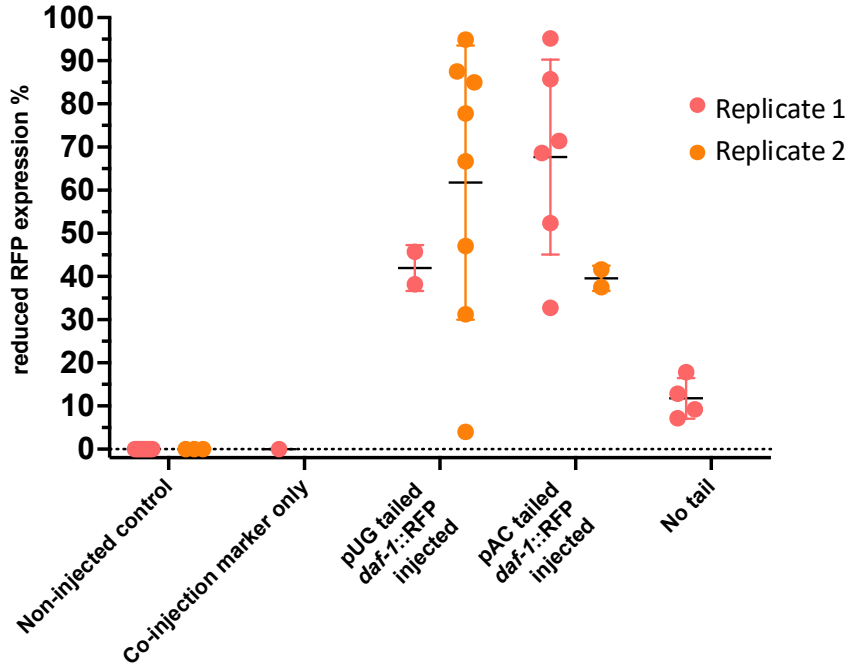
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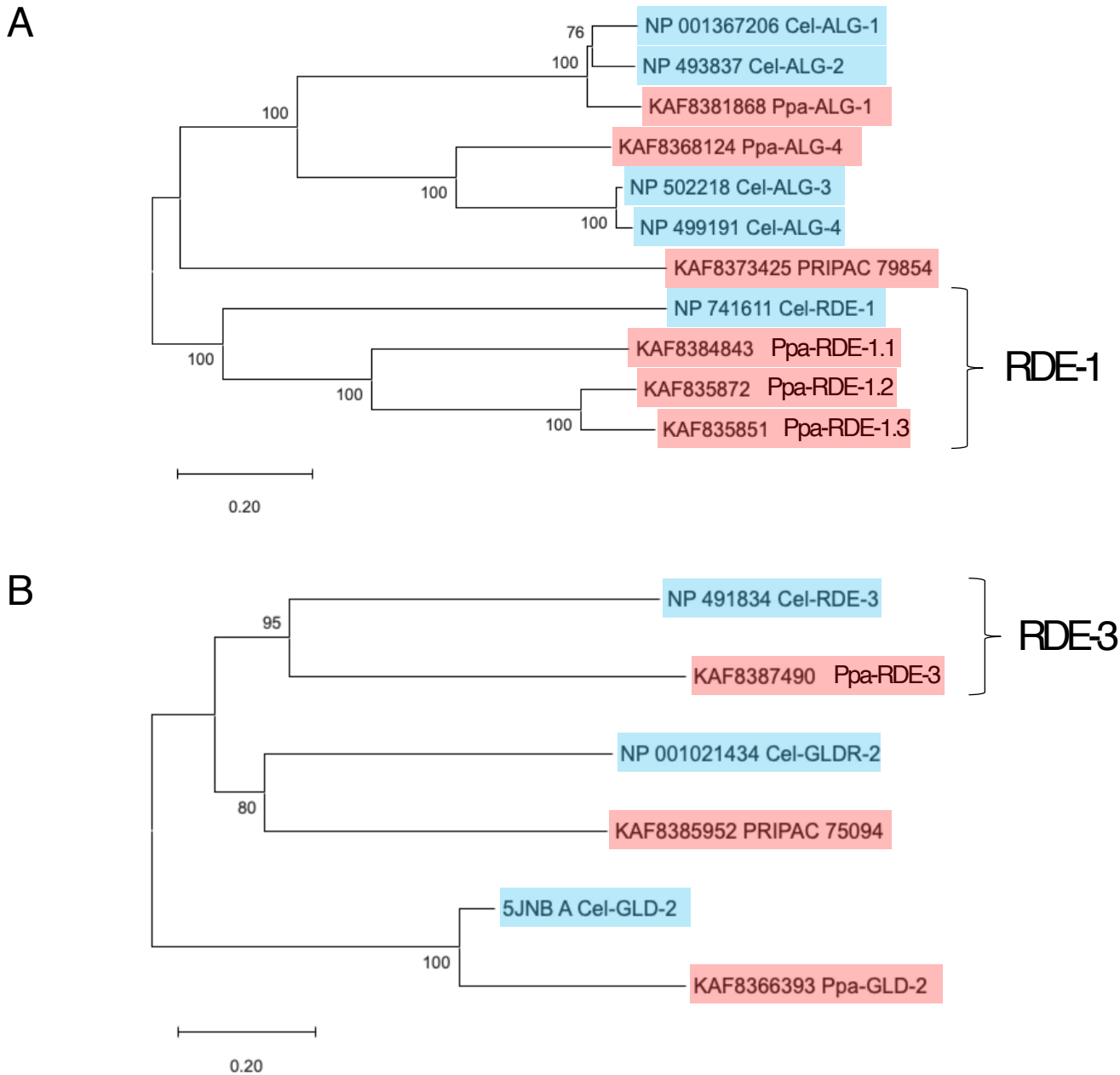
Figure 3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KAF8373425 PRIPAC 79854
NP 741611 Cel-RDE-1
KAF8384843 Ppa-RDE-1.1
KAF835872 Ppa-RDE-1.2
KAF835851 Ppa-RDE-1.3
RDE-1
76
100
100
100
100
100
100
100
0.20
B
NP 491834 Cel-RDE-3
KAF8387490 Ppa-RDE-3
NP 001021434 Cel-GLDR-2
KAF8385952 PRIPAC 75094
5JNB A Cel-GLD-2
KAF8366393 Ppa-GLD-2
RDE-3
95
80
100
0.20

Figure 4

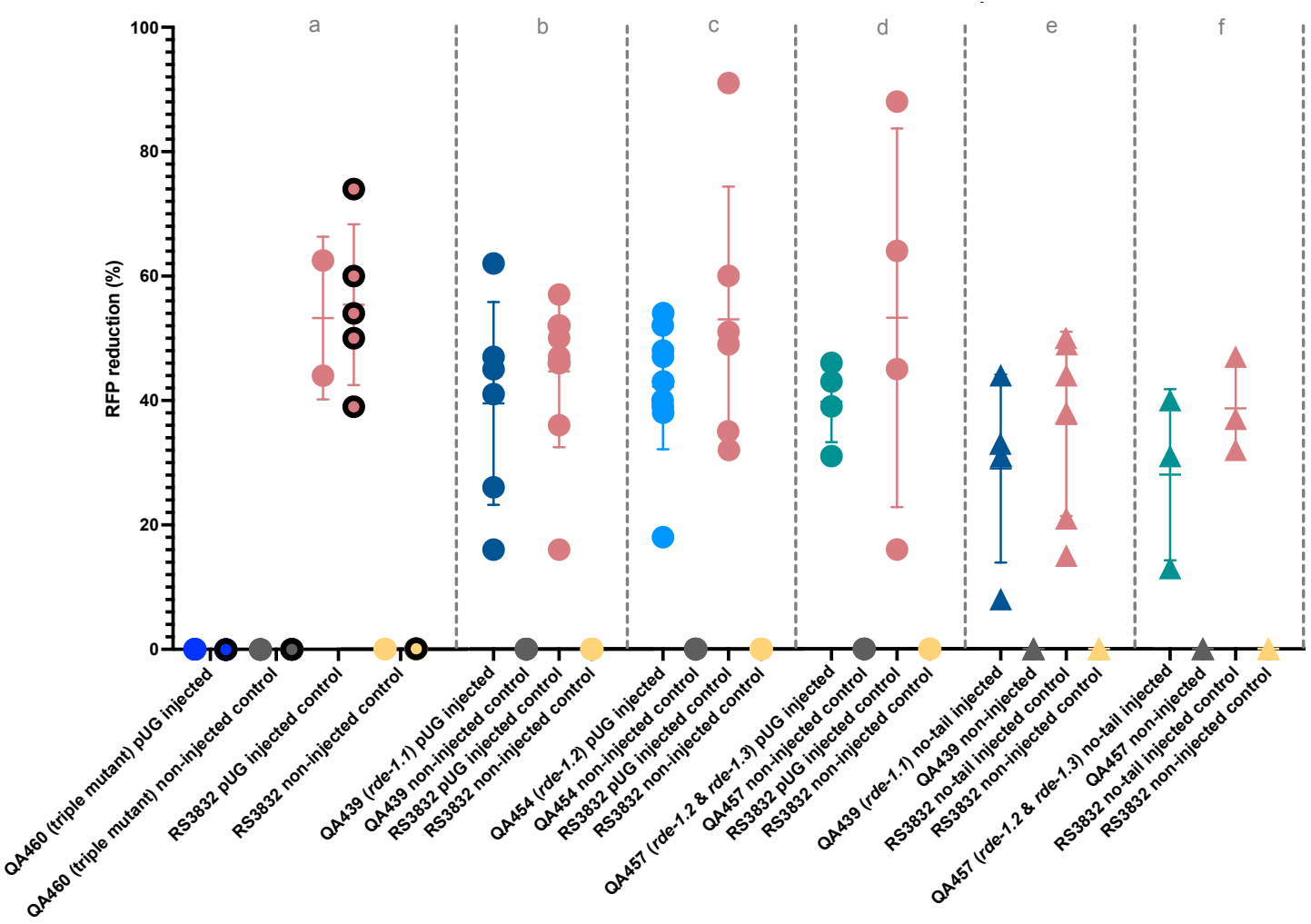
a
b
c
d
e
f
RFP reduction (%)
100
80
60
40
20
0
QA460 (triple mutant) pUG injected
QA460 (triple mutant) non-injected control
RS3832 pUG injected control
RS3832 non-injected control
QA439 (rde-1.1) pUG injected
QA439 non-injected control
RS3832 pUG injected control
RS3832 non-injected control
QA454 (rde-1.2) pUG injected
QA454 non-injected control
RS3832 pUG injected control
RS3832 non-injected control
QA457 (rde-1.2 & rde-1.3) pUG injected
QA457 non-injected control
RS3832 pUG injected control
RS3832 non-injected control
QA439 (rde-1.1) no-tail injected
QA439 non-injected
RS3832 no-tail injected control
RS3832 non-injected control
QA457 (rde-1.2 & rde-1.3) no-tail injected
QA457 non-injected
RS3832 no-tail injected control
RS3832 non-injected control

Figure 5

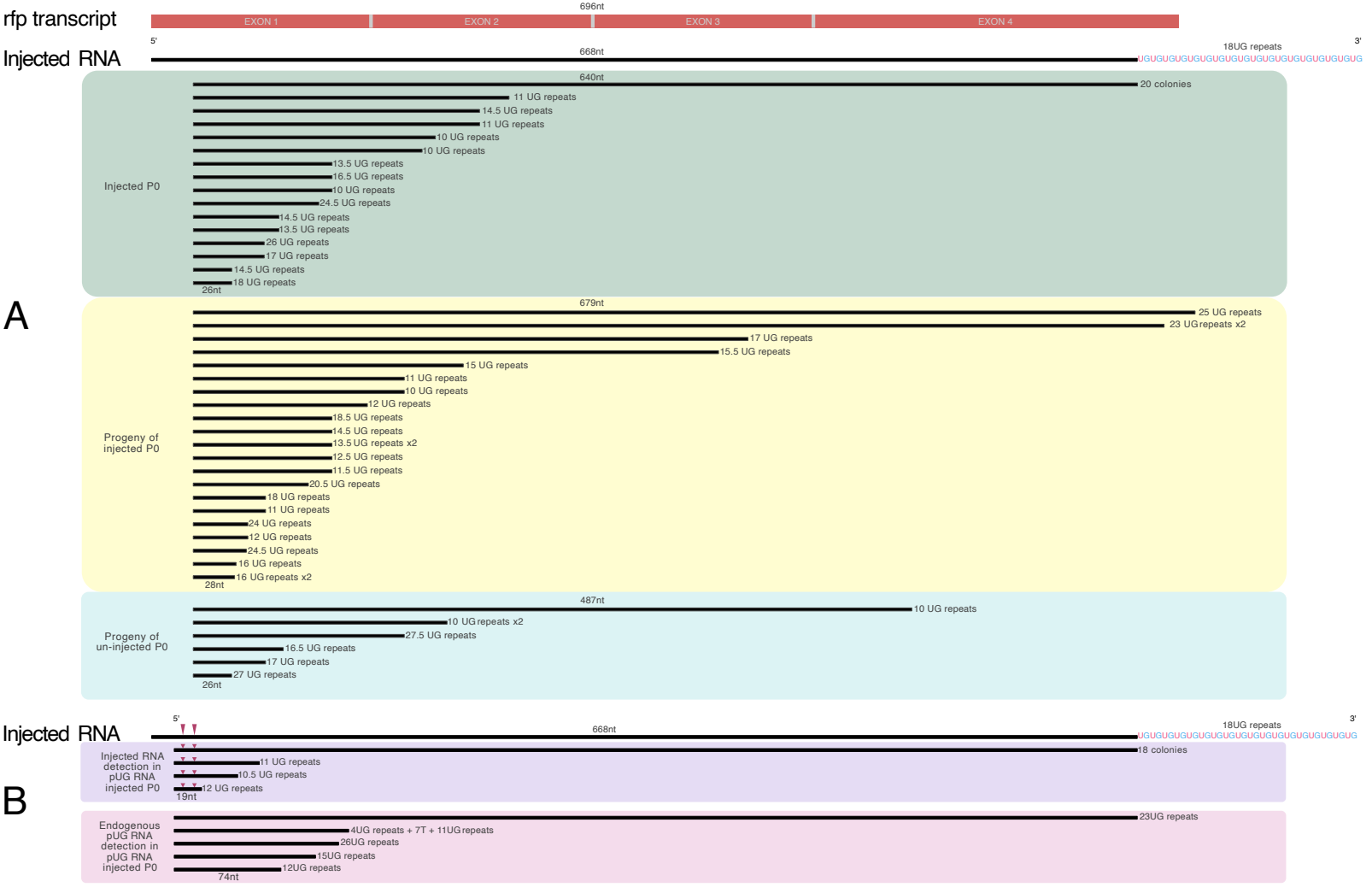
rfp transcript
696nt
EXON 1
EXON 2
EXON 3
EXON 4
Injected RNA
5'
668nt
18UG repeats
3'
UGUGUGUGUGUGUGUGUGUGUGUGUGUGUG
A
640nt
Injected P0
20 colonies
11 UG repeats
14.5 UG repeats
11 UG repeats
10 UG repeats
10 UG repeats
13.5 UG repeats
16.5 UG repeats
10 UG repeats
24.5 UG repeats
14.5 UG repeats
13.5 UG repeats
26 UG repeats
17 UG repeats
14.5 UG repeats
18 UG repeats
26nt
679nt
Progeny of injected P0
25 UG repeats
23 UG repeats x2
17 UG repeats
15.5 UG repeats
15 UG repeats
11 UG repeats
10 UG repeats
12 UG repeats
18.5 UG repeats
14.5 UG repeats
13.5 UG repeats x2
12.5 UG repeats
11.5 UG repeats
20.5 UG repeats
18 UG repeats
11 UG repeats
24 UG repeats
12 UG repeats
24.5 UG repeats
16 UG repeats
16 UG repeats x2
28nt
487nt
Progeny of un-injected P0
10 UG repeats
10 UG repeats x2
27.5 UG repeats
16.5 UG repeats
17 UG repeats
27 UG repeats
26nt
Injected RNA
5'
668nt
18UG repeats
3'
UGUGUGUGUGUGUGUGUGUGUGUGUGUGUG
B
Injected RNA detection in pUG RNA injected P0
18 colonies
11 UG repeats
10.5 UG repeats
12 UG repeats
19nt
Endogenous pUG RNA detection in pUG RNA injected P0
23UG repeats
4UG repeats + 7T + 11UG repeats
26UG repeats
15UG repeats
12UG repeats
74nt

Figure 6

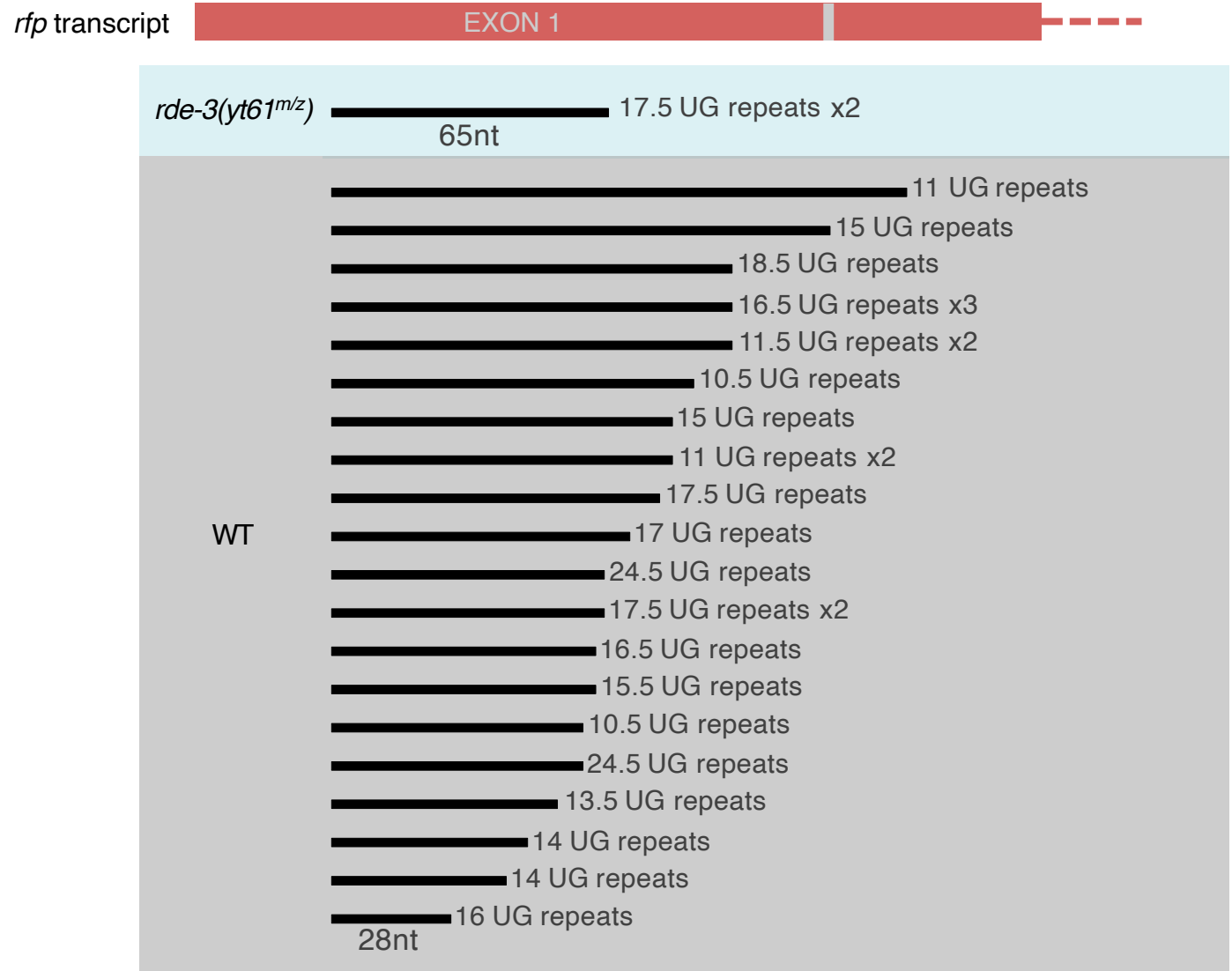
rfp transcript
EXON 1
rde-3(yt61^m/z)
17.5 UG repeats x2
65nt
WT
11 UG repeats
15 UG repeats
18.5 UG repeats
16.5 UG repeats x3
11.5 UG repeats x2
10.5 UG repeats
15 UG repeats
11 UG repeats x2
17.5 UG repeats
17 UG repeats
24.5 UG repeats
17.5 UG repeats x2
16.5 UG repeats
15.5 UG repeats
10.5 UG repeats
24.5 UG repeats
13.5 UG repeats
14 UG repeats
14 UG repeats
16 UG repeats
28nt

Table 1: CRISPR/Cas9 induced mutant alleles in *Ppa-rde-1.X* and *Ppa-rde-3*

gene model	gene name	CRSPR target sequence	allele number	molecular lesion[1]	Strain[2]
PPA12342	*Ppa-rde-3*	CCCTCTCAAGCTGGTTATTA	*yt58*	45 bp insertion + 5 bp deletion	QA441
PPA12342	*Ppa-rde-3*	,,	*yt59*	11 bp deletion	QA442
PPA12342	*Ppa-rde-3*	,,	*yt60*	3 bp deletion	QA443
PPA12342	*Ppa-rde-3*	,,	*yt61*	11 bp deletion	QA444
PPA12342	*Ppa-rde-3*	,,	*yt62*	7 bp deletion	QA445
PPA12342	*Ppa-rde-3*	,,	*yt63*	5 bp deletion	QA446
PPA12342	*Ppa-rde-3*	,,	*yt64*	1 bp insertion + 11 bp deletion	QA447
PPA37777	*Ppa-rde-1.1*	GGGTTTTGATGGTCTACTAC	*yt53*	288 bp deletion	QA436
PPA37777	*Ppa-rde-1.1*	,,	*yt54*	288 bp deletion + 402 bp insertion	QA437
PPA37777	*Ppa-rde-1.1*	,,	*yt55*	29 bp insertion	QA438
PPA37777	*Ppa-rde-1.1*	,,	*yt56*	11 bp deletion	QA439
PPA37777	*Ppa-rde-1.1*	,,	*yt57*	31 bp insertion	QA440
PPA00739	*Ppa-rde-1.2*	GTAGACAAGTGTAAATCCCG	*yt65*	11 bp deletion	QA454
PPA00739	*Ppa-rde-1.2*	,,	*yt66*	10 bp deletion	QA455
PPA00739	*Ppa-rde-1.2*	,,	*yt67*	27 bp deletion	QA456
PPA36770	*Ppa-rde-1.3*	AAGTTGAATGTCAAGCTGGG	*yt68*	30 bp insertion + 16 bp deletion	QA457
PPA36770	*Ppa-rde-1.3*	,,	*yt69*	3 bp insertion + 14 bp deletion	QA458
PPA36770	*Ppa-rde-1.3*	,,	*yt70*	22 bp deletion	QA459

[1]For the sequences see suppl Tab. 1; [2]for the full genotypes see Tab. 2.

Table 2: Full genotypes of *P. pacificus* strains used

Strain number	full genotype[1]
PS312	wt
RS3832	*tuEx333 [daf-1p::TurboRFP]*[1]
QA441	*Ppa-rde-3(yt58)/+; tuEx333 [daf-1p::TurboRFP]*
QA442	*Ppa-rde-3(yt59)/+; tuEx333 [daf-1p::TurboRFP]*
QA443	*Ppa-rde-3(yt60/+; tuEx333 [daf-1p::TurboRFP]*
QA444	*Ppa-rde-3(yt61)/+; tuEx333 [daf-1p::TurboRFP]*
QA445	*Ppa-rde-3(yt62)/+; tuEx333 [daf-1p::TurboRFP]*
QA446	*Ppa-rde-3(yt63)/+; tuEx333 [daf-1p::TurboRFP]*
QA447	*Ppa-rde-3(yt64)/+; tuEx333 [daf-1p::TurboRFP]*
QA436	*Ppa-rde-1.1(yt53); tuEx333 [daf-1p::TurboRFP]*
QA437	*Ppa-rde-1.1(yt54); tuEx333 [daf-1p::TurboRFP]*
QA438	*Ppa-rde-1.1(yt55); tuEx333 [daf-1p::TurboRFP]*
QA439	*Ppa-rde-1.1(yt56); tuEx333 [daf-1p::TurboRFP]*
QA440	*Ppa-rde-1.1(yt57); tuEx333 [daf-1p::TurboRFP]*
QA454	*Ppa-rde-1.2(yt65); tuEx333 [daf-1p::TurboRFP]*
QA455	*Ppa-rde-1.2(yt66); tuEx333 [daf-1p::TurboRFP]*
QA456	*Ppa-rde-1.2(yt67); tuEx333 [daf-1p::TurboRFP]*
QA457	*Ppa-rde-1.2(yt65) Ppa-rde-1.3(yt68); tuEx333 [daf-1p::TurboRFP]*
QA458	*Ppa-rde-1.2(yt65) Ppa-rde-1.3(yt69); tuEx333 [daf-1p::TurboRFP]*
QA459	*Ppa-rde-1.2(yt65) Ppa-rde-1.3(yt70); tuEx333 [daf-1p::TurboRFP]*
QA453	*Ppa-rde-3(yt61)/+*
QA460	*Ppa-rde-1.1(yt56); Ppa-rde-1.2(yt65) Ppa-rde-1.3(yt68); tuEx333 [daf-1p::TurboRFP]*

Although labelled as extrachromosomal array, the transgene *tuEx333* behaves like a chromosomal transgene (Mendelian segregation in crosses and 100% transmission when homozygous).