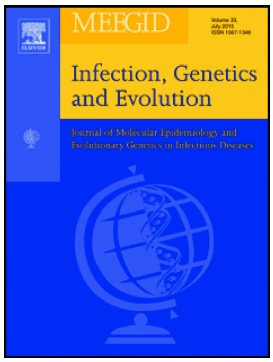

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Very low intraspecific sequence variation in selected nuclear and mitochondrial *Parascaris univalens* genes

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Abstract

Equines were over decades considered to be infected by two morphologically virtually indistinguishable ascarid species, *Parascaris univalens* and *Parascaris equorum*. Reliable species discrimination is only possible using enzyme isoelectric focussing and karyotyping with *P. univalens* having one and *P. equorum* two chromosome pairs. However, presumably the complexity of both methods prevented their routine use in nearly all previous studies about prevalence and drug resistance of *Parascaris* spp. These have barely been performed on the species level although most studies stated presence of one or the other species. Recently, only *P. univalens* has been identified by karyotyping and the last published study identifying *P. equorum* dates back to 1989. In order to improve species-specific detection, molecular markers are required. Here, partial 12S rRNA, cytochrome oxidase I (COI) and complete internal transcribed spacer (ITS)-1 and -2 sequences were obtained from 24 karyotyped *Parascaris* specimens from Poland and 6 German specimens (not karyotyped) and used in phylogenetic analyses with orthologous sequences from GenBank. All karyotyped specimens were identified as *P. univalens*. In the phylogenetic analysis, they formed very homogenous clusters for all target genes and in a multi-locus analysis. Within this cluster, almost all sequences from GenBank were also included, no matter if they had been assigned to *P. univalens* or *P. equorum*. However, a small number of *P. univalens* ITS and COI sequences originating from donkeys from a single farm in China formed a highly supported sister cluster suggesting that they might represent another *Parascaris* genotype or species. Our data also strongly suggest that nearly all ITS and COI sequences previously deposited in GenBank and assigned to *P. equorum* actually represent *P. univalens*. The fact that significantly different sequences can be found in *Parascaris* spp. suggests that PCR-based species diagnosis will be possible once molecular markers have been identified for *P. equorum* from karyotyped specimens.

Keywords: *Parascaris equorum*, karyotyping, internal-transcribed-spacer, cytochrome oxidase 1, ascarid, molecular detection

1. Introduction

Gastrointestinal nematodes and particularly roundworm species belonging to the superfamily Ascaroidea represent highly prevalent infectious pathogens in many mammalian hosts, predominantly affecting the health of younger individuals. While in other grazing animals, such as cattle or sheep, this group of parasites does not occur very frequently, in horses it is highly prevalent, particularly on stud farms (Fritzen et al., 2010; Hautala et al., 2019; Laugier et al., 2012; Relf et al., 2013; Saeed et al., 2019). The ascarid species of *Equus* hosts all belong to the genus *Parascaris* and historically in *Equus caballus*, *Parascaris equorum* and *Parascaris univalens* have been found most frequently. These two species cannot be distinguished morphologically but differ concerning their karyotype with *P. univalens* (previously named *Ascaris megalocephala univalens*) having one pair (2n = 2) and *P. equorum* (previously named *Ascaris megalocephala bivalens*) two pairs (2n = 4) of chromosomes as already noticed in the early days of cytology (van Beneden, 1883; Nussbaum, 1884). Despite the fact that based on electrophoretic (isoelectric focusing of enzymes) and cytological/developmental findings both species were confirmed as distinct species (Bullini et al., 1978; Goday and Pimpinelli, 1986), neither concerning their life cycle, the morphology of any of their life cycle stages or the clinical consequences they cause, any explicit differences are known. Thus, the exact species identification is challenging and only karyotyping (Goday and Pimpinelli, 1984) or electrophorectic analyses (Bullini et al., 1978) as well as faint morphological differences of the ending of the spicula (Biocca et al., 1978) have been described for this purpose. Such tools are not feasible for field application and thus in most of the studies dealing with *Parascaris* infections in horses, performed during the past decades, neither was employed. Actually, we were not able to find any publication using the

latter method, while the former has been used in several cytological studies but has to the best of our knowledge never been employed in *Parascaris* field studies investigating prevalence or anthelmintic resistance. Within a literature survey employing the Pubmed database searching for articles listing the term *Parascaris* 9243 references were obtained (date: 25.03.2021). By examining each publication concerning the addressed parasite species 147 papers remained which actually studied *Parascaris* specimen. When selecting only publications which appeared following the year 1978, as the year at which *P. equorum* and *P. univalens* were considered to be distinct species and not subspecies (Biocca et al., 1978; Bullini et al., 1978), a total of 106 papers remained. Two Polish studies (Gundlach et al. 2004; Studzińska et al. 2017), which were not included in the initial search since the respective journal is not included in the Pubmed database, were added due to their regional relevance. Of the resulting list of publications approximately two thirds referenced *P. equorum* as their study subject, while in none of these publications karyotyping was employed (Table S1).

The reason for this is certainly the relatively time-consuming and complex analytical procedure (compared with the process of assessing the quantification of *Parascaris* egg expulsion in horse faeces) and biological constraints associated with the karyotyping procedure due to the process of chromatin diminution. This occurs in all precursors of somatic cells during metaphase of the second to fifth cleavage, which leads to chromosome fragmentation into 64 small chromosomes (Niedermaier and Moritz, 2000) and the elimination of ca. 90% of DNA (i.e. the heterochromatin) from the nucleus (Boveri, 1887, for review see Pimpinelli and Goday, 1989; Tobler et al., 1992). Accordingly, either adult worms or eggs isolated freshly from faeces are required to be able to successfully perform the karyotyping procedure.

As a consequence of the above mentioned lack of species-specific detection concerning *Parascaris* infections, researchers and veterinarians are currently unaware of the prevalence

of these two species (Nielsen et al., 2014). Within numerous studies performed during the past decades the authors referred to *P. equorum* as the species they detected when they found *Parascaris* spp. eggs in the horse faeces or (pre-) adult stages in the intestines, without having conducted a proper species identification (e.g. von Samson-Himmelstjerna et al., 2007; Armstrong et al., 2014; Beasley et al., 2015). More recently, attempts were made to provide genetic data allowing the molecular species identification, as it has been established for example for the trichostrongyloids in ruminants, where similarly the eggs shed by a range of species cannot be reliably assigned to the respective species or even genus. Two independent investigations described the mitochondrial genome of *P. univalens* from karyotyped specimens (Jabbar et al., 2014; Nielsen et al., 2014) resulting in identical genetic maps and highly similar sequences. Unfortunately, to date no respective data are available for *P. equorum*. According to our assessment, this is due to the fact that all recently performed studies using the karyotyping method for species identification did only reveal the presence of *P. univalens* and thus it appears that *P. equorum* material is currently simply not available. In this situation the present study aimed at providing additional information on the genetic variability within the species of *P. univalens* by providing sequence information on a set of genetic loci including both nuclear and mitochondrial genes. These data will be of particular interest following the first description of respective sequence data clearly assigned to *P. equorum* and will hopefully assist in the identification of species specific markers.

2. Material and Methods

2.1. Sample collection

Live female worms of *Parascaris* sp. were collected in three slaughterhouses located in southern Poland and one in the Czech Republic. In Lublin, female worms were collected from

11 horses (female worm counts were 10, 8, 7, 6, 6, 3, 3, 2, 2, 1 and 1). Only a single positive horse containing 26 female worms was found in Kraków. In Wroclaw, from two positive horses two and 54 worms were collected, respectively. In Brno, 9 worms were collected from a single horse. After washing, worms in total were transferred into prewarmed saline and kept alive at 37°C. By opening the worms longitudinally, the uterus was removed and stored in 0.7% NaCl or 0.1 N H_2SO_4 for long time storage. As not all samples could be examined directly, several samples were stored at 4 °C for a maximum of one year. In addition, 4 worms with German origin were collected in a necropsy study in the Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, in 2015/2016. These worms were frozen immediately after collection and therefore karyotyping was not possible anymore. Animal experiments were conducted in accordance with European (EU directive 2010/63/EU) and German law (Tierschutzgesetz) and ethical approval for this study was obtained from the Landesamt für Gesundheit und Soziales (LAGeSo) Berlin (reference A0237/14 and A0287/15).

2.2. Karyotyping

In order to obtain fertilized eggs, the proximal part of the uterus was opened and washed out with fresh saline, before the eggs were collected in tubes. Eggs were pelleted using a manual centrifuge (Hettich, Germany) and the supernatant was discarded. After the addition of 6-8 ml 0.5 M NaOH, eggs were left at room temperature for 1 h for decorication. A protocol, identical to the one described for karyotyping eggs collected from faecal samples (Nielsen et al., 2014) was carried out to induce embryonic development and to visualize chromosomes. After staining with orcein or DAPI (both from Carl-Roth, Karlsruhe, Germany), every sample was assessed by light microscopy or fluorescence microscopy to enumerate the number of chromosomes, respectively. Subsequently, at least three different eggs at the first mitotic

division were photographed from each sample. This was successful for all worms except of five worms collected in Wroclaw.

2.3. DNA extraction

Genomic DNA was extracted from uteri (also used for isolation of eggs for karyotyping) of adult female *P. univalens* using the InnuSPEED Tissue DNA Kit (Analytic Jena) according to the manufacturer's instructions. Extracted DNA was eluted in a total volume of 300 µl DEPC-treated water (Carl-Roth) and stored at −20 °C until further use.

2.4. PCR assays and sequencing

PCRs were conducted with published primer pairs either for the cytochrome c oxidase I gene (COI), small subunit of mitochondrial rRNA (12S rRNA), internal transcribed spacer 1 (ITS-1) or the internal transcribed spacer 2 (ITS-2) region of the rRNA gene (Table S2).

All PCRs for amplification of the target regions were performed in parallel with a no-template (negative) and a positive control (plasmid DNA containing the target sequence as insert). PCR reactions contained 0.2 mM dNTPs, 250 nM of each primer, 0.4 U Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific) and 2 µl template DNA in 50 µl 1×HF buffer. The reaction involved an initial denaturation step at 98 °C for 30 s, followed by 40 cycles of denaturation at 98 °C for 10 s, annealing at a primer specific temperature (Table S2) for 30 s and extension at 72 °C for 30 s and a final elongation step at 72 °C for 5 min. Table S2 provides information regarding primer sequences, annealing temperatures, target genes, fragment sizes and number of PCR cycles conducted.

PCR products were analysed in parallel to the 100 bp or 1kb size marker (Thermo Scientific) using electrophoresis in 1.0- 1.5% agarose gels. Positive PCR amplicons were purified from additional agarose gels using the DNA Gel Recovery Kit (Zymo Research, Germany) and were ligated into the StrataClone Blunt PCR Cloning Vector pSC-B-amp/kan (Agilent) and

transformed into StrataClone SoloPack Competent *Escherichia coli* cells according to the protocol of the manufacturer. Plasmids were screened by restriction with EcoRI for presence of an insert and sent for sequencing to LGC Genomics. Sequences were trimmed to remove vector and primers.

2.5. Phylogenetic analyses

Sequences were compared to sequences in GenBank using Blastn (Altschul et al., 1990) against the non-redundant database (limited to the genus *Parascaris*) or by Blastn against the genomes of *P. univalens* (Bioproject PRJNA386823), *Ascaris lumbricoides* (Bioproject PRJNA515325) and *Ascaris suum* (Bioproject PRJNA62057). Hits were downloaded but since there were multiple hits for ITS sequences per genome, only the best hit was used. Accession numbers for all sequences used in the analyses together with the country where the worm was collected and the host species are provided in Table S3. The table also details information if karyotyping was performed on the samples and to which species the samples were assigned to in GenBank®.

Multiple sequence alignments of ITS-1, ITS-2 and 12S rRNA sequences were constructed using the MAFFT online server (Katoh et al., 2019) applying the Q-INS-I iterative refinement method with (ITS1, ITS-2) or without the --leavegappyregion option (12s rRNA). Codon-wise alignment in Muscle (Edgar, 2004) as implemented in Mega7 (Kumar et al., 2016) was used for COI sequences. DAMBE6 (Xia, 2017) was used to conduct a test for substitution saturation (Xia and Lemey, 2009). Since the test revealed no evidence for saturation, DAMBE6 was further used to split the COI dataset into two blocks (Codon positions 1 and 2 vs. codon position 3). Both blocks were joined and a partition file was created using FASconCAT-G (Kück and Longo, 2014).

Maximum likelihood phylogenetic trees were calculated on the IQ-TREE (Nguyen et al., 2015) webserver (Trifinopoulos et al., 2016). IQ-TREE was set to autoselect the optimal

substitution model (including models with FreeRate heterogeneity) using Modelfinder (Kalyaanamoorthy et al., 2017). Trees were calculated from 1000 ultrafast bootstrapping replicates (Hoang et al., 2018) and the Shimodaira–Hasegawa modification of the approximate maximum likelihood ration test (SH-aLRT) as well as an a Bayesian-like transformation of the approximate LRT (aBayes) were calculated to provide node support in addition to bootstrap values. Phylograms were visualised and re-rooted using the *Ascaris* sequences as outgroup in FigTree v1.4.4. Final editing of trees was performed in CorelDraw 2018.

3. Results and Discussion

3.1. Karyotyping identified only Parascaris univalens

Karyotyping was performed on eggs which were recovered from worms of three different locations in Poland (in total 130 worms), one location in the Czech Republic (9 worms) and one location in Germany (4 worms). Representative pictures of stained eggs are shown in Fig. S1. All specimens showed only two large chromosomes and were therefore assigned to the species *P. univalens* while no *P. equorum* was found. DNA was extracted and used for PCRs from 24 karyotyped *P. univalens*. Basically, these were the samples of those worms that were karyotyped first. Therefore, samples from three horses from Brno and Wroclaw were not used for molecular analysis.

3.2. Internal transcribed spacers

A phylogram showing the relations between ITS-1 (Fig. 1) revealed very close relationships between most of the sequences, no matter if they were deposited in GenBank as *P. univalens* or *P. equorum*. The only exception was a group of three sequences, assigned by Peng et al. (2019) without performing karyotyping as *P. univalens* genotypes M4, M11 and M15. All

were coming from donkeys in China with >99% identity among each other and they formed a distinct subcluster with very high statistical support (Peng et al., 2019). This subcluster was rooted within the larger cluster of sequences containing all karyotyped *P. univalens* samples (Fig. 1). Identity to sequences from the major cluster was 96.0 – 96.9% while identity among the sequences in the major cluster was in the range 99.12 – 100%. Unfortunately, all these three ITS-1 sequences were shorter (79% query coverage) than the sequences from the present project since they represented only partial ITS-1 sequences. However, this does not explain the localisation in a distinct subcluster since the same query coverage was obtained for isolates B12 and J20 that were placed in the main cluster (Fig. 2). In contrast, query coverage for all included sequences was 100% for the ITS-2 analysis. Again, the phylogenetic analysis placed the vast majority of sequences in a single cluster (Fig. 2). Of the 31 sequences in this cluster, 32 were identical while the other 2 showed 99.7% identity. Three sequences from isolates M4, M11 and M15, all from donkeys from China and assigned by Peng et al. (2019) as *P. univalens* without karyotyping were located outside of the main cluster as a highly supported sister cluster rooted in a basal position to the main group (Fig. 2). These three sequences were 99.5 – 99.7% identical to each other and showed only about 94% of the length of the main cluster sequences due to an internal 20 bp gap. Identity of these sequences to the sequences of the main cluster was in the range 90.1 – 90.6%. All of the *P. univalens* sequences originating from karyotyped samples (all *P. univalens*) were placed in the large cluster. This cluster also contained all sequences assigned to the species *P. equorum* in GenBank (none of them karyotyped). In both ITS trees, the branches connecting the isolates from Chinese donkeys (isolate names starting with M) with the remaining *Parascaris* sequences were much longer than the branches connecting *A. suum* and *A. lumbricoides* and 36% (ITS-1) and 41% (ITS-2) of the length connecting *Ascaris* spp. with *Parascaris* spp.

3.3. Mitochondrial genes

For the COI sequences, 46 of the sequences (including all specimens except of M14, M15, M17, M18 and M19) were highly similar with 97.4 – 100% identity in pairwise comparisons and they formed a highly supported cluster in the phylogenetic analysis (Fig. 3). This group contained both *P. univalens* and *P. equorum* sequences and all of the karyotyped *P. univalens* were included here. A highly supported subcluster, rooted within this large group, was represented by five additional sequences, *P. univalens* isolates M14, M15, M17, M18, M19 (all not karyotyped), again all coming from donkeys in China (Peng et al., 2019) showed 99.4 – 100% identity among each other but only 92.5 – 94.3% identity to the members of the large group. Again, the branch length connecting the Chinese isolates from donkeys with the other *Parascaris* specimens was much longer than the branches connecting the two *Ascaris* species. Comparison of branch length between the *Ascaris* and the *Parascaris* cluster with the branch connecting the two *Parascaris* clusters revealed that the latter had 54% the length of the branches connecting the genera.

For the 12S rRNA, only very few sequences were available that were not generated within this project. All of these sequences fell into the same cluster (Fig. S2) showing a sequence identity of 99.2 – 100%. Again, sequences from karyotyped samples identified as *P. univalens* and GenBank® sequences assigned to the species *P. equorum* were included in this cluster.

3.4. Multi-locus analysis

Independently of the gene analysed, all karyotyped *P. univalens* samples were placed in a single group which had low variability. The same group included also most of the non-karyotyped samples as well as most of the sequences deposited in GenBank® including all those assigned to *P. equorum*. In three of the analyses there were sequences that were considerably distinct from these main clusters. All of these sequences were derived from samples collected from donkeys in China (isolate names starting with M) and assigned to *P. univalens* without performing karyotyping (Peng et al., 2019). Peng et al. (2019) already

noted that their sequences obtained from donkeys were considerably different than the sequences obtain from zoo-kept mountain zebras (isolates names starting with B) and horses (isolate names starting with J) and that there was virtually no gene flow between these groups. Unfortunately, the authors did not include sequences from the published mitochondrial *P. univalens* genomes in their analyses and instead of concluding that there might be different species, they annotated all their sequences as *P. univalens*.

In order to confirm that there are two sister clades in the samples from the genus *Parascaris*, a multi-locus analysis was conducted. Despite the fact that Peng et al. (2019) deposited multiple sequences in GenBank®, there was only one isolate from donkeys for which ITS-1, ITS-2 and COI sequences were available, i.e. M15. Since no 12S rRNA sequences were available for any of the samples from donkeys, the multi-locus analyses focused initially on the remaining three markers. Fig. 4 reveals a large cluster of 25 sequences, 21 of those from samples karyotyped as *P. univalens*, showing 92.9%, 0.997 and 81% node support according to the SH-aLRT, aBayes and ultrafast bootstrapping. In a sister position to this cluster, the single M15 sequence from a specimen isolated from a donkey was placed. One of the explanations for this phylogeny might be different *P. univalens* genotypes in horses and zebras on one and donkeys on the other hand. However, it may also well be that the M-type isolates represent in fact *P. equorum* or even *P. trivalens*, which was initially described from Chinese horses (Li, 1934, 1937; Tchou, 1937). Since *E. asinus* and *E. zebra* are in fact much closer related to each other than to *E. caballus* (Vilstrup et al., 2013), the latter explanation might even be more persuasive than assuming a donkey-specific lineage of *P. univalens*. This is even more the case since the donkeys and horses from which the samples were collected by Peng et al. (2019) came from different farms and the farm might be an alternative explanation than the equine species for the distribution of genotypes in their data.

4. Conclusions

Presence of *P. equorum* has not been confirmed in any studies using karyotyping since 1989, but the number of studies using karyotyping is much too small to be epidemiologically representative. Nevertheless, a potentially reduced prevalence of *P. equorum* might have been caused by the frequent use of anthelmintics in horses, particularly foals. Since evidently *P. univalens* evolved resistance to macrocyclic lactones, this species potentially may have outcompeted *P. equorum*. Nevertheless, the genetic distance of the *Parascaris* spp. found in donkeys in China compared to the highly homogenous group of karyotyped *P. univalens* specimen from Europe and North America suggests that other genotypes or possibly species of *Parascaris* can still be found. Even compared with the length of the branches connecting *Ascaris* and *Parascaris*, the genetic distance is quite high suggesting that these specimens belong to another species than *P. univalens*. The most likely candidate is *P. equorum* but even *P. trivalens* cannot be excluded. Only combined karyotyping/molecular analyses will allow to actually identify species-specific markers in the future. The molecular data presented here can be used to design screening strategies to find dissimilar markers followed by karyotyping of populations from farms where such genotypes were found. Importantly, our data also strongly suggest that nearly all ITS and COI sequences previously deposited in GenBank and assigned to *P. equorum* were actually derived from *P. univalens* specimen.

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Figure legends

Fig. 1. Phylogenetic reconstruction of relationship between *Parascaris* spp. using ITS-1 sequences. Maximum-likelihood phylograms were calculated using IQ-TREE and rooted using sequences from *Ascaris lumbricoides* (*Alu*) and *Ascaris suum* (*Asu*) as outgroup. Sequences from karyotyped samples are shown in bold. The species as deposited in GenBank is abbreviated as *Pun*, *Parascaris univalens*; *Peq*, *Parascaris equorum*; *Par*, *Parascaris* spp. Isolate information is highlighted in blue. In case the isolate was not named, the GenBank® accession number was indicated. GenBank® accession numbers are available in Table S3. For sequences extracted from whole genomes, the Bioproject accession number is shown. If the host was a not horse (or unknown), the host species is provided. The country of origin is indicted using two letter ISO 3166-1 codes: CN, China; DE, Germany; PL, Poland; US, U.S.A. For a few samples, the geographic origin was not available in GenBank®. Node support was calculated using the Shimodaira–Hasegawa and a Bayesian-like transformation modification of the approximate maximum likelihood ration test and ultrafast bootstrapping and are provided in this order at the branches. Branch support is not shown is values for ultrafast bootstrapping were <50%. The scale bar represents 0.02 substitutions per site.

Fig. 2. Phylogenetic reconstruction of relationship between *Parascaris* spp. using ITS-2 sequences. Maximum-likelihood phylograms were calculated using IQ-TREE and rooted using sequences from *Ascaris lumbricoides* (*Alu*) and *Ascaris suum* (*Asu*) as outgroup. Sequences from karyotyped samples are shown in bold. The species as deposited in GenBank is abbreviated as *Pun*, *Parascaris univalens*; *Peq*, *Parascaris equorum*; *Par*, *Parascaris* spp. Isolate information is highlighted in blue. In case the isolate was not named, the GenBank® accession number was indicated. GenBank® accession numbers are available in Table S3. For sequences extracted from whole genomes, the Bioproject accession number is shown. If the host was a not horse (or unknown), the host species is provided. The country of origin is

indicted using two letter ISO 3166-1 codes: CN, China; DE, Germany; PL, Poland; US, U.S.A. For a few samples, the geographic origin was not available in GenBank®. Node support was calculated using the Shimodaira–Hasegawa and a Bayesian-like transformation modification of the approximate maximum likelihood ration test and ultrafast bootstrapping and are provided in this order at the branches. Branch support is not shown is values for ultrafast bootstrapping were <50%. The scale bar represents 0.02 substitutions per site.

Fig. 3. Phylogenetic reconstruction of relationship between *Parascaris* spp. using mitochondrial cytochrome c oxidase I sequences. Maximum-likelihood phylograms were calculated using IQ-TREE and rooted using sequences from *Ascaris lumbricoides* (*Alu*) and *Ascaris suum* (*Asu*) as outgroup. Separate substitution models were fitted for codon positions 1 and 2 and for codon position 3. Sequences from karyotyped samples are shown in bold. The species as deposited in GenBank is abbreviated as *Pun*, *Parascaris univalens*; *Peq*, *Parascaris equorum*; *Par*, *Parascaris* spp. Isolate information is highlighted in blue. In case the isolate was not named, the GenBank® accession number was indicated. GenBank® accession numbers are available in Table S3. For sequences extracted from whole genomes, the Bioproject accession number is shown. If the host was a not horse (or unknown), the host species is provided. The country of origin is indicted using two letter ISO 3166-1 codes: CN, China; DE, Germany; PL, Poland; US, U.S.A. For a few samples, the geographic origin was not available in GenBank®. Node support was calculated using the Shimodaira–Hasegawa and a Bayesian-like transformation modification of the approximate maximum likelihood ration test and ultrafast bootstrapping and are provided in this order at the branches. Branch support is not shown is values for ultrafast bootstrapping were <50%. The scale bar represents 0.04 substitutions per site.

Fig. 4. Multilocus phylogenetic reconstruction of relationship between *Parascaris* spp. using combined data from ITS-1, ITS-2 and mitochondrial cytochrome c oxidase I sequences. Sequences were aligned separately and then alignments were concatenated. Maximum-likelihood phylograms were calculated using IQ-TREE and rooted using sequences from *Ascaris lumbricoides* (*Alu*) and *Ascaris suum* (*Asu*) as outgroup. Separate substitution models were fitted for ITS-1, ITS-2 and for codon positions 1 and 2 and for codon position 3 of cytochrome oxidase. Sequences from karyotyped samples are shown in bold. The species as deposited in GenBank is abbreviated as *Pun*, *Parascaris univalens*, *Peq*, *Parascaris equorum*; *Par*, *Parascaris* spp. Isolate information is highlighted in blue. In case the isolate was not named, the GenBank® accession number was indicated. GenBank® accession numbers are available in Table S3. For sequences extracted from whole genomes, the Bioproject accession number is shown. If the host was a not horse (or unknown), the host species is provided. The country of origin is indicted using two letter ISO 3166-1 codes: CN, China; DE, Germany; PL, Poland; US, U.S.A. For a few samples, the geographic origin was not available in GenBank®. Node support was calculated using the Shimodaira–Hasegawa and a Bayesian-like transformation modification of the approximate maximum likelihood ration test and ultrafast bootstrapping and are provided in this order at the branches. Branch support is not shown is values for ultrafast bootstrapping were <50%. The scale bar represents 0.02 substitutions per site.

Fig. S1. Karyotyping of early *Parascaris* embryos. Orcein-stained eggs, at one-cell stage (left) and two-cell stage (right), showing the presence of one pair of chromosomes (arrows) which identifies the specimens as *Parascaris univalens* (2n=2). Bars, 15 µm.

Fig. S2. Phylogenetic reconstruction of relationship between *Parascaris* spp. using mitochondrial 12S rRNA I sequences. Maximum-likelihood phylograms were calculated using IQ-TREE and rooted using sequences from *Ascaris lumbricoides* (*Alu*) and *Ascaris suum* (*Asu*) as outgroup. Sequences from karyotyped samples are shown in bold. The species as deposited in GenBank is abbreviated as *Pun*, *Parascaris univalens*; *Peq*, *Parascaris equorum*; *Par*, *Parascaris* spp. Isolate information is highlighted in blue. In case the isolate was not named, the GenBank® accession number was indicated. GenBank® accession numbers are available in Table S3. For sequences extracted from whole genomes, the Bioproject accession number is shown. If the host was a not horse (or unknown), the host species is provided. The country of origin is indicted using two letter ISO 3166-1 codes: CN, China; DE, Germany; PL, Poland; US, U.S.A. For a few samples, the geographic origin was not available in GenBank®. Node support was calculated using the Shimodaira–Hasegawa and a Bayesian-like transformation modification of the approximate maximum likelihood ration test and ultrafast bootstrapping and are provided in this order at the branches. Branch support is not shown is values for ultrafast bootstrapping were <50%. The scale bar represents 0.02 substitutions per site.

Conflict of interest statement

Manuscript submitted for publication in the journal 'Infection, Genetics and Evolution'

Very low intraspecific sequence variation in selected nuclear and mitochondrial *Parascaris univalens* genes

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Georg von Samson-Himmelstjerna declares on behalf of all co-autors that there are no conflicts of interest to declare.

Credit author statement:

Georg von Samson-Himmelstjerna: conceptualization, funding acquisition, project management, supervision, writing-original draft, writing-review and editing; I. Jana I. Janssen: investigation, writing-review and editing; Sabrina Ramünke: investigation, writing-original draft, writing-review and editing; Clara Goday: investigation, writing-review and editing; Fernando de A. Borges: investigation, writing-review and editing; Bretislav Koudela: investigation, resources, writing-review and editing; Artur Niedźwied: investigation, writing-review and editing; Krzysztof Tomczuk: investigation, resources, writing-review and editing; Maria Bernadeta Studzińska: investigation, writing-review and editing; Slawomir Kornas: investigation, resources, writing-review and editing; Jürgen Krücken: conceptualization, funding acquisition, formal analysis, supervision, writing-original draft, writing-review and editing

Highlights

- Genetic characterisation of karyotyped *Parascaris univalence* on four marker regions
- No evidence for *Parascaris equorum* in samples
- Highly supported homogenous clusters of *P. univalens* sequences for all loci
- Current annotations of *P. equorum* sequences in GenBank probably misleading
- Few unkaryotyped *P. univalens* denoted sequences with high diversity to *P. univalens*

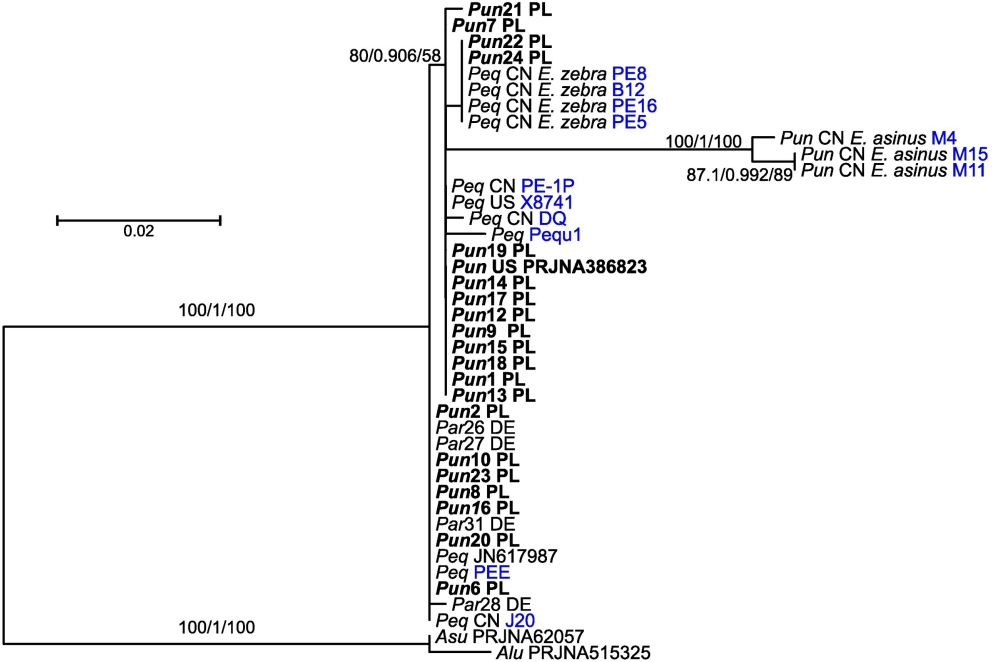
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Figure 1

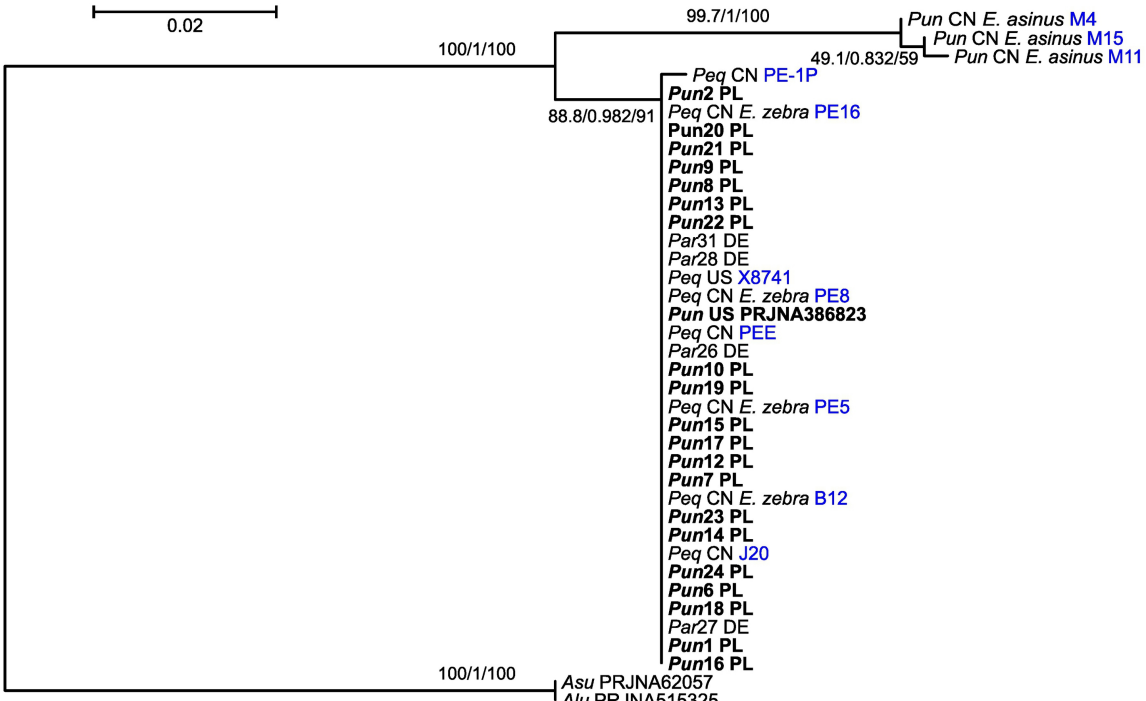
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Figure 2

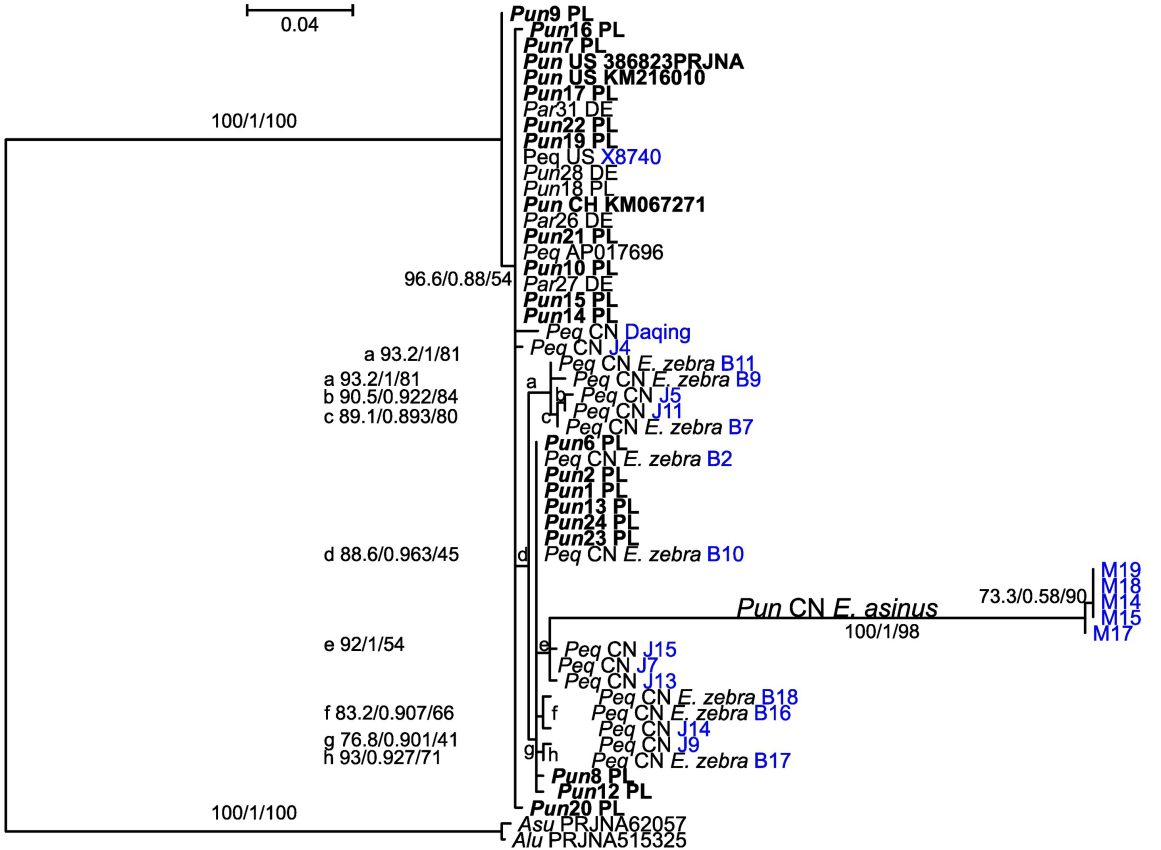
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Figure 3

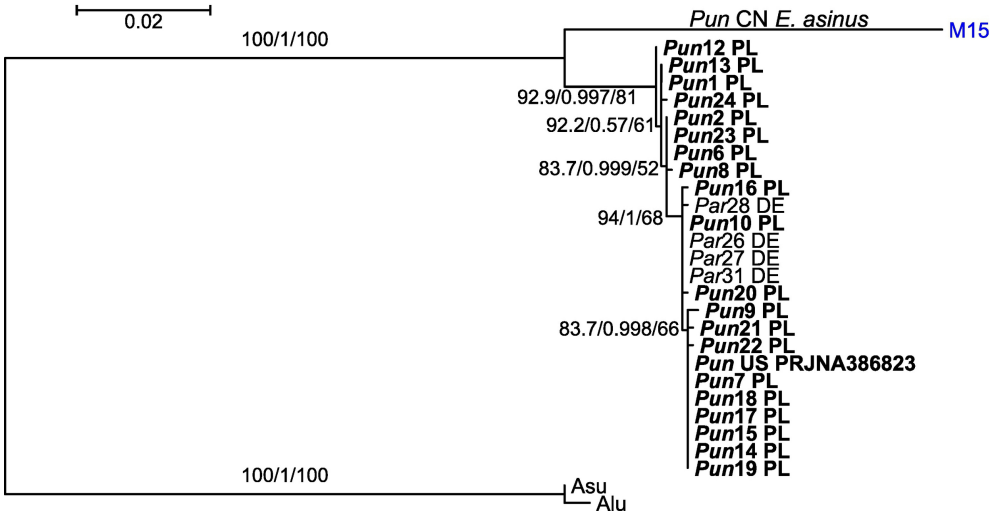
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Figure 4