The population genetics of *Strongyloides papillosus* in Pakistani goats revealed by whole genome sequencing.

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Abstract

Strongyloides nematodes are parasites of livestock, and *S. papillosus* infects ruminant livestock that can cause disease. Recent genomic analysis of several *Strongyloides* species is now facilitating the population genomic analyses of natural *Strongyloides* infections, for example finding that *S. ratti* in wild UK rats exists as an assemblage of long-lived, asexual lineages. Here we have investigated the population genomics of *S. papillosus* in goats in Pakistan. We find that *S. papillosus* is common, with a prevalence of 28 %; that the population is genetically diverse and that individual goats commonly have mixed-genotype infections; and that there is evidence of only limited admixture. These results now provoke further questions about the host range of different *S. papillosus* genotypes that can in the future be investigated by further population genomic analyses.

Introduction

Strongyloides (Rhabditida, Strongyloididae) is a genus of gut parasitic nematode that infects a wide variety of terrestrial vertebrates. *Strongyloides papillosus* has been reported to parasitise sheep, goats, and cattle and can be experimentally maintained in rabbits (Pinn *et al.,* 2022). However, the species identity of *Strongyloides* infecting these host species has been questioned. Sequence analysis of the rRNA coding gene of *Strongyloides* from cattle and sheep found two different forms that appeared to be substantially (though not completely) host specific (Eberhardt *et al.,* 2008). The names *S. papillosus* and *S. vituli* for parasites from sheep and from cattle, respectively, were suggested (Eberhardt *et al.,* 2008), with subsequent further evidence of *S. vituli* infection in cattle (Ko *et al.,* 2019). More generally, the host specificity of *Strongyloides* species remains unexplored (Al-Jawabreh *et al*., 2023). Most *Strongyloides* species have been described morphologically, often from only some stages of the life cycle, which makes it hard to not only define a *Strongyloides* species, but also its host range. However, population genetic analyses of *Strongyloides* has the potential to resolve such questions.

The parasitic phase of *S. papillosus* (in common with other species of *Strongyloides*) consists of adult female worms only that live in the mucosal epithelium of the small intestine and reproduce by parthenogenesis. Eggs are shed in the host faeces, ultimately resulting in infective, filariform third-stage larvae (iL3s). These iL3s can develop either, directly, via two larval moults, or, indirectly, as the progeny of a facultative adult dioecious free-living generation that reproduces sexually (Viney, 2017). Hosts become infected by percutaneous infection by iL3s (Turner *et al.,* 1960). The parthenogenetic reproduction of the parasitic female worms means that the larvae passed in faeces are genetic copies of their mother that is resident in the host gut. The only opportunity for sexual reproduction is when free-living males and female worms are present (presumably in faeces or nearby soil). The factors that control the occurrence of this free-living adult generation are not fully understood, but in *S. ratti* in rats this varies among *S. ratti* genotypes, and is affected both by the temperature external to the host and by the host immune response (Viney & Lok 2007). From a population genetic perspective the facultative nature of sexual reproduction may mean that the population genetic structure of *Strongyloides* deviates from that predicted by Hardy-Weinberg expectations.

S. papillosus can be a common infection of ruminants, with a prevalence of up to 58 % reported in lambs, though the prevalence of infections differs widely geographically and among different livestock management systems (Thamsborg *et al*., 2017). *S. papillosus* can cause diarrhoea, dehydration, anorexia, anaemia and malnutrition, especially in young animals (Thamsborg *et al*., 2017). High intensity infections can cause serious strongyloidiasis, which can be fatal (Pinn *et al.,* 2022). Interdigital dermatitis and fatal ventricular fibrillation resulting from *S. papillosus* infection have been described in sheep and cattle (Turner *et al.,* 1960; Nakamura *et al.,* 1994; (Thamsborg *et al*., 2017). However, healthy animals tolerate infection with high faecal

egg counts. Juvenile animals are most susceptible to heavy infection upon first exposure, but rapidly develop acquired immunity to future *S papillosus* infections (Turner *et al.,* 1960). Case reports of strongyloidiasis due to naturally acquired infection are extremely rare, with only a few confirmed records (Romero *et al.,* 2022; Onyali e*t al.,* 1989; Round, 1963; Taira and Kato, 1986; Turner and Wilson, 1958; Woodhouse, 1948). However, there are also case reports of high-level and rapid death of calves, apparently due to *Strongyloides* infection, thought to be due to hyperinfection (Thamsborg *et al*., 2017). *S. papillosus* can be treated with anthelminthic drugs such as macrocyclic lactones (*e.g.* Ivermectin) and albendazole (Thamsborg *et al*., 2017). Rabbits can also be infected with *S. papillosus* and as such can be used as an experimental model for *S. papillosus* (Thamsborg *et al*., 2017).

S. papillosus has a karyotype consisting of 4 diploid (2n = 4) chromosomes. One diploid pair are autosomes, while the other diploid pair is an ancestral fusion of an autosome and an X chromosome. Sex determination occurs by male specific chromatin diminution, in which one copy of the X chromosome part of the autosomal–X chromosome fusion is lost, resulting in the generation of 2 autosomal fragments and the retention of the autosome–X chromosome fusion, such that in males 2n = 5, but in females 2n = 4 (Streit, 2017). The genome of *S. papillosus* has been sequenced, together with 3 other species of *Strongyloides* (Hunt *et al*., 2016). The *S. papillosus* genome is estimated to be 60 Mbp, though it is less well assembled than the other *Strongyloides* species such that the estimate of its genome size is not very accurate. Notwithstanding, the *S. papillosus* genome assembly can be used for population genomic analyses of this species.

Population genetic analyses of parasitic nematodes of livestock has found that there is very high gene flow and an absence of parasite population structure (Blouin *et al*., 1995). This was thought to be due to both the often very large effective population sizes of the nematodes, and because of extensive host movement that occurs as part of commercial farming. For nematode parasites of species of wildlife then there are a diversity of observed parasite population structures, with this dependant on host and parasite specific biology (Cole and Viney 2018). There has been previous population genomic analysis of *Strongyloides*. Specifically, *S. ratti* infecting wild rats in the UK was found to exist as a mixture of mainly asexual lineages parasites that were widely dispersed across the host population, findings that were consistent with earlier, UK-wide, studies (Fisher & Viney, 1998; Cole *et al*., 2023).

Here we report a population genomic analysis of *S. papillosus* in goats in Pakistan where we have used single worm, whole genome sequencing, and we find evidence of extensive mixed-genotype infections in hosts.

Materials and Methods

Study area. This study was conducted in the Rawalpindi district, Punjab state, which is part of the Pothohar plateau. The Pothohar plateau depends on natural rain fall and is the main agricultural zone of Pakistan, where the majority of the population are engaged in rearing livestock.

Faecal sampling and culturing. This study was approved by the bioethical committee of Quaid-i-Azam University, Islamabad, Pakistan. Faecal samples were collected directly from the rectum of 150 slaughtered goats using sterile disposable plastic gloves, with fresh gloves used for each goat to avoid cross-contamination. The faeces were cultured by mixing a few grams of fresh faeces with an approximately equal volume of wet charcoal, which was then kept at room temperature for up to 13 days when individual iL3s were collected, which were identified as *Strongyloides* by morphological examination (Zhou *et al.,* 2019). The larvae were washed in distilled water and then stored in 70 % v/v ethanol prior to whole genome sequencing.

DNA extraction and genome sequencing of single larvae. The DNA extraction protocol is based on the method of Cole *et al*., 2023 but adapted for ethanol-stored worms after Zhou *et al*., 2019. Pools of larvae from each host were processed separately; pools of iL3s in ethanol were transferred into distilled water and then individual larvae were placed into wells of a 96 well plate, with each well containing 50 µL TE, which was then stored at -80ºC. Later, 70 µL of lysis buffer was added to each well, giving a final concentration of 200 mM NaCl,100 mM Tris-HCl (pH8.5), 50 mM EDTA (pH8), 0.5 % w/v SDS, 0.9 mg /mL Proteinase K, and 45 mM Dithiothreitol (DTT). Plates were then sealed and held at 60°C for 2 hours with gentle agitation, after which they were held 85°C for 15 minutes to inactivate the Proteinase K. Lysates were then stored at -80°C before whole genome sequencing.

Whole genome sequencing was performed by the Centre for Genomic Research at the University of Liverpool. Illumina libraries were prepared using the NEB FS Ultra 2 DNA kit at half of the manufacturer's specified reaction volumes. In brief, samples were incubated with the Fragmentation System enzyme at 37 ºC for 8 minutes to fragment the DNA, then end repaired and ligated to adapters, and barcoded primers added via a 13 cycle amplification. After purification with ampure beads, the samples were pooled equally and treated with free adapter blocking reagent to remove surplus adapter. The quality of the pool was assessed by Qubit and Bioanalyzer and subsequently by qPCR using the Illumina Library Quantification Kit from Kapa on a Roche Light Cycler LC480II according to the manufacturer's instructions. The libraries were sequenced on the Illumina NovaSeq 6000 platform following the XP workflow on one lane of an S1 flow cell at the configuration 2 x 150bp.

Sequence analyses. We whole genome sequenced 114 *S. papillosus* iL3s and mapped the sequence reads using Bowtie (bowtie2/2.3.5.1/gcc-5.5.0) to the *S. papillosus* reference genome (PRJEB525, WBPS18 available from WormBase-

ParasiteSite; parasite.wormbase.org), and calculated the average depth and coverage across the genome of each alignment using SAMtools (v1.9). Of these 114, 37 had a genome coverage of at least 70 % and an average depth of at least 10, and these samples were then analysed further.

SNP calling was performed using SAMtools (v.1.9). We used VCFtools to filter SNPs using the criteria that (i) they had a mean mapping quality of at least 20, (ii) they had a QUAL score of at least 20, (iii) SNP loci had a minor allele frequency of ≥ 0.02, and (iv) their mean depth was between the 10 and 90 % percentiles of the whole data set, which was 6 and 21, respectively. Among the 37 *S. papillosus* genome sequences, nucleotides that were identical among all samples (but different from the PRJEB525 reference genome) were removed.

Using the filtered SNP set of the 37 *S. papillosus* iL3s we performed population structure analysis; specifically, by (i) using principal component analysis (PCA) in PLINK and Tassel v.5 with plots visualized in R studio v.4.2.1; (ii) constructing a neighbour-joining tree and calculating pair-wise genetic distance in Tassel v.5, visualising the tree in ITOL (https://itol.embl.de/); and (iii) using ADMIXTURE version 1.3.0 (Alexander and Large, 2011) analysis, with linkage disequilibrium (LD)-based pruning, which was done with window sizes of 50 kb, step sizes of 10 SNPs, and with a r^2 threshold of 0.1. ADMIXTURE was run for k = 2 – 15 and results plotted in R studio v.4.2.1.

We measured LD among the 37 iL3s, initially phasing the genotype data into haplotypes using Beagle version (5.0), and using VCFtools to compare each SNP to every other SNP within a 500 bp window, and then calculated Pearson's coefficient of correlation, r^2, for each pair. We did this for the three largest *S. papillosus* scaffolds. After reviewing initial results we excluded 6 samples that were genetically most divergent (**Figure 1A**), such that LD results for 31 larvae are presented here.

The *S. papillosus* reference mitochondrial genome was obtained from NCBI GenBank (Accession Number NC_028622.1). We generated maximum likelihood trees for the mitochondrial genomes of the 37 larvae, producing consensus fasta sequences for each; where an individual was heterozygous the reference allele was applied. Sequences were aligned with MAFFT version 7 (Katoh *et al*., 2009) using the fast alignment method FF-NST-1. Maximum likelihood tree estimation was carried out utilizing RaxML version 8.1.15 (Stamatakis, 2006), applying a general time reversible gamma model of substitution rate heterogeneity, and rapid bootstrapping with 100 replicates and visualized in ITOL (https://itol.embl.de/).

It has been suggested that there are two species of *Strongyloides* that infect ruminants, *S. papillosus* and *S. vituli*, that can be differentiated by their rRNA sequence (Eberhardt *et al.,* 2008; Ko *et al*., 2019), and a complete mitochondrial genome sequence has also been reported for *S. vituli* (Ko *et al.,* 2023). For the 37

samples we analysed the rRNA and mitochondrial sequences to confirm their species identity.

Results

We sampled 150 goats of which 42 (28 %) were faecal culture positive for *Strongyloides*. In all we obtained 526 iL3s, ranging from 7 – 15 iL3s per infected goat. We sequenced 114 individual iL3s of which 37 generated sufficiently good data and which were taken forward for further analysis. For these 37 this resulted in sequence data with an average genome coverage of 89 % (range 74 – 94 %), and an average read depth of 21 (range 9.7 – 69), giving an average genome coverage size of 53.6 Mb. We detected 11,023 filtered SNPs in the nuclear genome, which gives an average SNP density of 0. 2 SNPs per kb. This alignment of reads to the *S. papillosus* genome is consistent with identifying these larvae as *S. papillosus* rather than *S. vituli*. We also analysed the rRNA and mitochondrial genome sequence of the 37 samples, which confirmed their identity as *S. papillosus.*

To investigate the genetic relationship among the 37 iL3s we constructed a neighbour joining tree of the nuclear genome (**Figure 1A**). This showed that there was genetic heterogeneity among the worms. Most (33 worms) were fairly closely related, but 2 pairs of worms (two of which were from a single host, the other two from two different hosts) were much more divergent from the remaining 33. A PCA also showed three clusters of iL3s, with 33 iL3s in a single cluster, and then two smaller clusters each consisting of the same two pairs of divergent iL3s (**Supplementary Figure 1**). We investigated the data for evidence of admixture, with the results for k = 10 (CV = 0.13) showing that only eight worms had evidence of admixture (**Figure 1B**). Examining admixture results for other values of k, also showed low levels of admixture; specifically for k = 3, 7, 15 then six, three, and six worms showed admixture, respectively (**Supplementary Figure 2**). We mapped the k = 10 ADMIXTURE results onto the neighbour joining tree and found the these ADMIXTURE-identified groups are congruent with the main clades in the tree, and with the PCA analysis (**Figure 1A**).

Among the 21 hosts, 11 each had more than one worm sequenced, giving a total of 27 worms (five hosts with three worms; six hosts with two worms). We asked whether worms from a single host were in one or more k = 10 ADMIXTURE-defined groups and / or were admixtured. We found that 10 of the 11 hosts contained worms from more than one group and / or were admixtured. This suggests that multi-genotype *S. papillosus* infections are common in goats in Pakistan.

For the mitochondrial genome we detected 357 SNPs and used these to construct a maximum likelihood tree of the 37 iL3 and the reference *S. papillosus* mitochondrial genome. This showed that there were three *S. papillosus* clades (**Figure 2**). Two clades contained most (16 and 9 iL3s) iL3s, with the third clade containing 12 iL3s that were more divergent. This third clade also contained the *S. papillosus* reference mitochondrial genome (**Figure 2**).

As for the nuclear genome, we considered the 11 hosts that each had more than one sequenced worm. We found that nine hosts contained worms that were in more than

one mitochondrial clade. This is further evidence of mixed *S. papillosus* genotype infections in these hosts. Interestingly, the four iL3s with divergent nuclear genomes (**Figure 1A**) were within mitochondrial clade 1 (**Figure 2**). This result therefore shows some divergence in the genetic relationship among iL3s as judged by nuclear and mitochondrial data.

We compared the nuclear and mitochondrial trees. This showed that, with the exception of one nuclear clade, the three mitochondrial clades grouped related nuclear clades (**Figure 1A**). The exception is the highly divergent nuclear clade, which belonDgs to mitochondrial clade 1, whereas its closest neighbours belong to mitochondrial clade 2.

We analysed the LD for the largest three scaffolds of the *S. papillosus* genome (size range 1.1 – 1.6 Mb), finding that it was generally low and declined slowly with large genomic distances (**Supplementary Figure 3**), reminiscent of LD in wild *S. ratti* (Cole *et al*., 2023).

Discussion

This work has used whole genome sequencing to investigate the population genetics of *S. papillosus* in goats in Pakistan. We have found that *S. papillosus* is a common infection of goats in Pakistan, with a prevalence of 28 %. *S. papillosus* can infect a number of species of ruminants, and it remains to be discovered what its prevalence is in these other species. Given that *S. papillosus* is able to infect a number of different potential host species, there is the possibility for among-host species transmission of *S. papillosus*. Such sharing of parasites among different host species has the potential to genetically structure the parasite population, with this possibility being dependant on the amount of within, verses between, host species transmission.

The population genomic analysis revealed evidence of extensive genetic diversity among the parasites sampled, for both the nuclear and mitochondrial genomes. These data show that mixed-genotype infections are common in the goats that were sampled, which is likely due to goats being repeatedly exposed to infection from a genetically diverse *S. papillosus* population. This pattern is broadly consistent with patterns of population genetics seen in other parasitic nematodes (Blouin *et al*., 1995) Among the parasites sampled, a minority were genetically relatively divergent. Specifically, by analysis of the nuclear genome 4 of the 37 worms were distinct. This might point to the existence of even higher levels of genetic diversity in the *S. papillosus* population, which might be revealed by more extensive sampling in goats. Further, because *S. papillosus* is able to infect different ruminant species, the more-divergent genotypes we observed may be because these are parasites whose host preference (but not restriction) is for species other than goats. However, further sampling and genetic analysis is needed to understand the distribution of *S. papillosus* among different ruminant host species and the relationship of *S. papillosus* and *S. vituli* (Eberhardt *et al*., 2008). The results of the analysis of the mitochondrial genome are broadly consistent, showing 3 distinct clades with the third clade containing mitochondrially diverse worms (and the *S. papillosus* reference genome). Again, these results may potentially indicate a diversity of parasites that infect a range of host species, of which only a small portion has been sampled in the current study. However, it is notable that the grouping of parasites by their nuclear and mitochondrial genomes is not fully concordant. This is indicative of different histories of these two genomes in some of the parasites that we have sampled.

The *Strongyloides* life cycle is obligatory parthenogenetic with facultative sexual reproduction, with the degree of sexual reproduction varying between species, geographical location, and genotype (Viney & Lok 2007). The amount of sexual reproduction in *S. papillosus* that we have sampled is unknown, but during the culture of faeces we did not observe any free-living adult stages, suggestive of rare or no sexual reproduction. A previous analysis of wild *S. ratti* in the UK found that it consisted of a mixture of long-lived asexual lineages (Cole *et al*., 2023), and a similar pattern may exist among the *S. papillosus* that we have sampled, though further study is required to confirm this.

There is evidence of relatively low LD in the *S. papillosus* genomes, which declined slowly over large distances, which is suggestive of limited recombination. There was evidence of limited admixture among the *S. papillosus* samples, despite the high levels of genetic diversity in the population. Together, the limited recombination and limited admixture is suggestive of a population undergoing relatively little sexual reproduction. This pattern is reminiscent of LD in UK *S. ratti* populations (Cole *et al*., 2023). However, these results should be treated with some caution since the *S. papillosus* genome is not as well assembled as those of other *Strongyloides* species, and errors of assembly would affect estimates of LD. Notably, syntenic comparison of the *S. papillosus* scaffolds used in the LD analysis with other *Strongyloides* genomes may indicate incorrect a partially assembly and / or genome rearrangement of the *S. papillosus* genome compared with other *Strongyloides* species (Hunt *et al*., 2016).

To extend and develop this work and to better understand the population genomics of *S. papillosus* it would be desirable to more extensively sample larvae from a diversity of sympatric host ruminants. This will allow an analysis of the degree of partitioning, if any, of *S. papillosus* genetic diversity among different host species. Future work would also be facilitated by a better, more complete assembly of the *S. papillosus* genome, which could be achieved by long-read DNA sequencing. Analysis of diverse, wild genotypes of *S. ratti* has discovered clusters of parasitism genes (Hunt *et al*., 2016) that are hyperdiverse compared with the rest of the *S. ratti* genome (Cole *et al*., 2023). It would be interesting to study this in *S. papillosus* too, but this will also require a more complete genome assembly.

Figure legends

Figure 1. Population genomic analyses of 37 *S*. *papillosus* larvae from 21 goat hosts. (A) Neighbour-joining tree of the nuclear genome (full view to the left, and the central region enlarged to the right), with individual worms coloured according to the ADMIXTURE groups, with the eight admixtured worms shown as dotted circles and the mitochondrial clades shown as numbered (mt1, mt2, mt3) arcs and (B) ADMIXTURE analysis showing the degree of ancestry on the y-axis, with K = 10.

Figure 2. A maximum likelihood tree of the mitochondrial genome of the 37 samples and the *S*. *papillosus* reference mitochondrial genome.

Figure 1

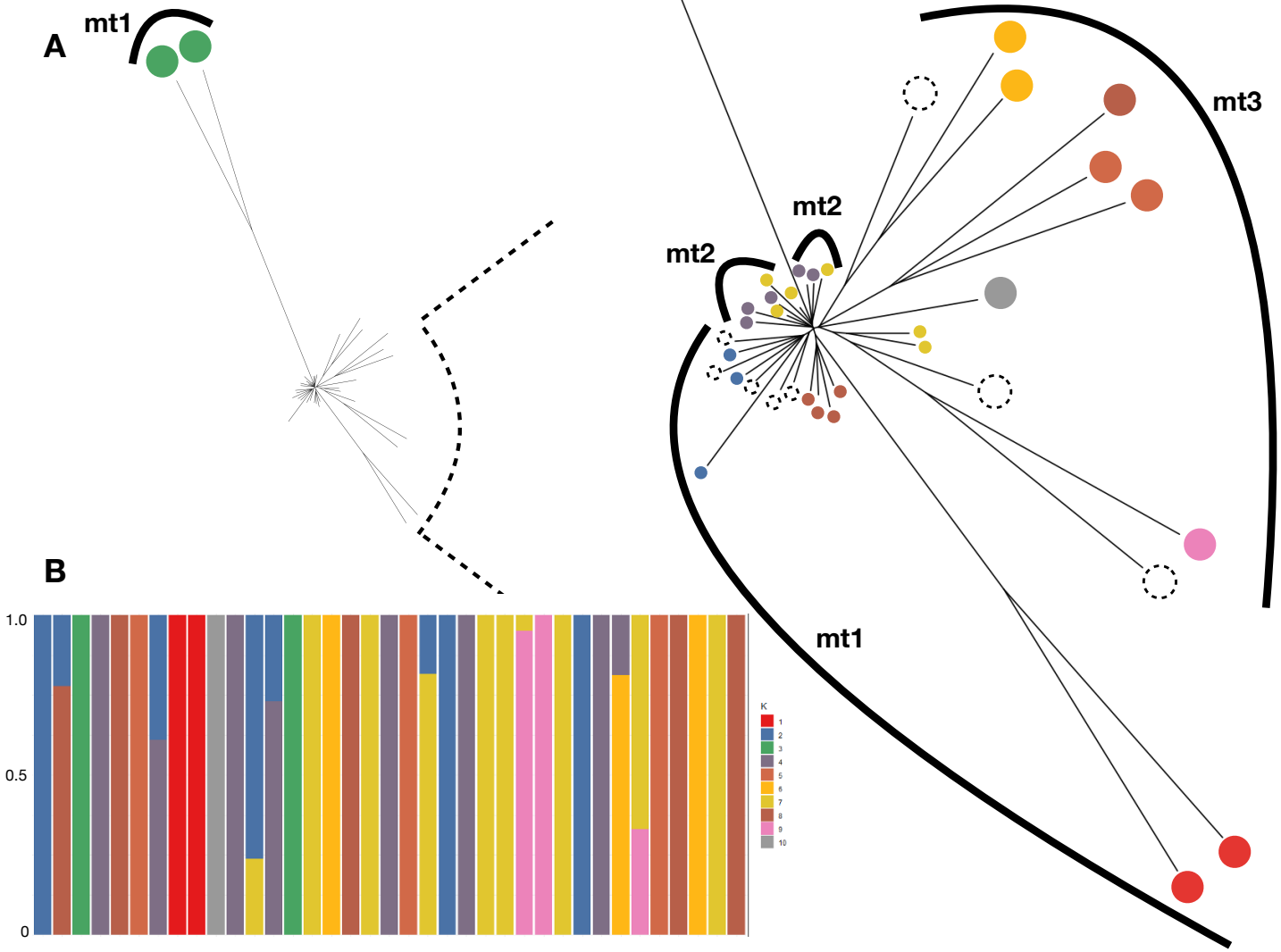
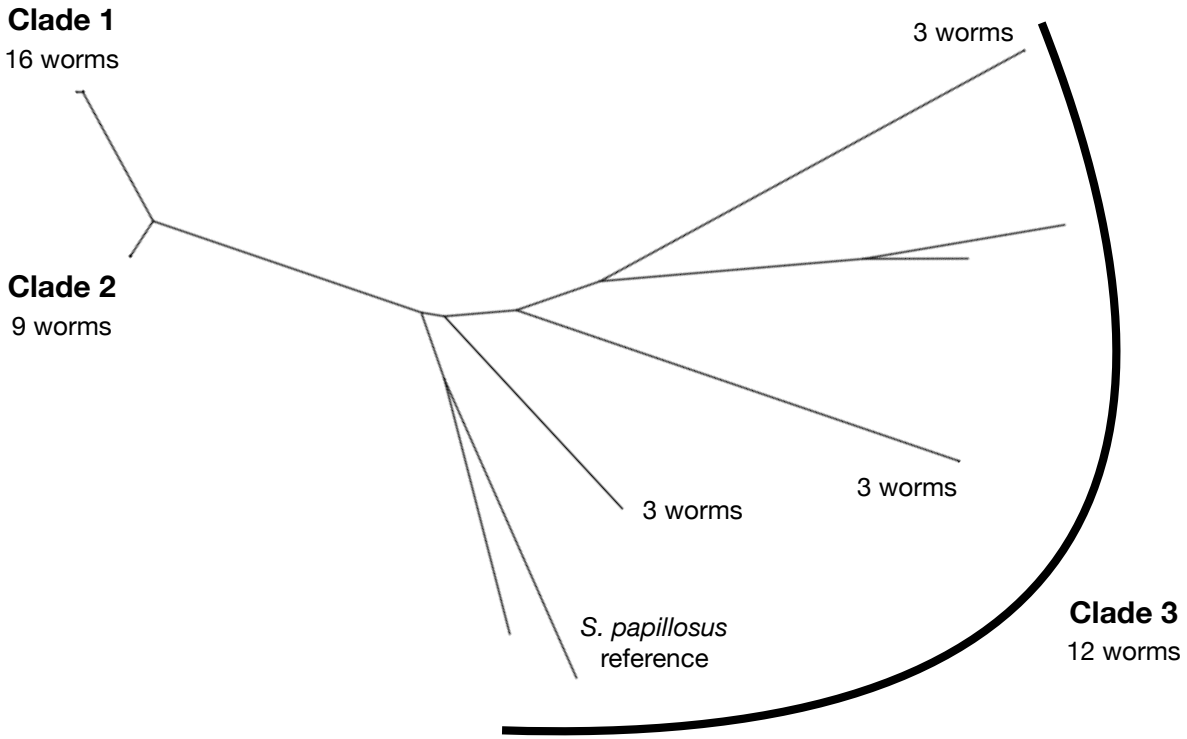


Figure 2


Clade 1
16 worms
Clade 2
9 worms
3 worms
3 worms
3 worms
S. papillosus
reference
Clade 3
12 worms

Supplementary Figures

Supplementary Figure 1. Principal component analysis showing the the first two principal components, which accounts for 38 % of the variance.

Supplementary Figure 2. ADMIXTURE analyses for k = 3, 7 and 15

Supplementary Figure 3. LD for the three largest scaffolds of the *S. papillosus* genome assembly**.**

Acknowledgments
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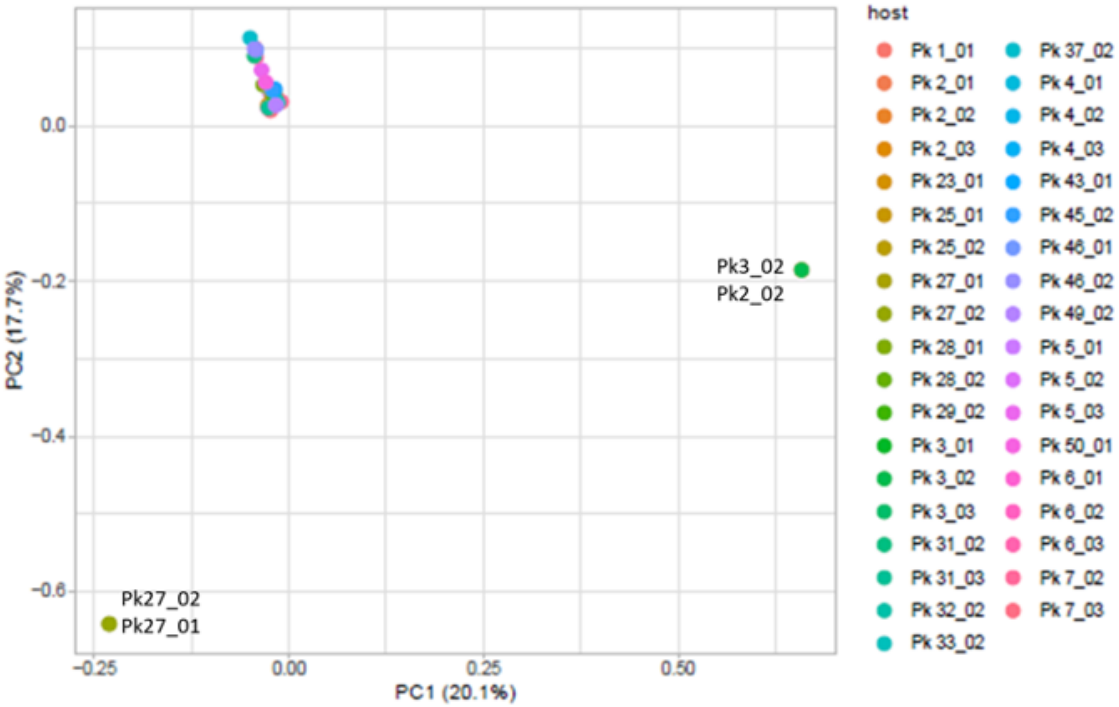
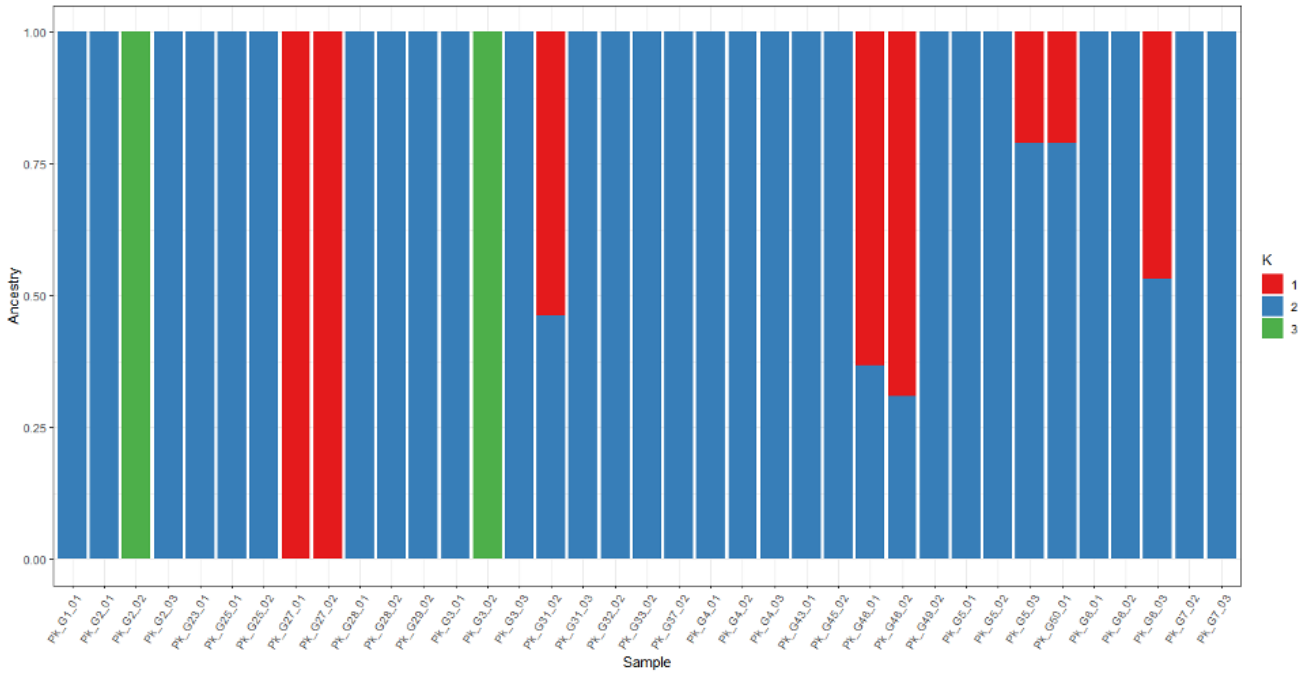
Supplementary Figure 1

Supplementary Figure 1



Supplementary Figure 2

k = 3



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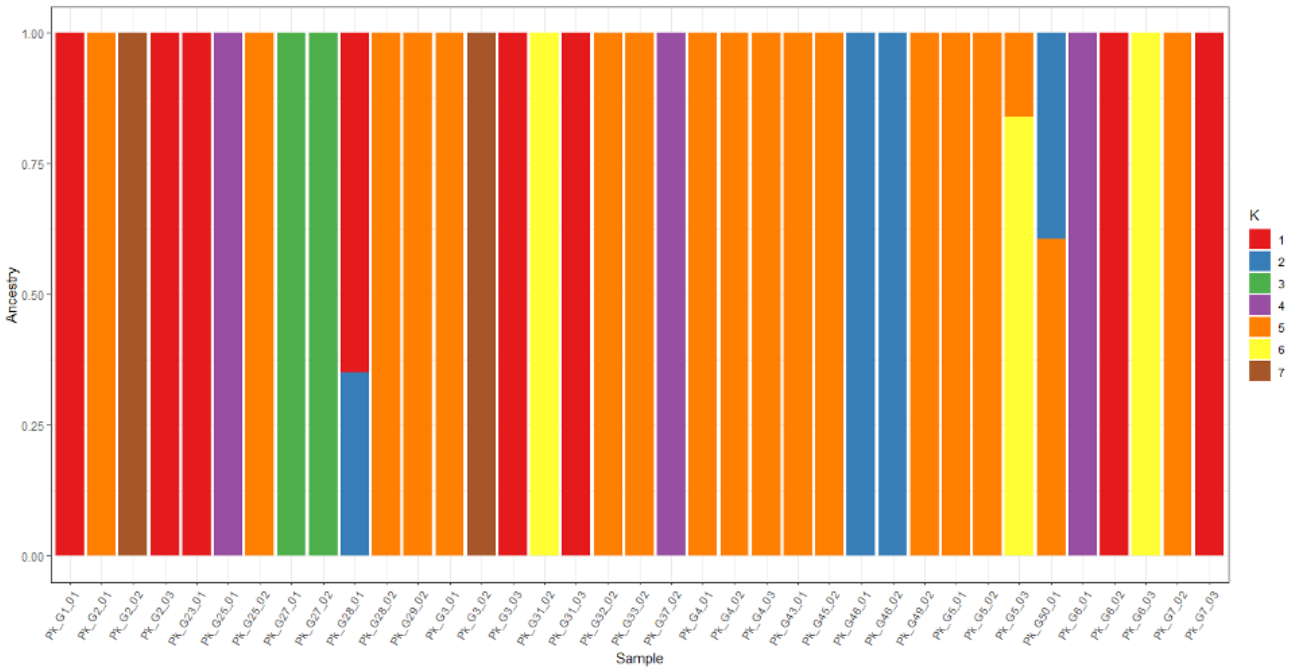

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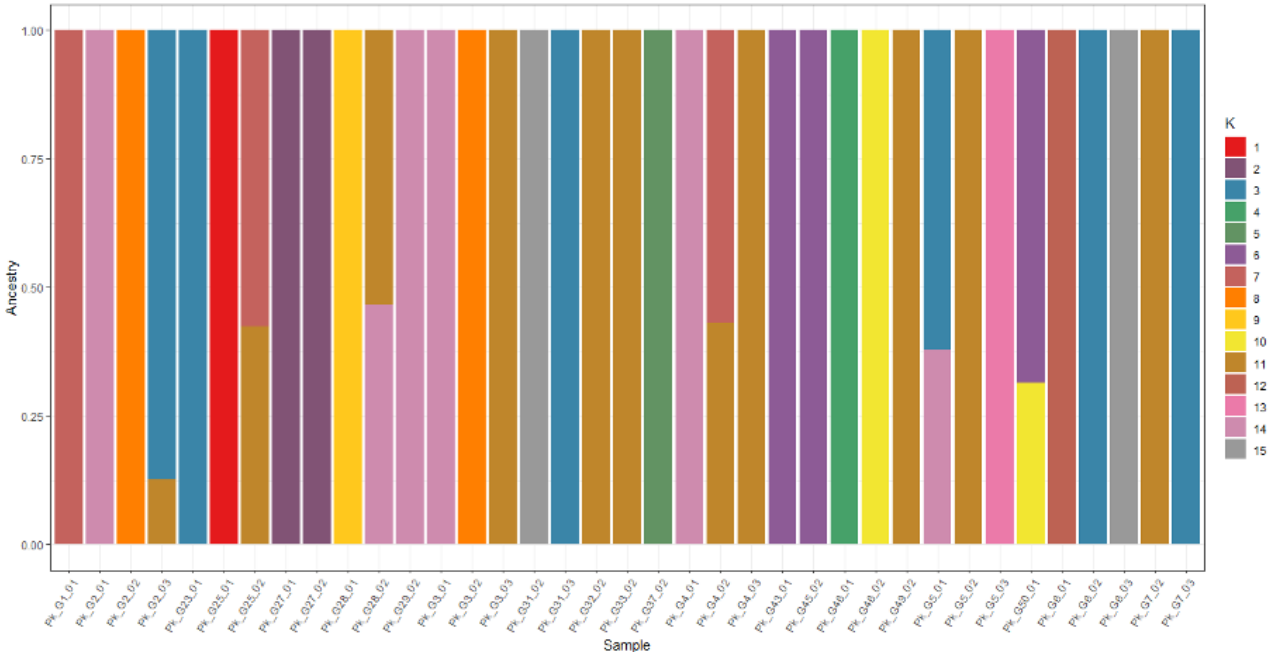
1.00
0.75
0.50
0.25
0.00
Ancestry
Sample
K
1
2
3
4
5
6
7
8
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10
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12
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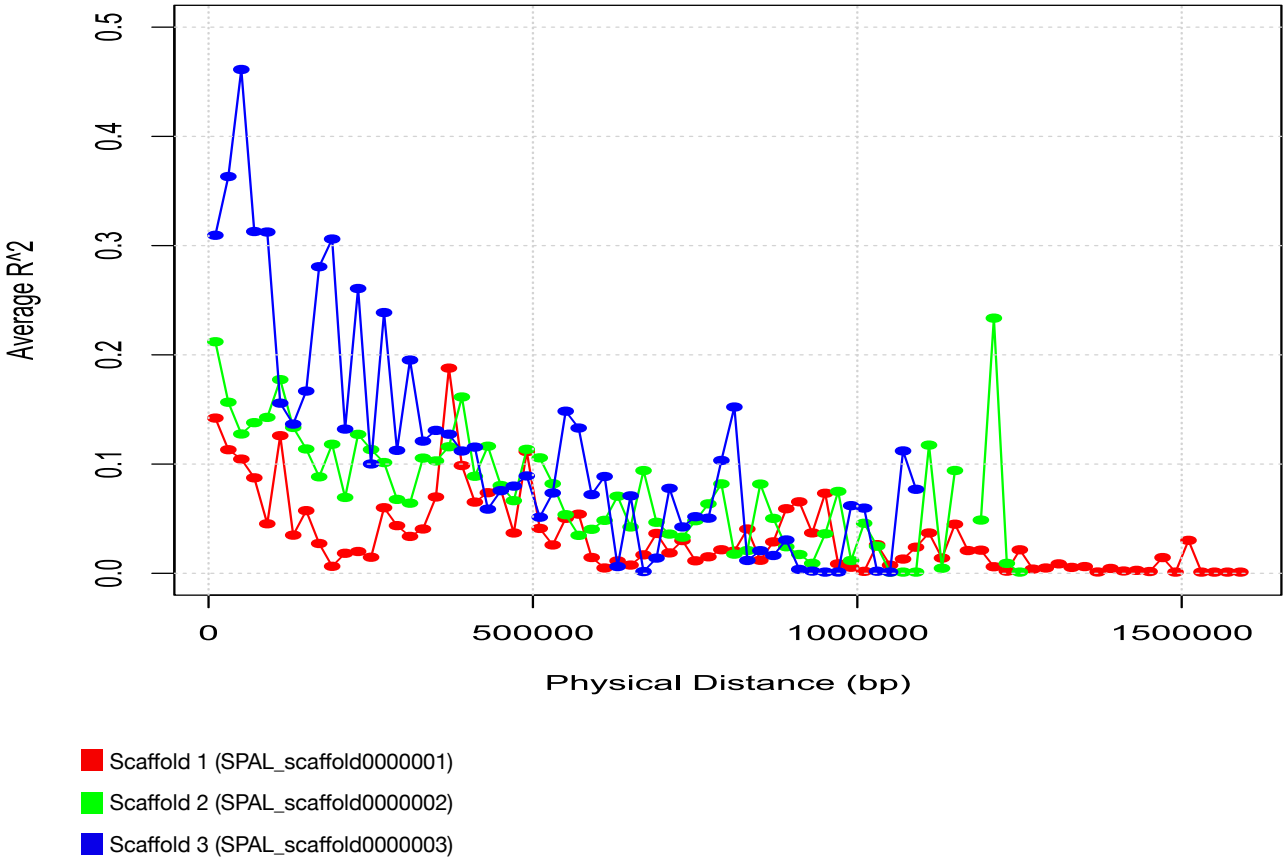
15

Supplementary Figure 3

Supplementary Figure 3