

Fig. 1. (A) Life cycle of *Strongyloides ratti*. Parasitic females produce eggs by parthenogenesis in the host's small intestine and the eggs are excreted into the environment in the faeces. Eggs develop into infective larvae by two alternative routes. In the homogonic route, eggs develop directly into infective larva. In the heterogonic route, eggs develop into free-living forms, reproducing sexually, after which the resultant eggs develop into infective larva. (B) Life cycle of *Strongyloides venezuelensis*. *S. venezuelensis* may lack the heterogonic developmental route in its life cycle.

(Brumpt, 1934; Wertheim and Lengy, 1964; Little, 1966; Hasegawa *et al.* 1988). *Strongyloides venezuelensis*, alongside *S. ratti*, is one of the most widely used laboratory models to study *Strongyloides* infection and mucosal immunity (Sato and Toma, 1990; El-Malky *et al.* 2013). Although both *S. ratti* and *S. venezuelensis* have rodents as hosts, the individual mechanisms by which they establish parasitism are thought to differ from each other. Phylogenetic analysis with ribosomal RNA gene suggested that *S. venezuelensis* is not as closely related to *S. ratti* as to *S. papillosus* and the primate parasite *Strongyloides fuelleborni* (Dorris *et al.* 2002). The migration patterns of the two rodent parasites in the host (Takamura, 1995), as well as mechanisms of host immunomodulation for successful parasitism, differ from each other as well (Wilkes *et al.* 2007; Matsumoto *et al.* 2013). More interestingly, observation of free-living adults is less common in *S. venezuelensis* as compared with *S. ratti* (Hasegawa *et al.* 1988; Harvey *et al.* 2000), suggesting that reproduction strategies used by the two species may also be different. Thus, the parasitic abilities of the two species may have evolved independently from each other, and a comparison between the two may provide invaluable insights into understanding the parasitic mechanism and its evolution in *Strongyloides* nematodes.

Though *S. venezuelensis* is a widely used laboratory model, some aspects of its basic biology remain unclear. In this report, we re-examined the phylogenetic position of *S. venezuelensis* within the genus *Strongyloides*, using 18S rRNA and the D3 expansion segment of 28S rRNA genes. We also examined the developmental routes of progeny from the parasitic females, chromosome behaviour in the germ cells and

early embryos in *S. venezuelensis*. Our analyses suggest that acquisition of rodent parasitism occurred independently in *S. venezuelensis* and *S. ratti*.

MATERIALS AND METHODS

Culturing and handling nematodes

Strongyloides venezuelensis HH1 and *S. ratti* TDI isolates were used in this study. They were isolated from Okinawa, Japan (Hasegawa *et al.* 1988) and Tokyo, Japan, respectively, and maintained in the Parasitology laboratory of the University of Miyazaki, using male Wistar rats. Infectious aliquots were prepared by faecal culture using filter paper at 27 °C for 2 days and 5 days for *S. venezuelensis* and *S. ratti*, respectively (Sato and Toma, 1990). The nematodes were washed three times in distilled water, and administered by subcutaneous injection. *Strongyloides papillosus* iL3 was supplied from Dr Ayako Yoshida of University of Miyazaki. *Parastromyloides trichosuri* DNA (strain KNP from Warwick Grant's lab) was supplied by Berriman lab from Wellcome Trust Sanger Institute, UK. *Strongyloides stercoralis* was collected in Yangon, Myanmar.

PCR conditions and DNA sequencing

Nearly full-length 18S ribosomal DNA (rDNA) was amplified with primers 988F–1912R and 1813F–2646R from a lysate of a single nematode as described previously (Holterman *et al.* 2006). These primers amplify 1652 bp out of 1754 bp of full-length 18S rDNA of *C. elegans* (NR_000053.1). The D3 region of 28S rDNA was amplified using primers D3A–D3B

(Nunn *et al.* 1996). PCR amplifications were carried out in 30 μ L reaction mixtures containing 15 μ L GoTaq Green Master Mix (Promega), 0.5 μ M of each primer, and 1 μ L of appropriately diluted nematode lysate under thermal-cycling conditions of 94 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min. PCR products were purified before sequencing using a MinElute 96 UF PCR purification plate (QIAGEN). DNA sequencing was performed using the BigDye Terminator 3.1 kit and ABI PRISM 3700 or 3130 Genetic Analyzer (Applied Biosystems).

Phylogenetic analyses

Nearly full-length 18S rRNA gene and D3 expansion segments of 28S rRNA gene of *Strongyloides* species were used for phylogenetic reconstruction. *Parastrongyloides trichosuri* was used as an outgroup taxon. The newly obtained and published sequences for each gene were aligned using MAFFT (Kato *et al.* 2002) with iterative refinement method (FFT-NS-i). Both datasets were concatenated using Concatenator v.1.1.0. (Pina-Martins and Paulo, 2008). Phylogenetic analyses of the sequence dataset were performed with maximum likelihood (ML) using PAUP*4b10 (Wilgenbusch and Swofford, 2003) and Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). The best-fitted model of DNA evolution was obtained using ModelTest v.2 (Darriba *et al.* 2012) with the Akaike Information Criterion (AIC). The Akaike-supported model, base frequency, proportion of invariable sites, gamma distribution shape parameters and substitution rates in the AIC were then used in phylogenetic analyses. BI analysis under the TVM+G model for D3 expansion segment of 28S and the TVM+I+G model for 18S rDNA was initiated with a random starting tree and run with four Metropolis-coupled Markov chain Monte Carlo (MCMC) for 1×10^6 generations in the combined dataset. The MCMC were sampled every 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority rule consensus tree. Trees were visualized using TreeView (Page, 1996). In ML analysis the estimation of the support for each node was obtained by bootstrap analysis with 1000 heuristic search replicates using the previously obtained models. Posterior probabilities and bootstrap support are given on respective clades.

Free-living occurrence observation

Three rats were infected with 500 iL3s of *S. venezuelensis* or *S. ratti*. Faeces samples from

each rat were collected at 8, 12, 14, 16 and 24 days post-infection (d.p.i.) for *S. venezuelensis* and at 8 d.p.i. for *S. ratti*. Faeces (approx. 0.5 g) were cultured on a 2% (w/v) agar plate at various temperatures (19, 25, or 30 °C) for 3 days and free-living male and female nematodes were counted. Three agar plates for each day and each condition were used. A portion of the faecal samples (*c.* 1.5 g) was diluted in distilled water to determine the eggs/larvae per gram (epg/lpg) of faeces. All statistical analyses were performed using the software package R version 2.15.2 (<http://www.r-project.org>).

Early embryogenesis

Parasitic females were obtained from rat intestine at 5–10 d.p.i. Fresh parasites were transferred to a pre-warmed (37 °C) agarose pad (4% (w/v) agarose) prepared on a microscope slide (Shaham, 2006) and covered with a silicon grease-rimmed cover slip and viewed under a Nomarski microscope (IX71, Olympus, Japan). All procedures involving the microscopic observations were performed at 37 °C.

DAPI staining/microscopy

Parasitic females were fixed with ice-cold methanol and stained with DAPI as previously described (Nemetschke *et al.* 2010). To collect the eggs, parasitic females were allowed to lay eggs in PBS, the eggs were then squashed and stained as previously described with the exception of using methanol instead of acetic acid for fixation (Albertson *et al.* 1979). Microscopic observations were carried out using a confocal laser scanning microscope (LSM700, Zeiss).

RESULTS

Phylogenetic position of *S. venezuelensis*

In order to determine the phylogenetic position of *S. venezuelensis* in the genus, we used nearly full-length 18S rRNA and D3 expansion segments of 28S rRNA genes. We sequenced these regions in *S. venezuelensis*, *S. papillosus*, *S. stercoralis*, *S. ratti* and *P. trichosuri* for phylogenetic analysis. 18S sequences from other *Strongyloides* species obtained from the public database were also included in the analysis. Our phylogenetic tree using *P. trichosuri* as an outgroup indicated that the *Strongyloides* species can be divided into two clades (Fig. 2): one clade comprising of *S. papillosus*, *S. fuelleborni*, *S. venezuelensis*, *Strongyloides callosciureus*, *Strongyloides robustus* and *Strongyloides cebus*, and the other including *S. stercoralis*, *Strongyloides procyonis* and *S. ratti*. Within the former clade, *S. venezuelensis* was further clustered into a subclade with *S. papillosus* and *S. fuelleborni*.

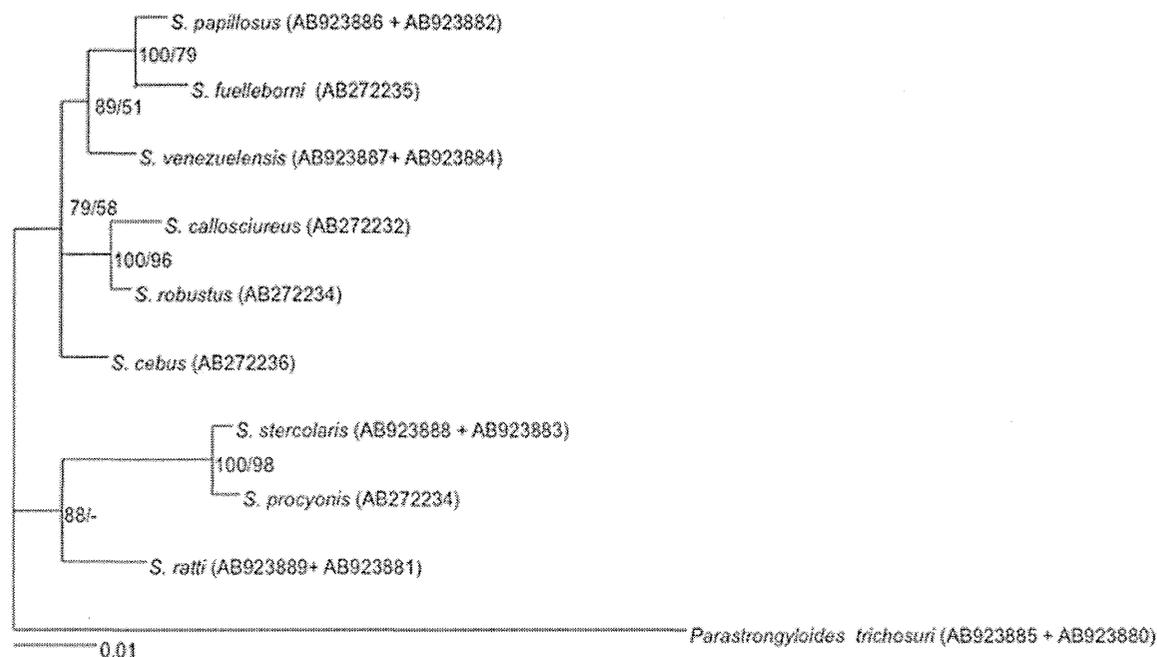


Fig. 2. The molecular phylogenetic relationship between *Strongyloides* species inferred from concatenated nearly full-length 18S rRNA gene (1640 positions from edited alignment) and D3 expansion segments of 28S rRNA gene (289 positions from edited alignment). TVM+G and TVM+I+G were used for D3 expansion segment of 28S and 18S, respectively. Posterior probabilities (first number in the node label) more than 65% are given for appropriate clades; bootstrap values greater than 50% are given on appropriate clades in ML analysis (second number in the node label).

Occurrences of free-living adults in *S. venezuelensis*

As the ratio of free-living females/males *vs* females developing homogonically into iL3s is influenced by several factors, including host immunity and other environmental conditions (temperature, pH etc.), in other *Strongyloides* species (Arizono, 1976; Moncol and Triantaphyllou, 1978; Nwaorgu, 1983; Viney, 1996; Harvey *et al.* 2000; Minato *et al.* 2008; Sakamoto and Uga, 2013), we used two parameters (days post infection and faeces incubation temperature) to examine the occurrence of free-living nematodes (FLNs) in *S. venezuelensis*.

In a total of 162 attempts, comprising of six d.p.i.s (8, 12, 14, 16, 20 and 24) and three temperature conditions (19, 25 and 30 °C), each with three rats and triplicate, we observed FLNs of *S. venezuelensis* in only 19 cases. As this frequency is much lower than that reported in *S. ratti* (Harvey *et al.* 2000; Minato *et al.* 2008; Sakamoto and Uga, 2013), we validated our method using *S. ratti* in place of *S. venezuelensis*. In the *S. ratti* experiment we observed FLNs in all culture plates tested (Table S1) and the numbers of FLNs (8.32 to 17.4 FLNs per 1000 eggs) were similar to those reported previously (Minato *et al.* 2008). Therefore we confirmed that this low frequency (19 out of 162 cases) was not due to errors in handling of the samples. Of note is that in some culture plates (52 cases) we did not observe the homogonically developed nematodes (iL3s) although the faeces

contained a sufficient number of eggs, which may suggest that the development of *S. venezuelensis* is more sensitive to environmental conditions than that of *S. ratti*. Furthermore, free-living males were never detected in *S. venezuelensis* (Table 1) while approximately half of FLNs in the *S. ratti* experiment were males (Table S1). Consequently, no hatching was observed in eggs derived from the free-living females (data not shown).

In the 19 plates with *S. venezuelensis* FLNs, 7 plates (17 FLNs in total) were from 25 °C culture and 12 plates (46 FLNs) were from 30 °C culture (Table 1). We did not observe any FLNs in 19 °C cultures (Table 1).

We observed *S. venezuelensis* FLNs in 1, 3, 4, 3, 2, and 2 cases at 8, 12, 14, 16, 20 and 24 d.p.i., respectively (Fig. 3) and the number of FLNs on each d.p.i. was 3, 6, 6, 43, 4, and 4, respectively. Although the number of eggs per gram of faeces (epg) decreased as the d.p.i. increased (Fig. 3), the number of instances that FLNs were observed did not change significantly (Generalized Linear Model with binomial error distribution: $df = 52$, $P > 0.9$). Then, we sought to investigate the relationship between epg and occurrence of FLNs. Figure S1 shows a plot of epg and number of FLNs per gram of faeces. The trend observed was that fewer epg showed more numbers of FLNs per egg (Fig. S1).

The highest number of FLNs was obtained with faeces collected at 16 d.p.i. and cultured at 30 °C

Table 1. Total number of free living nematodes observed in *S. venezuelensis* faeces samples

	19 °C	25 °C	30 °C
Free-living female	0 (0/54)	17 (7/54)	46 (12/54)
Free-living male	0 (0/54)	0 (0/54)	0 (0/54)

Fifty-four culture plates were used at each temperature. Numbers in parentheses represent numbers of incidents of free-living observation (i.e. 17(7/54) indicates a total number of 17 free-living nematodes found in 7 out of 54 culture plates).

(34 FLNs, 6.58 per 1000 eggs). However this was an outlier as the other two plates under the same conditions (same rat, d.p.i. and temperature) showed much lower numbers of FLNs (0.24 or 0 per 1000 eggs).

Germ cells in the parasitic female

Strongyloides venezuelensis parasitic females have two elongated (didelphic) gonads extending from the vulva to the head or the tail directions, reflexed around the beginning of the intestine or anus, continuing around the vulva (Little, 1966) (Fig. 4A, B). Egg development and embryogenesis progress as stages move along the tract from the germinal tissue to the vulva. Both gonads spiral about themselves around the intestine (Fig. 4A, B, E). As with many nematodes the distal part of the germ line was a syncytium; nuclei were located at the inner surface of the germ line (Fig. 4C–E). Chromosomes in all nuclei in the distal region dispersed peripherally, and nuclei were condensed in the 'germinal zone' (Fig. 4F). As nuclei moved away from the germinal zone they were packed one by one into a cell in the 'growth zone' and became oocytes (Fig. 4G). Germinal vesicles appeared and chromosomes became condensed during the oocyte movement towards the proximal region, and they seemed to be arrested at this stage until they passed through the oviduct (Fig. 4G). We detected four chromosomes and this state could be prometaphase of the 'maturation division' (see below). We did not identify any sperm or sperm nuclei in the gonad in either bright-field or DAPI-stained observations. From these results, we conclude that parasitic females of *S. venezuelensis* reproduce parthenogenetically.

Early embryogenesis of eggs from the parasitic female

To see if the embryo starts development without fertilization, we observed oocyte maturation and early embryogenesis in the parasitic female sequentially with light microscopy. After the mature oocyte passed through the oviduct into the uterus, the eggshell was formed (Fig. 5A). The embryo shape was oval and the long axis of the embryo in the uterus

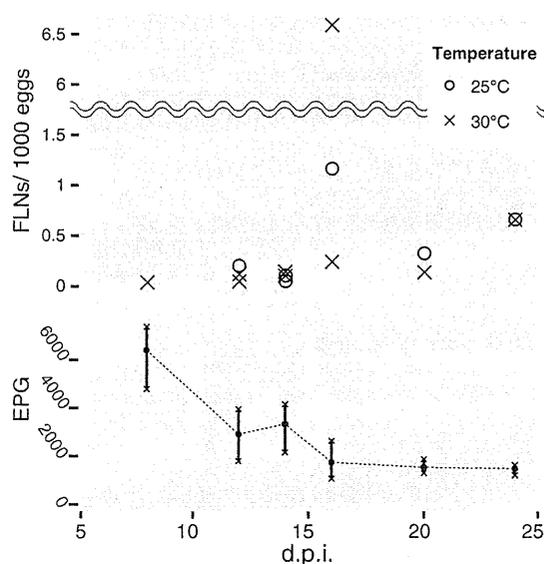


Fig. 3. (Upper) Free-living occurrences in *S. venezuelensis*. Nineteen positive cultures (in which free-living nematodes were found) out of 162 were plotted by number of free-living nematodes (FLNs) per 1000 eggs and day post infection (d.p.i.). Faeces were cultured at 19, 25 or 30 °C. No free-living nematodes were observed in 19 °C cultures. (Bottom) EPG change by d.p.i. The black dot represents the mean and crosses represent the highest and the lowest in each d.p.i.: $n = 3$.

was in parallel to the anterior-posterior axis of the mother. The germinal vesicle disappeared, and then only one pronucleus appeared at the lagging side-pole of the embryo (Fig. 5A, B). At this stage a protruded polar body-like structure was observed adjacent to the pronucleus (Fig. 5B). Pseudocleavage furrow and cytoplasmic streaming were observed (Fig. 5C), the pronucleus moved towards the middle of the cell (Fig. 5D) and its membrane broke down (Fig. 5E, F). Subsequently the embryo divided to form the two-cell stage (Fig. 5G, H) and the four-cell stage (Fig. 5I). All embryos ($n = 7$) observed in these experiments developed to first-stage larvae and hatched successfully (not shown), suggesting that the process observed was normal development.

Chromosome structure and behaviour during early embryogenesis

To clarify the mechanism by which ploidy is maintained, we observed chromosome structure and behaviour during early embryogenesis by DAPI staining. Developmental stages of the fixed and stained embryos were decided by comparison to the living embryos observed above. After passing through the oviduct into the uterus, the oocyte resumed mitotic cell division (Fig. 6A). This division always occurred asymmetrically at the lagging side-pole of the embryo (Fig. 6A) to produce one polar body. Polar bodies stained clearly as blue dots, but often disappeared

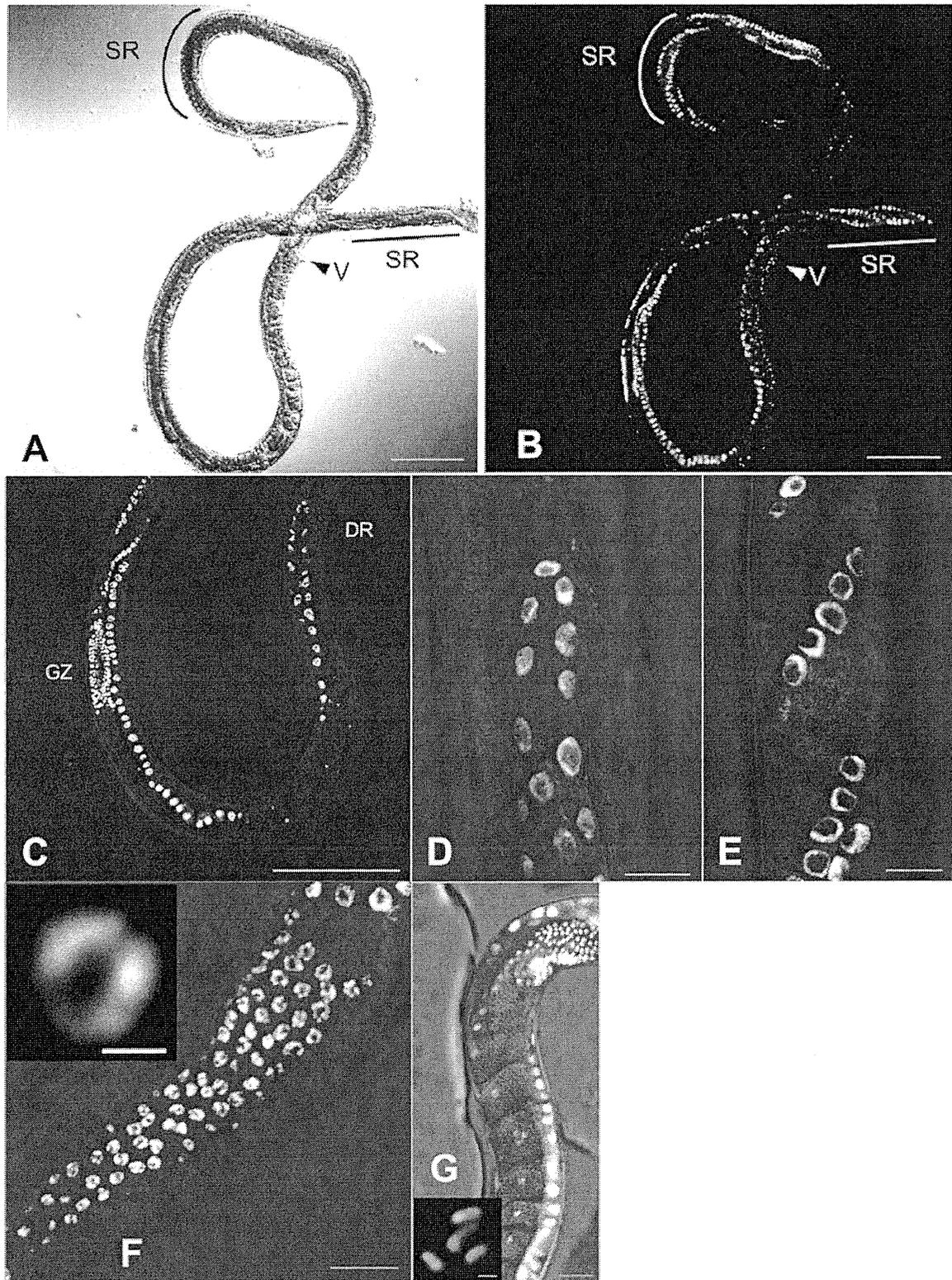


Fig. 4. Germ cells in a parasitic female of *S. venezuelensis*. (A, B) Whole body of a parasitic female under bright field and DAPI fluorescence microscopy. V, vulva; SR, spiralled region; (C) Distal side of a gonad. DR: distal region GZ: germinal zone; (D) Oogonium in distal end of a gonad; (E) Oogonium in spiralled region of a gonad; (F) Condensed nucleus in the germinal zone; (G) Condensed chromosomes in the growth zone. (Scale bars: A, B, C = 100 μm ; D, E, F = 10 μm ; G = 20 μm ; boxes in F, G = 1 μm).

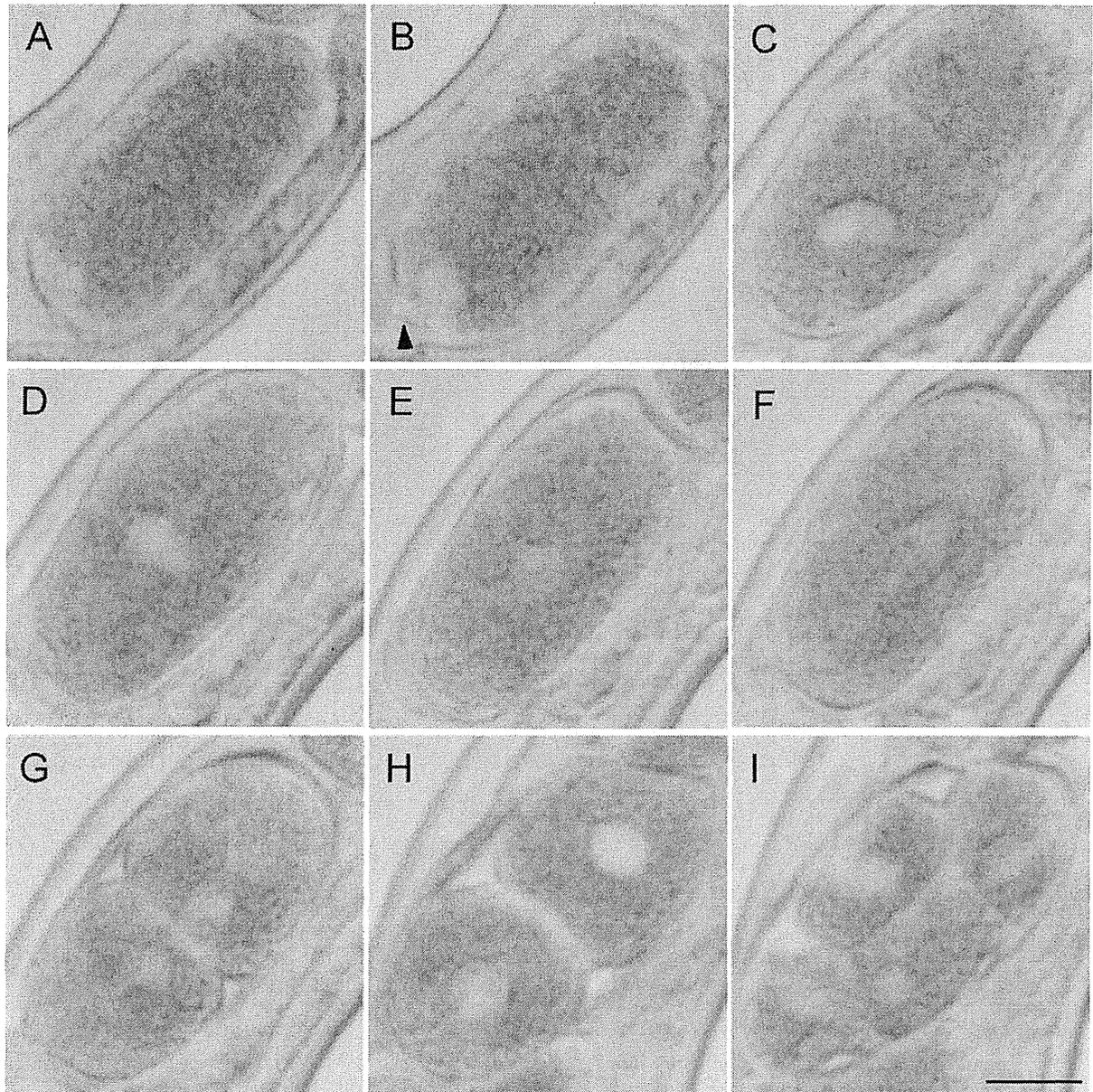


Fig. 5. Early embryogenesis in a live *S. venezuelensis* parasitic female. The leading edge of the embryo is arranged to be upper right. (A) An embryo that has passed through the oviduct; (B) An embryo under maturation division; (C) Pseudocleavage with one pronucleus; (D) Nucleus moving to the centre of the cell; (E) Nucleus dispersed at the centre of the cell; (F, G, H) Cell division to form a two-cell embryo; (I) Four-cell embryo (Arrow head indicates a polar body, Scale bar = 10 μ m). Time frame of the development is shown in Table S2.

as embryogenesis progressed. One pronucleus was reconstructed with vesicle (Fig. 6B), moved to the centre, and chromosomes were visible in the prophase stage (Fig. 6C). Chromatids fused and aligned along the metaphase plate (Fig. 6D) and migrated to each pole (Fig. 6E) subsequently forming two-cell stage embryos (Fig. 6F). The cleavage occurred asymmetrically with a bigger blastomere in the leading pole than the lagging one, which is contrary to the observations in *Caenorhabditis elegans* (Wallenfang and Seydoux, 2000) and other *Caenorhabditis* nematodes (Brauchle *et al.* 2009). Most eggs were laid as two-cell stage embryos. Then, we collected the laid

eggs, squashed and stained with DAPI. Four chromosomes, two longer than the others, were observed in prophase cells in the four-cell stage embryos (Fig. 6G, H), suggesting that the karyotype of *S. venezuelensis* is $2n = 4$. We confirmed in nine eggs, all of which had four chromosomes, and no specimens showed different compositions of chromosomes.

DISCUSSION

Strongyloides spp. are unique among parasitic nematodes in having both parasitic and free-living stages

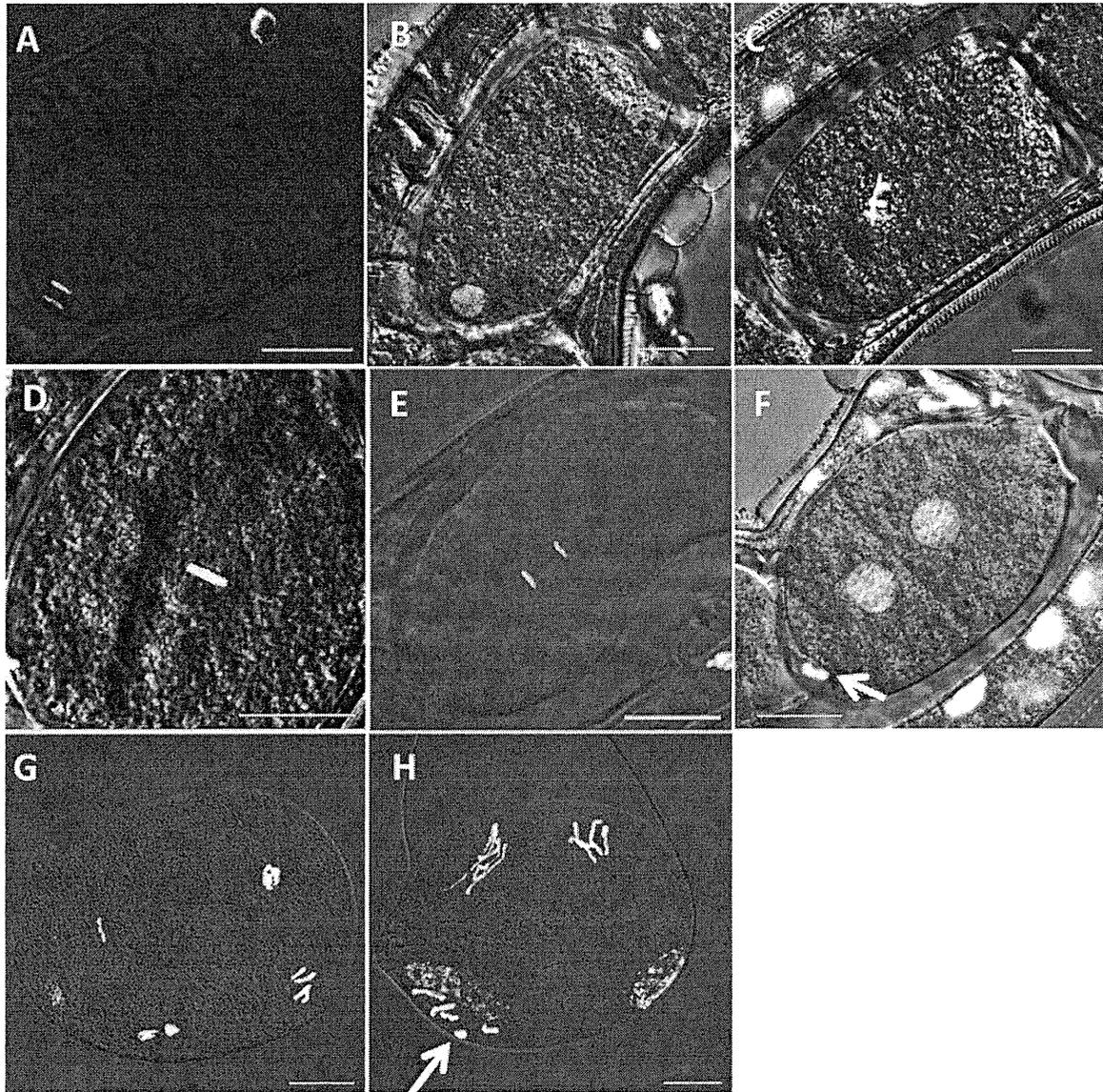


Fig. 6. Chromosome behaviour after passing through oviduct into the uterus. The leading edge of the embryo is arranged to be upper right. (A) Anaphase of maturation division; (B) A pronucleus reconstructed after maturation division; (C) A pronucleus moves to the centre and chromosomes are formed; (D) Metaphase of the first embryonic cell division; (E) Anaphase of the first embryonic cell division; (F) Telophase of the first embryonic cell division; (G, H) Four cell embryos. Newly laid eggs were squashed and stained for the observation. (Arrows indicate polar bodies. All scale bars = 10 μ m).

in their life cycle. Since the parasitic females produce eggs by parthenogenesis, the free-living stage is the only occasion for them to exchange genetic materials with other individuals. Eggs that are parthenogenetically produced by the parasitic females can develop into three morphs: free-living males, free-living females or iL3. In *S. ratti* the frequency of free-living development is as high as 60% (Minato *et al.* 2008), which suggests frequent exchanges of genetic materials take place in this species. In this study we observed a much lower frequency of free-living development in *S. venezuelensis* than *S. ratti*. Furthermore, we did not find any free-living males. This suggests that *S. venezuelensis*

rarely, or possibly never, exchanges genetic materials with other individuals in its life cycle. It is still possible, however, that there are unknown triggers to stimulate the nematode to have more free-living males and enable them to perform genetic exchanges. This could be an artificial loss of ability due to the stress of prolonged maintenance in laboratory, though absence of free-living males was reported previously in a recent field isolate of *S. venezuelensis* (Hasegawa *et al.* 1988). It would be of value to investigate free-living occurrence rates in other strains or wild isolates to confirm if this is a general characteristic of the species or the observation in this study is an exceptional case.

We noted that the number of free-living females increased with days after infection, although it was not clear which parameter was more influential, days post infection (i.e. ageing of worms and effect of host immunity) or dispersed density of eggs in the faeces. We also observed a higher number of free-living females in 30 and 25 °C cultures than at 19 °C. A similar trend for temperature conditions was also observed with other *Strongyloides* species (Arizono, 1976; Nwaorgu, 1983; Minato *et al.* 2008; Sakamoto and Uga, 2013).

We observed four chromosomes in *S. venezuelensis* somatic cells, suggesting *S. venezuelensis* has a karyotype of $2n=4$. It is known that *S. ratti* has three pairs of chromosomes ($2n=6$) and one of them is a sex chromosome. On the other hand, *S. papillosus* females have two pairs of chromosomes ($2n=4$). The lengths of the two are significantly different and the longer one is suggested to be the result of a fusion of a sex chromosome with one of the autosomes of $2n=6$ (Triantaphyllou and Moncol, 1977). The number of chromosomes in *S. venezuelensis* could also result from such a fusion. However, the lengths of the two pairs of the four *S. venezuelensis* chromosomes did not differ from each other to the extent seen in *S. papillosus* (Nemetschke *et al.* 2010). It would be very interesting to correlate the evolutionary history of reproduction in *Strongyloides* species with their chromosome structures.

Nemetschke *et al.* showed that *S. papillosus* employs chromatin diminution to exclude chromosome regions corresponding to the *S. ratti* sex chromosome in males (Albertson *et al.* 1979; Nemetschke *et al.* 2010). Although *S. venezuelensis* has the same number of chromosomes as *S. papillosus* and chromosome diminution could generate males in this species, we did not observe such events or any different chromosome composition in this study. This correlates with the absence of males in *S. venezuelensis*, although the basis for the lack of chromatin diminution is unclear: it could be that we were unable to generate *S. venezuelensis* males via this procedure or that males were not observed because *S. venezuelensis* is not capable of chromatin diminution.

Because embryogenesis occurred without fertilization and the chromosome number in mature oocytes, which are located proximal to the oviduct, was the same as that of the somatic cells it is likely the *S. venezuelensis* reproduces via mitotic parthenogenesis. Even without sperm stimulation, an embryo of *S. venezuelensis* had a polarity and produced the polar body on the lagging pole of the embryo. This orientation is opposite from *C. elegans* (Wallenfang and Seydoux, 2000). Parasitic stages of *Strongyloides* species including *S. ratti* or *S. papillosus* have been reported to have only female sex and reproduce by mitotic parthenogenesis (Zaffagnini, 1973; Triantaphyllou and Moncol, 1977; Viney, 1994). Our observation is consistent with these reports.

In order to confirm this, molecular studies would be useful by checking the progenies have the same genotype (clones) or have genetic variations due to cross-overs of sister chromosomes.

Our phylogenetic analysis using genes for 18S and D3 of 28S ribosomal RNA showed two well-supported clusters in *Strongyloides* species, namely a group including *S. ratti* and *S. stercoralis* and another including *S. papillosus* and *S. venezuelensis* (Fig. 2). This is consistent with the gonad morphologies of these species, wherein members of the *S. papillosus* group have spiral morphology, while those of the *S. ratti* group have straight morphology (Bartlett, 1995; Little, 1966; Sato *et al.* 2007). The number of chromosomes observed for *Strongyloides* species was also consistent with the phylogeny; karyotypes known thus far are $2n=6$ for *S. ratti* and *S. stercoralis* and $2n=4$ for *S. papillosus* and *S. venezuelensis*. *Strongyloides ratti* and *S. venezuelensis* are both parasites of rodents. However they are clearly different in terms of rRNA phylogeny, chromosome number and gonad morphology. Therefore, it is likely that they acquired their ability to parasitize rodents independently from each other.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit <http://dx.doi.org/S0031182014001036>.

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DATA DEPOSITION

Sequence data has been deposited in GenBank/EMBL/DBJ under accession numbers AB923880–923889.

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Assessment of Helminth Biodiversity in Wild Rats Using 18S rDNA Based Metagenomics

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Abstract

Parasite diversity has important implications in several research fields including ecology, evolutionary biology and epidemiology. Wide-ranging analysis has been restricted because of the difficult, highly specialised and time-consuming processes involved in parasite identification. In this study, we assessed parasite diversity in wild rats using 18S rDNA-based metagenomics. 18S rDNA PCR products were sequenced using an Illumina MiSeq sequencer and the analysis of the sequences using the QIIME software successfully classified them into several parasite groups. The comparison of the results with those obtained using standard methods including microscopic observation of helminth parasites in the rat intestines and PCR amplification/sequencing of 18S rDNA from isolated single worms suggests that this new technique is reliable and useful to investigate parasite diversity.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files. Sequencing data are available from the DDBJ Sequence Read Archive database (DRA) under the BioProject PRJDB3050.

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Introduction

Parasitism is one of the most common and successful lifestyles on the Earth [1] and has evolved independently at least 60 times during the evolutionary history of animal life [1] [2]. Several parasite lineages have diversified greatly over geological time. As a result, parasites outnumber their free-living relatives in some taxonomic groups in animal kingdom [1].

Studying parasite diversity is important for at least 3 major reasons. First, parasites are now recognised as playing important roles in ecosystem fractions [3] by influencing the populations and communities of their hosts [4]. Second, many parasite species are medically and agriculturally important. Although little is known about their evolutionary origins, several human parasites may have evolved by switching to humans from wild or domestic animals [5,6]. Additionally, species interactions involving parasites are a key to understanding many biological invasions and emerging infectious diseases [3]. Finally, because of the many independent transitions to parasitism within taxonomic groups, researchers can study the processes of evolution as the phenomena is related to speciation rates and diversification [1]. Therefore, the number of studies investigating the patterns of parasite diversity among/within host species and among geographical regions has been increasing in recent years [4]. However, the traditional approach of identifying all individual parasitic worms based on microscopic observation and PCR amplification/sequencing of

18S rDNA from isolated single parasites is time consuming, and requires highly specialised experience in morphology. In addition, morphological identification is simply impossible in some cases. As a result, parasite communities are not well classified, leaving diversity analysis ambiguous and non-holistic. Recent advances in high-throughput massively parallel sequencing, also called 'next generation sequencing' (NGS), are revolutionising the description of microbial diversity within and across complex biomes from the human body to the Earth's biosphere [7,8]. The greater sequence coverage and lower per-base sequence cost offered by NGS instruments including Illumina sequencers and 454 pyrosequencers have been greatly contributing to this progress.

Most of the metagenomic studies performed to date have targeted the biodiversity of prokaryotic communities using 16S ribosomal RNA gene (rDNA) sequences [9,10,11]. Attempts to assess eukaryotic diversity using NGS techniques have just begun for fungi [12], nematodes [13,14] and marine microbes [15]. In this study, we performed eukaryotic 18S rDNA-based metagenomics to assess biodiversity of helminth parasites (i.e. Nematoda, Cestoda and Trematoda) in the alimentary tract of wild rats. We analysed massive numbers of sequence reads obtained by 18S rDNA-PCR amplification followed by Illumina sequencing. To evaluate accuracy, sensitivity and resolution power of the method, we compared these results with those from the standard methods including extraction of helminth parasites from the intestine, microscopic observation and single-worm PCR amplification/

sequencing. Our results suggest this new technique is useful for the identification of animal parasites and the assessment of parasite diversity.

Materials and Methods

Collecting wild rats

Nine wild rats (7 *Rattus norvegicus* and 2 *R. rattus*), which were captured under the rodent extermination programmes at 2 locations of Miyazaki City, Japan, from November 2013 to January 2014 (Table 1) were received from Miyazaki City Phoenix Zoo or Miyazaki Pest Control Association. The rats were transported to the laboratory and maintained in clean cages for 12 hr before sacrificing by ether inhalation. Faecal pellets they excreted *ad libitum* were collected for metagenomic analysis.

Ethical statement

Animal experiments were performed in accordance with the procedures approved by the Animal Experiment Committee of the University of Miyazaki under an approval no. 2009-506-6, as specified in the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan, 2006.

Isolation of parasitic worms from rat intestines

Parasites were isolated from rat intestines as previously described with some modification [16]. Briefly, whole intestines were extirpated from freshly sacrificed rats and separated into 2 parts (20 cm from the pylorus ring and the remainder). They were dispeared, washed and incubated separately in PBS (phosphate buffered saline) at 37°C for 2 h to let worms emerge. PBS was then centrifuged at 3000×g for 5 min to concentrate the isolated parasites, which were observed using light microscopy.

DNA extraction, sequencing from individual parasites and phylogenetic analyses

DNA from nematodes was extracted into DirectPCR Lysis Reagent (Viagen) containing 20 mM dithiothreitol (Wako) and 0.5 mg/mL proteinase K (Qiagen). Individual nematodes were transferred to a 10-μL aliquot of the lysis buffer and incubated at 60°C for 20 min followed by 95°C for 10 min. DNA extraction from cestodes was performed using the QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen). One microlitre of the extract was used for PCR amplification of the 18S ribosomal RNA gene. These PCR reactions contained primers 988F and 1912R [17] for nematodes and wormA and wormB for cestodes [18], along with GoTaq Green Master Mix (Promega). PCR products were purified using the MinElute 96 UF Kit (Qiagen) and sequenced using BigDye Terminator v3.1 and an ABI 3130 sequencer (Applied Biosystems).

Phylogenetic analyses were performed with obtained and published 18S rDNA sequences. Sequences were aligned with MAFFT v6.864b [19] using '-auto' option, and the alignments were cleaned with Gblocks v.0.91b [20] using flags '-b4=10 -b5=n -b6=y -s=y'. Phylogenetic analyses were performed with RAxML v.7.2.8 [21]. The trees were bootstrapped 100 times for support.

Illumina library construction and sequencing

DNA extraction from individual faecal pellets (approximately 0.1 g) obtained from wild rats as described above was performed using the PowerSoil DNA extraction kit (MoBio) as recommended

in the Earth Microbiome Project (<http://www.earthmicrobiome.org/>). Barcoded PCR products were generated according to the protocol of the Earth Microbiome Project [22] (<http://www.earthmicrobiome.org/emp-standard-protocols/18s/>). Briefly, the V9 region of the eukaryotic 18S rRNA gene was amplified in triplicates for each sample with 1391f and EukBr primers [23] containing Illumina adaptors and a unique 12 bp Golay barcode using Ex Taq Polymerase (Takara). PCR amplification was performed in the presence of the mammal blocking oligo [24] on a 30-μL scale under the following conditions: 94°C for 3 min; 22 cycles of 94°C for 45 s; 65°C for 15 s; 57°C for 30 s; 72°C for 90 s and 72°C for 10 min. PCR products from the 3 reactions were combined and purified using the MinElute 96 UF PCR Purification Kit (Qiagen). The final sequencing library was prepared by mixing equal amounts of PCR products and purifying the mixture using agarose gel electrophoresis and the QIAquick Gel Extraction Kit (Qiagen).

Libraries were sequenced with Illumina MiSeq using the MiSeq Reagent Kit v2 (500cycles) (Illumina) and custom sequencing primers (<http://www.earthmicrobiome.org/emp-standard-protocols/18s/>) according to the manufacturer's recommended protocol (<https://icom.illumina.com/>). The linearization, blocking and hybridization step was repeated in situ to regenerate clusters, release the second strand for sequencing and hybridise the R2 sequencing primer. This was followed by another 250 cycles of sequencing to produce paired-end reads. The sequencing data have been deposited to DDBJ sequence read archive (DRA) under the BioProject PRJDB3050.

Illumina data analysis

Illumina sequence data was processed using QIIME version 1.8.0 [25]. Paired-end reads were joined using the 'fastq-join' method in QIIME (join_paired_ends.py). After QIIME quality filtering and library splitting according to the Golay barcode sequences (split_libraries_fastq.py: -store_qual_scores -q 9 -max_barcode_errors 2 -sequence_max_n 1 -max_bad_run_length 2 -p 0.75), 18S rRNA OTUs were picked from the reads using a closed-reference OTU picking protocol against the SILVA 108 database (Eukarya_only) [26] at 95% identity with 'uclust' (pick_otus.py: -max_accepts 1 -max_rejects 8 -stepwords 8 -word_length 8).

Results

Identification of parasites from rat intestines

Seven and two wild rats were collected at 2 contrasting locations in Miyazaki City, Japan: a restaurant downtown in the middle of the city (TR) and a zoo in the suburbs (ZR), respectively (Table 1). The rat IDs were named with the header TR or ZR according to the location from where they were collected. The sample was composed of 3 males and 6 females from 2 species (*R. norvegicus* and *R. rattus*) with varying body sizes (body weight ranging from 95 g to over 300 g) (Table 1). The parasite isolation protocol from the intestines detected nematodes in 8 out of the 9 rats (Table 2). On the other hand, cestodes were identified in only 2 *R. rattus* rats collected in the zoo (*R. rattus*). They were more than 20 cm in length and difficult to distinguish from each other by their morphology (Table 2, Figure 1). The sequences of their 18S rRNA genes were highly similar (99.9% identity) to those of *Hymenolepis diminuta* (JX310720) (Table 3, 4, Figure S1E). We found no flukes in the wild rat intestines.

Table 1. Wild rats used in this study.

Rat ID*	Species	Gender**	Body length (cm)	Tail length (cm)	Body weight (g)	Location	Collection Date
TR2	<i>Rattus norvegicus</i>	F	18	NA	NA	NishiTachibana St., Miyazaki City	6-Nov-13
TR3	<i>Rattus norvegicus</i>	F	19	NA	NA	NishiTachibana St., Miyazaki City	6-Nov-13
TR4	<i>Rattus norvegicus</i>	F	19	16	151.6	NishiTachibana St., Miyazaki City	8-Nov-13
TR5	<i>Rattus norvegicus</i>	M	23	21	311.2	NishiTachibana St., Miyazaki City	8-Nov-13
TR6	<i>Rattus norvegicus</i>	M	16.5	14.5	97.5	NishiTachibana St., Miyazaki City	8-Nov-13
TR7	<i>Rattus norvegicus</i>	F	20.5	18	191.2	NishiTachibana St., Miyazaki City	8-Nov-13
TR8	<i>Rattus norvegicus</i>	F	18	17	159	NishiTachibana St., Miyazaki City	9-Nov-13
ZR1	<i>Rattus rattus</i>	F	14	18	100.8	Phoenix zoo, Miyazaki City	28-Jan-14
ZR2	<i>Rattus rattus</i>	M	14	15	96.3	Phoenix zoo, Miyazaki City	28-Jan-14

*Rat IDs were assigned based on collection locations (TR or ZR).

**F: female, M: male.

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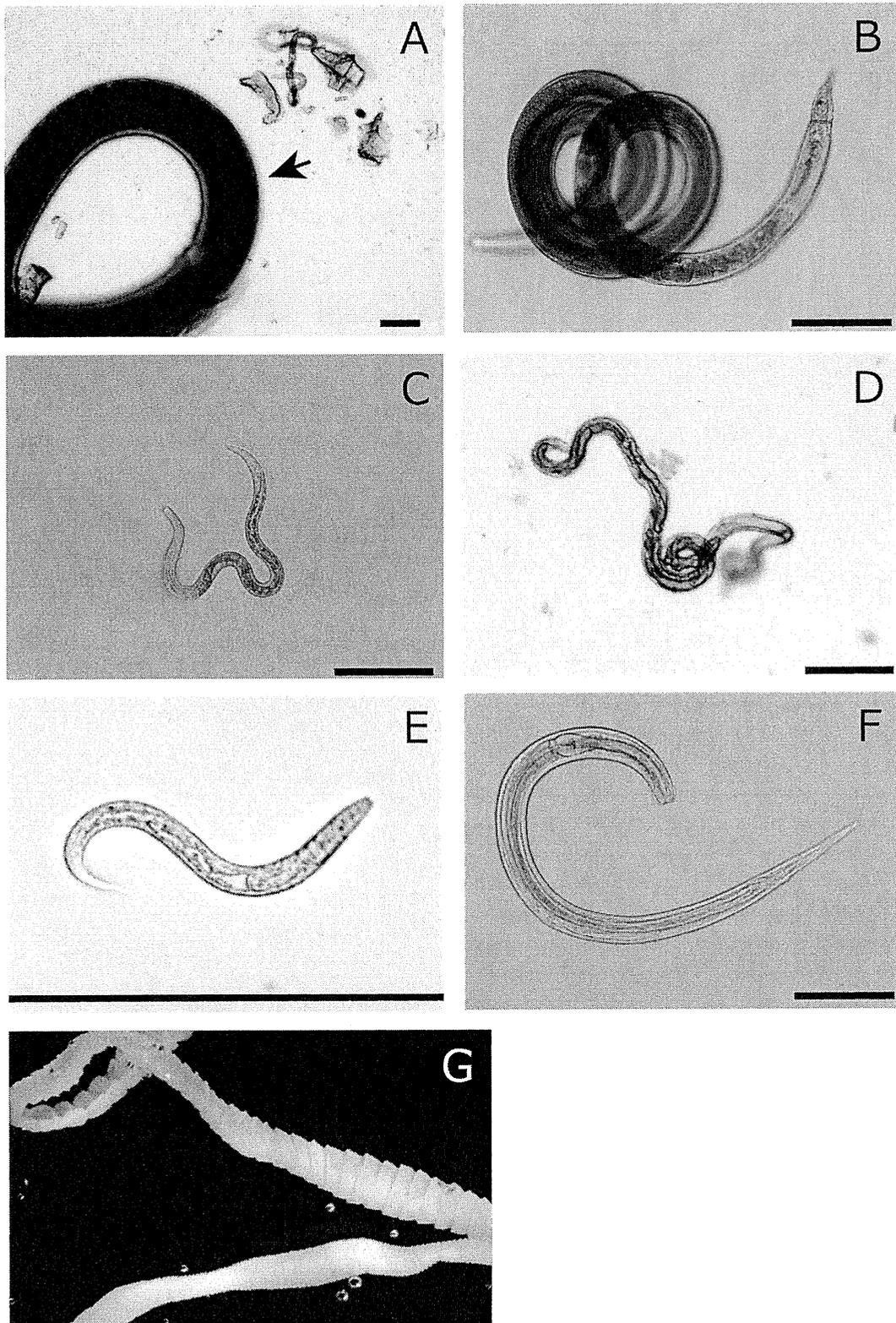


Figure 1. Seven morphological types identified in wild rat intestines. (A–F) Nematoda, (G) Cestoda (bar = 200 μ m).
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Table 2. Numbers of helminth parasites identified in rat intestines.

Rat ID	Nematodes						Cestodes		
	morphA	morphB	morphC	morphD	morphE	morphF			
TR2	0	75 (35)	1 (1)	85 (40)	11 (5)	0	0	0	0
TR3	2 (0)	19 (19)	6 (3)	142 (84)	7 (2)	0	0	0	0
TR4	0	134 (117)	2 (0)	42 (33)	0	0	0	0	0
TR5	0	177 (177)	0	128 (125)	0	0	0	0	0
TR6	0	37 (37)	2 (1)	165 (160)	0	0	0	0	0
TR7	0	344 (333)	6 (4)	273 (234)	0	0	0	0	0
TR8	0	130 (117)	0	94 (74)	0	0	0	0	0
ZR1	0	0	0	0	0	0	0	0	3
ZR2	0	0	0	0	0	0	0	2 (0)	3

Helminth parasites observed in the rat intestines were classified into 7 groups based on their morphological traits (Figure 1). The total number of parasites is shown in each cell. Values in parentheses are the number of parasites identified in the first 20 cm from the pylorus ring. doi:10.1371/journal.pone.0110769.t002

Nematode diversity

The total numbers of nematodes observed in each of the 8 rats varied, ranging from 2 in ZR2 to 623 in TR7. Most of the nematodes isolated were obtained from the first 20 cm of the intestine from the pylorus ring (Table 2).

Based on morphology, the nematodes were classified into 6 groups (Figure 1A–1F, Table 2). Briefly, morph A had a very large body size (more than 1 cm long) with a cylindrical shape and creamy-white colour, morph B had a differentiable red spiralled body, morph C had a medium body size (~1.0 mm in length) among the observed nematodes and a rhabditiform morphology, morph D was thin, 2–3 mm in length and characterised by the presence of a long pharynx, morph E was a small rhabditiform, probably first- or second-stage larvae and morph F was 1.4–2.0 mm long and differentiable by prominent and broad cervical alae (Figure 1A–1F). Those morphological characteristics are summarised in Table 3.

The 18S rDNA sequencing analysis of individual nematode suggested that these morphological groups consisted of at least 6 distinct species including those belonging to the genera *Ascaridia*, *Nippostrongylus*, *Heligmosomoides*, *Strongyloides* and *Aspiculuris* (Table 4, Figure S1). Morphological descriptions of the species are mostly consistent with the morphological characteristics observed. The 18S rDNA sequences of *N. brasiliensis* and *H. polygyrus* were very similar to each other. In morph B and morph C samples, we found 2 distinct sequence groups showing high similarity to the *N. brasiliensis* 18S rDNA sequence (99.2% and 97.5% identity, respectively). But one of them also showed comparable high similarity to *H. polygyrus* (97.5% identity) (Table 4). In the maximum-likelihood tree, those two sequences were classified together into a cluster which consisted of *N. brasiliensis* and *H. polygyrus* sequences, but they were sub-clustered separately from each other (Fig. S1B).

These results suggested the rats collected in the restaurant downtown were heavily infected by multiple parasitic nematode species including *Ascaridia*, *Nippostrongylus* (or *Heligmosomoides*) and *Strongyloides* nematodes. Of those, the majority of the infections were from *N. brasiliensis* and *Strongyloides* species. In contrast, rats collected in the zoo showed infrequent nematode infection; only a pin worm species was detected (Table 2, 3, 4).

18S rDNA Illumina sequencing

DNA was extracted from faecal samples collected from individual rats. The variable regions (V9) of the eukaryotic 18S ribosomal RNA genes present in each faecal community were amplified by PCR, and the resulting amplicons were sequenced on an Illumina MiSeq using 500 cycles.

Of the 6 million Illumina reads from the V9 regions of the eukaryotic 18S rRNA genes that passed the QIIME quality filters and were correctly assigned to each sample on the basis of the barcode sequences, 80.6% matched reference sequences in the SILVA 108 database at 95% sequence identity. They clustered into a total of 391 Operational Taxonomic Units (OTUs).

The QIIME level-2 OTU classifications (phylum level) are shown in Figure 2. In TR samples, 90–99% of the reads were assigned to Nematoda sequences. In TR6 samples, approximately 10% of reads were assigned to Apicomplexa (Figure 2).

Less than 10% of the reads in ZR samples were assigned to nematodes. Reads that were assigned to Streptophyta (Planta), Chordata (Animalia) or Dikarya (Fungi) were more represented. Reads assigned to Platyhelminthes were also found in ZR samples (approximately 2% in ZR1 samples and 3–49% in ZR2 samples) (Figure 2). Deeper classification revealed that most of the Streptophyta reads in ZR samples were assigned to corn (*Zea*),

Table 3. Morphological characters of isolated helminths.

Morphology Type	No. measured	Stage	Body length (mm) ^a	Body width (μm) ^a	Length of esophagus (μm) ^a	Descriptive characters
morph A	1	ND	210	294	ND	large body size, cylindrical shape, creamy-white colour
morph B	4	adult male	2.26 (1.74–2.76)	74.6 (61.1–92.7)	247 (192–294)	red spiralled body, a prominent umbrella-like bursa and two spicules at the posterior end
	4	adult female	2.81 (2.37–3.26)	68.71 (63.9–72.2)	269 (241–330)	red spiralled body, ellipsoidal eggs inside of the body, vulva opens at the posterior end
morph C	4	larva	0.83 (0.77–0.88)	29.0 (19.9–40.5)	178 (140–226)	middle size (~1.0 mm) rhabditiform
morph D	3	adult female	1.98 (1.83–2.10)	30.0 (28.7–31.0)	147 (146–147)	thin body, long pharynx, only females found, ellipsoidal eggs and vulva in the middle of the body
morph E	3	larva	0.19 (0.17–0.20)	14.4 (14.2–14.7)	66.2 (53.6–72.6)	small size (~0.20 mm) rhabditiform
morph F	1	adult male	1.57	74.5	258	braod cervical alae, oval esophageal bulb, slightly hooked tail with no clear spicules
	1	unmatured female ^b	1.32	60.1	254	braod cervical alae, oval esophageal bulb, pharynx plainly visible, conical shaped tail
morph G	2	adult	>200	1181 (1153–1231)	NA	flat segmented body, 4 suckers at the scolex, proglottids with both male/female sexual organs

^amean; range of the size in parentheses.^bprobably 4th stage larvae.

ND; Not determined, NA; Not applicable.

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Chordata reads were to assigned to pig (*Sus*) and Dikarya reads were assigned to yeast (*Saccharomyces* or *Candida*) (Table S1). Samples from each rat (different pieces of faecal pellets from the same day) showed similar contents although their ratios were

different (Figure 2). Other taxa to which more than 0.1% of the reads were assigned in any sample included Hexamitidae (approximately 0.04%–2.3% in ZR1 samples), Arthropoda (> 0.1% in ZR1 and ZR2, <0.1% in TR samples) and Trichomo-

Table 4. Species identification of isolated helminths based on 18S rDNA sequencing.

Morphology type	Number of sequences	Top hit in nematode 18S database	Sequence similarity (%)	Alignment length (bp)	sequence ID in Fig. S1
morph A	1	<i>Ascaridia galli</i> [EF180058]	98.2	895	A1
morph B	26	<i>Nippostrongylus brasiliensis</i> [AJ920356]	99.2	906	B1
	12	[AJ920356] or <i>Heligmosomoides polygyrus</i> [AJ920355]	97.5	906	B2
morph C	6	<i>Nippostrongylus brasiliensis</i> [AJ920356]	99.2	906	C1
	3	[AJ920356] or <i>Heligmosomoides polygyrus</i> [AJ920355]	97.5	906	C2
	1	<i>Ascaridia galli</i> [EF180058]	98.1	906	C3
morph D	12	<i>Strongyloides venezuelensis</i> [AB923887]	99.7	902	D1
	14	<i>Strongyloides ratti</i> [AB923889]	99.8	895	D2
morph E	8	<i>Strongyloides ratti</i> [AB923889]	99.8	895	E1
morph F	2	<i>Aspicularis tetraptera</i> [EF464551]	100	135	F1
morph G	2	<i>Hymenolepis diminuta</i> [JX310720]	99.9	2005	G1

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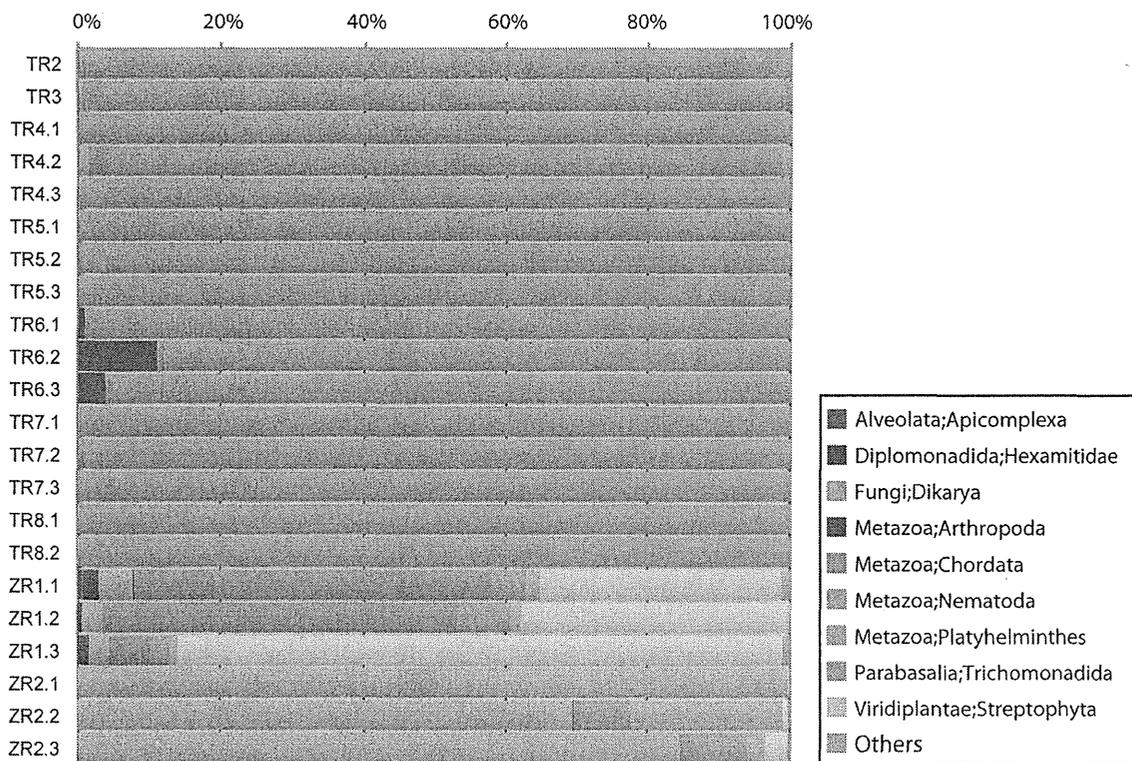


Figure 2. QIIME phylum-level classification of the 18S rDNA Illumina sequencing data.
doi:10.1371/journal.pone.0110769.g002

nadida (>0.1% TR2 and TR7 samples, <0.1% in other TR samples) (Figure 2).

The QIIME level-6 classifications (mostly superfamily level) clustered OTUs into 135 taxonomic groups (Table S1) and revealed that more than 90% of the total assigned reads in TR samples were classified into Panagrolaimoidea or Strongylida, which include *Strongyloides* species and *Nippostrongylus* species, respectively (Figure 3). Other nematode taxonomic groups in TR samples to which reads were assigned (with a minimum number of read filter of 4) include Ascaridiea, Heterakoidea, Oxyuroidea, Rhabditoidea and Acuarioidea although they were all rare OTUs (<0.1%, Figure 3, Table S1). In ZR samples, only 3 nematode superfamilies (Panagrolaimoidea, Strongylida and Oxyuroidea) had assigned reads (Figure 3, Table S1).

Because the SILVA 108 database contained only an essential number of nematodes reads, we sought to reclassify the reads assigned to Nematoda in the QIIME classification by BLAST similarity analysis using an in-house nematode 18S database. Although it was still difficult to classify them into deeper levels due to the short read length (~150 bp), the database enabled us to get a better insight into the nematode community. The reads assigned to Panagrolaimoidea or Strongylida in the QIIME classification showed the highest similarity to *Strongyloides* species and *N. brasiliensis* sequences in the nematode 18S database, respectively (Table 5). The reads assigned to Ascaridiea, Heterakoidea or Acuarioidea were highly similar to each other and showed the greatest similarity to *Ascaridia galli* or *Heterakis gallinarum*. The reads assigned to Oxyuroidea were most similar to *Aspiculuris tetraptera* (Table 5). These results were mostly consistent with the results of the standard method (direct microscopic observation)

(Table 2, 6). A trend was observed in the *Strongyloides/Nippostrongylus* ratio; samples with a lower ratio in the direct observation showed a lower reads ratio although *Strongyloides* reads ratios were always higher than the observed ratios (Table 2, 6, Figure 3). In addition to the species that we identified in the standard method, we found 18S rDNA reads which matched sequences from a free-living species *Oscheius spp.*

The Platyhelminthes reads both in TR and ZR samples were all classified into Hymenolepididae at the superfamily-level classification. The ZR2 rat had a higher percentage of Platyhelminthes reads (28%) than the ZR1 rat while they were rare in TR samples (<0.1%).

Reads assigned to the taxa that included parasitic Protozoa species were also identified in the QIIME classifications. Reads which were classified into Eimeriorina and Trichomonadidae were found in several samples (Figure 3). Notably, as much as 6.34% of the reads were classified into Eimeriorina in TR6 (Figure 3). Reads that were assigned to Giardiinae were found in TR8 and ZR1 samples (<0.01% and 1.30%, respectively), Trypanosomatidae in TR5 and TR8 and Acanthamoebidae in TR samples although they were rare (<0.1%) (Figure 3).

Discussion

Although 'metagenomics-based' studies of bacterial communities using 16S rDNA sequences have been extensively performed recently, there have been only few metagenomics reports targeting eukaryotic communities. In this study, we showed the power and usefulness of 18S rDNA Illumina sequencing for population studies of eukaryote parasites. Compared with traditional methods (isolation from rat intestines, microscopic observations and single-

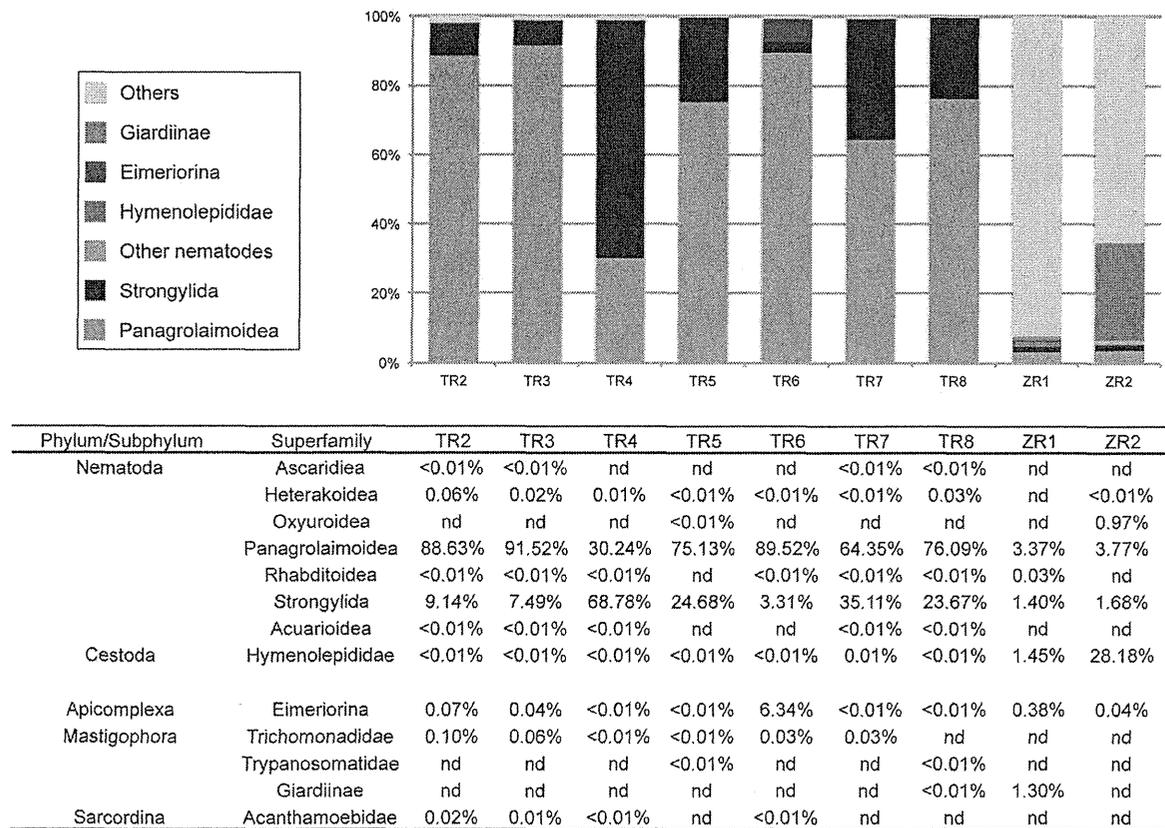


Figure 3. Parasite sequences in the QIIME superfamily-level classification.
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worm sequencing of 18S rDNA), the 18S metagenomics-based method is easy, quick to apply and sensitive. The 18S metagenomics-based method requires only faecal samples (no need to sacrifice hosts) and requires neither special techniques nor knowledge of parasite morphologies. Most importantly, 18S Illumina sequencing identified more varieties of parasite than the traditional methods in addition to all of the parasites identified using the traditional method.

The amount of parasite DNA in a faecal sample is expected to vary widely depending on their life cycles or conditions. The higher prevalence of *Strongyloides* species in the Illumina sequencing results than that in the result of the standard technique may have arisen because they produced and excreted more eggs/larvae into faeces than those by the other parasite species. We found a large Ascaridia-like nematode in the intestine of one of the rats (Figure 1). However, the number of reads that were assigned to this nematode group (Ascaridae) or a closely related group (Heterakoidea) in level 6 QIIME was very small (Figure 3). This suggests that the nematode was not very active in the rat body and did not produce eggs, possibly because it was sexually immature. Ascaridia/Heterakoidea nematodes are known as parasites of birds [27], and as far as we know, no Ascaridia/Heterakoidea nematodes have been reported from rodents. Therefore, the presence of Ascaridia/Heterakoidea nematode might reflect a recent accidental swallow of the parasite by the rat.

DNA extraction efficiencies from eggs or larvae of each species can also affect the number of sequence reads obtained. To reduce this kind of effects we used a well-established DNA extraction

protocol using MoBio PowerSoil kit, which was used as a standard method in the Earth Microbiome Project (<http://www.earthmicrobiome.org/>) and the Human Microbiome project [28]. The protocol, combining mechanical and chemical sample disruptions, has been widely used for various types of samples including soils and faeces, and it was shown that has been shown capable to extract DNA even from tough organisms such as spores or fungal mats (<http://www.mobio.com/>). The bias due to extraction efficiency in this study, therefore, can be low, but tests and optimisations of DNA extraction methods from parasites are still needed.

It is an interesting challenge to quantify parasites using 18S Illumina reads. To achieve the estimate, at least 2 normalisation steps seem to be needed. First, absolute read numbers need to be normalised using control DNA. Because DNA extracted from faeces was amplified by PCR and then appropriate amounts of the products for each sample were mixed and used for sequencing, we should not simply use the read numbers to estimate the amount of each parasite's DNA. Control DNA can be endogenous or exogenous (i.e. artificially added to each sample). For example, a large number of reads were assigned to corn (*Zea*) or pig (*Sus*) (Figure 3) in ZR samples. These probably resulted from the DNA of foods that the rats ingested. These reads were also present in TR samples although the ratios of read numbers were very small (< 0.01%). These kinds of reads, which can be assumed to always exist in relatively fixed amounts, could be a candidate for endogenous control DNA. Second, we need to use 'species factors' that are calculated on the basis of DNA emission in faeces per

Table 5. Nematode reads re-assigned with in-house nematode 18S database.

Order; Family	TR2	TR3	TR4	TR5	TR6	TR7	TR8	ZR1	ZR2	Possible nematode species
Rhabditida; Strongyloidea	119998	500677	208730	1085734	862840	292994	477358	129	526	<i>Strongyloides spp.</i>
Strongylida; Heligmonellidae	12381	40979	474806	356714	31943	159843	148495	55	238	<i>N. brasiliensis, H. polygyrus</i>
Ascaridida; Ascaridiidae, Heterakidae	87	134	78	5	0	41	208	0	0	<i>A. galli, H. gallinarum</i>
Oxyurida; Heteroxynematidae	0	0	0	0	0	0	0	0	137	<i>A. tetraptera</i>
Rhabditida; Rhabditidae	0	0	27	0	0	0	0	0	0	<i>Oscheius sp.</i>

Minimum 4-read filter was applied.
doi:10.1371/journal.pone.0110769.t005

Table 6. Comparison of the detection results from the standard method and the 18S Illumina method.

Standard method species	Illumina best classification	TR2		TR3		TR4		TR5		TR6		TR7		TR8		ZR1		ZR2		
		Std	Ilm																	
<i>A. galli, H. gallinarum</i>	<i>A. galli, H. gallinarum</i>				+	+														
<i>A. tetraptera</i>	<i>A. tetraptera</i>																		+	+
<i>N. brasiliensis</i> ^{a)}	<i>N. brasiliensis, H. polygyrus</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
<i>S. ratti</i>	<i>Strongyloides sp.</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
<i>S. venezuelensis</i>	<i>Strongyloides sp.</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
	<i>Oscheius sp.</i>							-	+											
<i>H. diminuta</i>	<i>Hymenolepis sp.</i>																+	+	+	+

"+" indicates worm/sequence detected. Std; Standard method, Ilm; 18S Illumina method.
^{a)}these samples can be divided into two groups based on the 18S sequences.
doi:10.1371/journal.pone.0110769.t006

individual parasite to estimate the number of parasites in a host body. In addition, copy number differences of rRNA genes between each parasite genome are also a subject to be considered [29].

We used the SILVA 108 database as a reference to classify 18S Illumina reads in QIIME. This study showed the database is very useful to provide a rough estimate of the structure of the helminth community in host bodies. However, the nematode and cestode sequences in the SILVA database were limited to only essential sequences and were not broad enough to cover nematode and cestode diversities. For example, the 18S rDNA sequences of rat parasites like *S. ratti*, *N. brasiliensis* and *H. polygyrus* do not exist in the SILVA 108 database. Therefore, we used an in-house nematode 18S rDNA database to obtain a better insight into the community structures. Additionally, because Illumina sequencing read lengths have been getting longer (up to 300 bp paired-end with the v3 kit), it will be able to use a region of the 18S rRNA gene longer than the V9 region (~150 bp) used in this study to obtain a better resolution during the classification step. Attempts to develop better primers to amplify and classify eukaryote organisms by metagenomics have already started [30,31]. Because parasites have considerably diverged within some small taxonomic groups and parasite species that researchers want to distinguish are often closely related to each other, superfamily-level classifications achievable with the QIIME software are sometimes not sufficient enough in depth to make fine distinctions. Therefore, developing a more complete database and using longer 18S sequencing reads are the next key steps to improve this technique as a more powerful tool to study parasite community structures.

Another important finding is that we identified a number of Eimeriorina sequences in the 18S Illumina data from TR rats (6.3% of total reads in TR6). Eimeriorina contains *Eimeria* species, which are protozoan parasites of animals [32]. These sequences were likely from parasites that have infected the rats. We also identified sequences that were assigned to taxa which include parasitic Protozoa species including *Trichomonas*, *Giardia*, *Trypanosoma* and *Acanthamoeba* spp. This suggests 18S Illumina sequencing is also useful to study protozoan parasite community structures. We also identified some fungal (yeast) sequences in a ZR rat. It is not clear whether they are parasitic or endosymbiotic species, but this suggests that 18S Illumina sequencing may enable us to investigate associations between helminths and protozoan parasites or parasites and other eukaryotic micro-organisms.

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Conclusions

Studying the diversity of parasites has been recognised as an interesting and useful approach in several research fields, such as those investigating the evolution of life, ecosystem fractions and invasion and migration of emerging diseases. However, the difficult and time-consuming processes required to identify parasites have restricted analysis covering a wide range of parasite species or dealing many samples. In this study, we showed the power and usefulness of 18S rDNA-based metagenomics in the investigation of parasite diversity. We also showed this approach still needs improvements in database completeness and read length in order to classify various parasites into a sufficient level. This approach with those improvements will enable us to analyse a large number of samples in a high throughput manner and can be the next standard to investigate parasite diversity.

Supporting Information

Figure S1 Maximum likelihood phylogenies based on 18S rDNA sequences to show relationships of the isolated worms with other nematode or cestode species/isolates. Selected nematode or cestode species were used in each of five trees; [A] with the sequences from morph A (A1) and morph C (C3), [B] morph B (B1, B2) and morph C (C1, C2), [C] morph D (D1, D2) and morph E (E1), [D] morph F (F1), [E] morph G (G1). Sequences obtained in this study were boxed in red, in which sequence group IDs (shown in Table 4) and individual sequence IDs (in parentheses) were given. Bootstrap values greater than 60% are shown on appropriate nodes. Genbank accession numbers were shown after the species names in each tip label.

(PDF)

Table S1 QIIME classification taxonomy summary (Level 6).

(XLSX)

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Author Contributions

Conceived and designed the experiments: HM TH TK. Performed the experiments: RT AH JEPR. Analyzed the data: IJT TK. Contributed reagents/materials/analysis tools: AY YO. Contributed to the writing of the manuscript: AH TK.

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