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The *Strongyloides* (Nematoda) of sheep and the predominant *Strongyloides* of cattle form at least two different, genetically isolated populations

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Abstract

Strongyloides sp. (Nematoda) are very wide spread small intestinal parasites of vertebrates that can form a facultative free-living generation. Most authors considered all *Strongyloides* of farm ruminants to belong to the same species, namely *Strongyloides papillosus* (Wedl, 1856). Here we show that, at least in southern Germany, the predominant *Strongyloides* found in cattle and the *Strongyloides* found in sheep belong to separate, genetically isolated populations. While we did find mixed infections in cattle, one form clearly dominated. This variety, in turn, was never found in sheep, indicating that the two forms have different host preferences. We also present molecular tools for distinguishing the two varieties, and an analysis of their phylogenetic relationship with the human parasite *Strongyloides stercoralis* and the major laboratory model species *Strongyloides ratti*. Based on our findings we propose that *Strongyloides* from sheep and the predominant *Strongyloides* from cattle should be considered separate species as it had already been proposed by [Brumpt, E., 1921. Recherches sur le determinisme des sexes et de l'evolution des Anguillules parasites (*Strongyloides*). Comptes rendu hebdomadaires des séances et mémoires de la Société de Biologie et de ses filiales 85, 149–152], but was largely ignored by later authors. For nomenclature, we follow [Brumpt, E., 1921. Recherches sur le determinisme des sexes et de l'evolution des Anguillules parasites (*Strongyloides*). Comptes rendu hebdomadaires des séances et mémoires de la Société de Biologie et de ses filiales 85, 149–152] and use the name *S. papillosus* for the *Strongyloides* of sheep and the name *Strongyloides vituli* for the predominant *Strongyloides* of cattle.

Keywords: *Strongyloides papillosus*; *Strongyloides vituli*; Nematodes; Host specificity

1. Introduction

The nematode genus *Strongyloides* consists of small intestinal parasites of vertebrates ranging from snakes to humans (Dorris et al., 2002; Speare, 1989). The parasitic form of *Strongyloides* sp. lives as a parthenogenetic female in the small intestine of the host. The progeny produced by these females can undergo either one of two fundamentally different life cycles. (1) They can form infective third-stage larvae (L3i) that infect a new host (homogonic or direct development). (2) They can give rise to a free-living generation that consists of females and males (heterogonic or indirect development). For review and discussion of the life cycle see for example Viney and Lok (2007) and Streit (2008).

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Speare (1989) has estimated that the more than 100 species names that can be found in the literature represent about 50 true species. Identifying the species, to which an individual of *Strongyloides* belongs to, based on morphological characteristics can be difficult or impossible, depending on the developmental stage and the species to be distinguished (*cf.* Augustine, 1940; Speare, 1989). Therefore, some species of *Strongyloides* were defined based on the host they were found in (Augustine, 1940). Further, in the literature, the species a *Strongyloides* isolated from the wild belonged to, was frequently deduced from the host it was found in (discussed by Speare, 1989). Augustine (1940) and Speare (1989) provide discussions of the usefulness and limitations of morphological characteristics for the species identification within the genus *Strongyloides*.

Recently, molecular markers like the sequence of the small ribosomal subunit (*SSU*) rDNA, have become more and more important as taxonomic and diagnostic features (Blaxter et al., 1998; Dorris et al., 2002; Eyualem and Blaxter, 2003; Floyd et al., 2002; Herrmann et al., 2006a, b; Holterman et al., 2006; Mayer et al., 2007).

Strongyloides infections are common in domestic ruminants (Bonfoh et al., 1995; Jäger et al., 2005; Lentze et al., 1999; Pienaar et al., 1999; Wymann et al., 2007). Although these infections often proceed without clinical symptoms (Lentze et al., 1999; Pienaar et al., 1999), they can also cause disease and they can even be fatal (Nakamura and Motokawa, 2000; Nakamura et al., 1994a,b; Pienaar et al., 1999; Taira and Ura, 1991).

Contrary to most more recent authors who considered all *Strongyloides* found in farm ruminants to be *Strongyloides papillosus* (Jäger et al., 2005; Lentze et al., 1999; Pienaar et al., 1999; van Wyk et al., 2004; Wymann et al., 2007; Zaffagnini, 1973), Brumpt (1921) distinguished between the *Strongyloides* of sheep (*S. papillosus*) and the *Strongyloides* of cattle (*Strongyloides vituli*).

The results we present here demonstrate that the *Strongyloides* in sheep and the predominant *Strongyloides* in cattle form distinct populations. We propose that the two forms should be considered separate species.

2. Materials and methods

2.1. Source of Strongyloides materials

Strongyloides spp. were isolated from rectal fecal samples from lambs and calves as described by Eberhardt et al. (2007). The reference isolate Lin is described in Eberhardt et al. (2007). For this study additional samples were taken in the years 2006 and 2007 from lambs in Tübingen, Strassberg and Breitenholz. Samples from calves were taken in Tübingen, Tübingen-Lustnau, Dettingen, Talheim and Breitenholz. All these locations are in Baden-Württemberg, southern Germany.

Samples from Mali: fecal samples from lambs and calves were taken in March (one calf) and June (two lambs and four calves) 2007 in the peri-urban Bamako, the capital of Mali. Samples were packed in the Central Veterinary Laboratory and delivered to Tübingen for analysis within 2 days of collection.

Samples from Pennsylvania: fecal samples from two calves infected with *Strongyloides* were taken on January 27, 2007 in the area of Philadelphia, U.S.A. These were cultured for one week and infective nematode L3is were collected. The samples were frozen in a volume of 40 μl of water and delivered to us for analysis in July 2007. These samples therefore contained a mixture of different nematode species that parasitized the two calves. As far as *Strongyloides* is concerned, these larvae are expected to be a mixture of L3is from the homogonic and the heterogonic cycle.

2.2. Culturing Strongyloides

Strongyloides was cultured as described by Eberhardt et al. (2007). In brief: The feces were crushed and mixed with sterile sawdust such that the sawdust contributed 1/3 to 1/2 of the total volume. The mixture was hydrated well and incubated at 25 °C for 1–2 days in a water saturated atmosphere. To collect the worms, the feces was wrapped in paper tissue (KIMTECH, Kimberly-Clark® product number 7101) and placed in a funnel filled with tap water (Baermann funnel) at room temperature. The worms that accumulated at the bottom of the funnel were picked individually and analyzed or used for the infection of rabbits. To maintain the culture of the reference isolate Lin the fecal pellets were treated as described above followed by culturing for 6–9 days in a Petri dish that was placed in a larger Petri dish with water. The L3i that accumulated in the water were collected and used for the infection of rabbits. Rabbits were infected by subcutaneous injection of the desired number of L3is in water.

2.3. Single worm lysis

Single worms were lysed and prepared for PCR analysis as described in Eberhardt et al. (2007) with

slight modifications. In brief, each worm was transferred into 4 μl of lysis buffer, which consisted of 10 mM Tris–HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% (v/v) NP-40, 0.45% (v/v) Tween-20 and 120 μg/ml proteinase K, frozen at −70 °C for 10 min and incubated at 65 °C for 60 min followed by a 15 min incubation at 95 °C to inactivate the proteinase K. Then 6 μl of water were added and 2 μl of this lysate were used as template for PCR amplification.

2.4. PCR amplification and sequencing of portions of the SSU sequence from single worms

PCR amplification with the primers RH5401 (5′-AAAGATTAAGCCATGCATG-3') and RH5402 (5′-CATTCTTGGCAAATGCTTTCG-3') and sequencing of the *SSU* with the primer RH5403 (5′-AGCTGGAATTACCGCGGCTG-3′) were done as described in Eberhardt et al. (2007). For this publication we refer to the 468 nt portion of the 18S rDNA that can be reliably read with this method as the *SSU* sequence (Fig. 1).

2.5. Genotyping at the SSU locus

Two different *S. papillosus SSU* sequences had been published and deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/), one by Dorris et al. (2002) (accession number AJ417027) and one by Eberhardt et al. (2007) (accession number EF066361). In Dorris et al. (2002) only 330 bp of the *SSU* were published. We did not find any differences in the remaining 131 nt (accession number EU885229). The genotype of individual worms at the *SSU* locus was determined by PCR amplification of the locus as described above followed by restriction analysis with *Psi* I as follows: 5–10 μl of the PCR reaction mix were digested with 0.5 U of *Psi* I (New England Biolabs) in

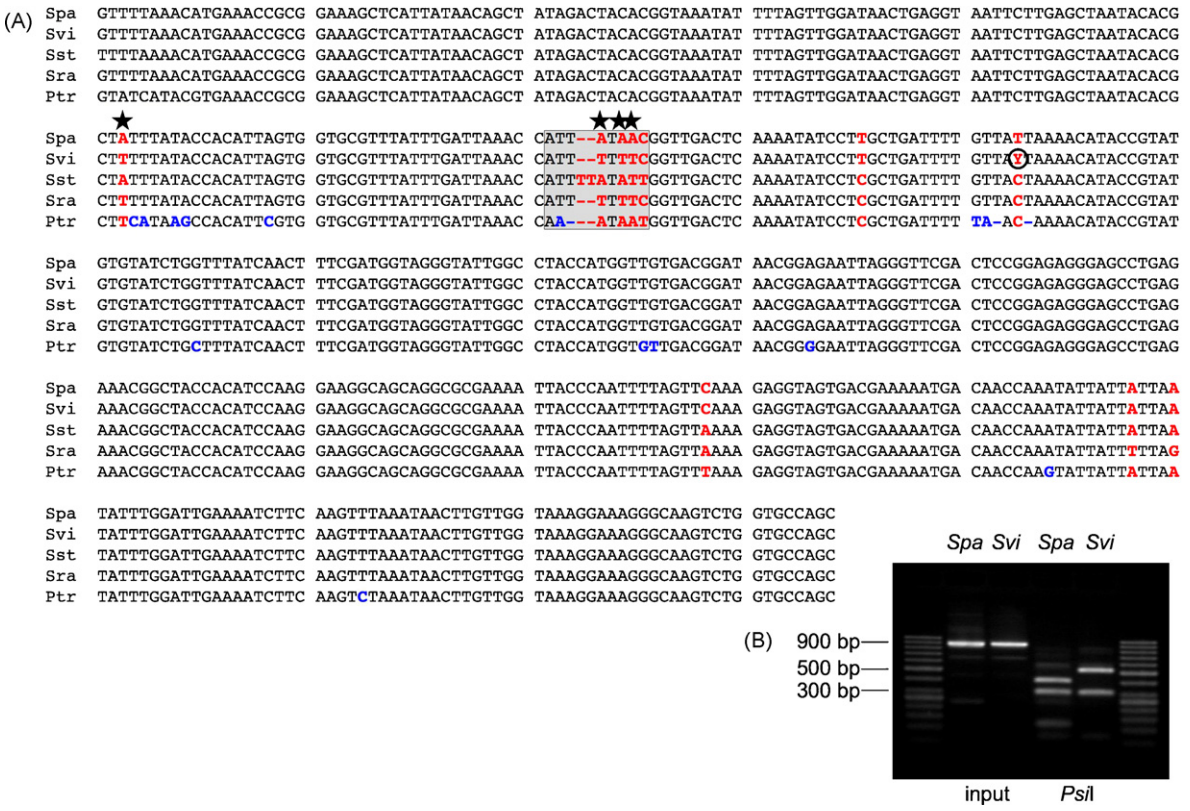


Fig. 1. (A) Alignment of the *SSU* sequences. *Spa*: *S. papillosus*, *Svi*: *S. vituli*, *Sst*: *S. stercoralis*, *Sra*: *S. ratti*, *Ptr*: *P. trichosuri*. Positions that differ between *S. papillosus* and *S. vituli* are marked with an asterisk. Positions with sequence differences within the four species of *Strongyloides* are highlighted in bold in all sequences (red). Positions that differ only in *P. trichosuri* are highlighted in bold in the *P. trichosuri* sequence (blue). The molecular synapomorphy according to Dorris et al. (2002) is boxed. A polymorphism that is likely to be a polymorphism between different copies of the rDNA within *S. vituli* (see Section 2) is circled. (B) Diagnostic *Psi* I restriction digest to distinguish *S. papillosus* and *S. vituli*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the manufacturer supplied buffer in a total volume of 20 μl at 37 °C for 2 h. The entire volume was analyzed by electrophoresis on a 2.5% agarose, 1× TAE gel (Sambrook and Russel, 2001). Digestion of the fragment produced by PCR with the primers RH5401 and RH5402 resulted either in products of 391 bp, 298 bp, 113 bp and 63 bp if the worm had the *SSU* sequence published by Eberhardt et al. (2007) or of 504 bp, 298 bp and 63 bp if the worm had the *SSU* sequence published by Dorris et al. (2002). From all sample sites the *SSU* of several worms was also analyzed by sequencing as described above. We noticed that the majority of the *Strongyloides* that otherwise had the sequence as published by Dorris et al. (2002) showed a mixture of T and C at the position indicated in Fig. 1. The C signal was variably strong but always weaker than the T signal. The remaining animals were monomorphic for T and none were monomorphic for C. This indicates that this is a polymorphism between different copies of the ribosomal DNA locus and not different alleles of the same locus. This polymorphism was detected in samples from all three continents. The polymorphism was treated as an ambiguity for the phylogenetic analysis.

2.6. Analysis of the samples from Philadelphia

The samples were thawed and 120 μl of lysis buffer was added followed by incubations at −70 °C for 10 min, 65 °C for 60 min and 95 °C for 15 min, after which 1040 μl of water was added. Four microliters of lysis buffer were processed in parallel (at the end 26 μl of water was added) to serve as templates for negative controls. Of each lysate or of negative control template 10 μl were used for the PCR amplification of the *SSU* in a total volume of 25 μl as described above (one reaction per sample plus two negative controls). PCR product was only detected in reactions derived from the two samples. The PCR products (4 μl per reaction) were cloned into TOPO TA (Invitrogen) and the resulting plasmids transfected into chemically competent TOP10 *Escherichia coli* bacteria (Invitrogen) following the manufacturer supplied protocols. For each sample plasmid DNA from 96 clones was isolated from 1 ml of overnight culture in LB supplemented with 50 μg/ml ampicillin (Sambrook and Russel, 2001) using the MagAttract 96 Miniprep Core Kit (Qiagen) according to the manufacturers instructions. One microliter of the resulting plasmid solution was used for sequencing with primer RH5403 as described above.

2.7. Cloning large subunit ribosomal protein (rpl) genes from S. vituli

Portions of *rpl-10*, *rpl-26* and *rpl-27* from *S. vituli* were cloned by degenerate PCR as described for *S. papillosus* by Eberhardt et al. (2007) using the same primers. The resulting sequences were submitted to GenBank. The accession numbers are: (EU885231, EU885228 and EU885230).

2.8. Phylogenetic analysis

The phylogenetic tree was determined from concatenated sequences including the three *rpl* sequences plus 468 nt of *SSU*, totaling 1475 nucleotides, 84 of which were parsimony-informative. As an out-group we used *Parastrongyloides trichosuri*, a parasite of Australian possums that has been established recently as an experimental system (Grant et al., 2006). The *rpl* sequences for *S. papillosus* (isolate Lin), *Strongyloides ratti*, *Strongyloides stercoralis* and *P. trichosuri* were taken from Eberhardt et al. (2007). A FASTA file with the concatenated sequences and a NEXUS file with the alignments are available as Online supplementary information. The phylogenetic tree was reconstructed using the heuristic search algorithm under the maximum parsimony criterion with the help of the PAUP*4.0b10 software (Swofford, 2002). Ambiguities were interpreted as polymorphisms and alignment gaps were used as a fifth character. For representation the tree was rooted at midpoint. The topological stability of the tree was assessed by 1000 bootstrap replications (Felsenstein, 1985).

2.9. Hybridization experiments

Two sets of experiments were done.

Set 1: Individual females from a culture derived from a calf from Mali and passed through a rabbit once (rabbit number 3 in Table 2) or from the reference isolate Lin were crossed to individual Lin males as described by Eberhardt et al. (2007). Two days after transferring the worms onto plates the presence or absence of larvae was determined and the *SSU* genotype of the parents was determined. If larvae were found, mating was considered to be successful. Only plates where both parents were still present and alive were considered.

Set 2: Individual females and males from either cultures from calves from Tübingen or Lin were crossed to Lin partners and analyzed as described above. As a control several females were cultured in absence of males. Not all of these unmated females were

genotyped but they were taken from a culture of which we genotyped a total of 61 free-living females. Of them, 56 were *S. vituli* and five of them were *S. papillosus*, therefore the vast majority of the females cultured without males must have been *S. vituli*. For confirmation, we genotyped five of them and all were *S. vituli*.

3. Results

3.1. Both published S. papillosus SSU sequences exist in the wild

We have identified a total of 31 lambs and 19 calves in southern Germany that were infected with *Strongyloides* and we have analyzed the *SSU* of a total of 291 individual *Strongyloides* of different stages derived from lambs and a total of 553 derived from calves (Table 1). The *SSU* of all individuals derived from lambs was as described by Eberhardt et al. (2007). The *SSU* of the vast majority (542) of the worms isolated from calves was as described by Dorris et al. (2002) but there were a small number of worms (10) with an *SSU* as described by Eberhardt et al. (2007). Only a single larva appeared to contain both sequences (see Section 4). This confirms that both sequences that had been published as *S. papillosus SSU* exist in naturally occurring *Strongyloides* populations that were generally considered to belong to the species *S. papillosus*.

3.2. Both sequences exist also in Africa and North America

We analyzed *Strongyloides* derived from fecal samples from two lambs and five calves that we collected in Mali, Africa. From the cultures derived from the lambs we genotyped a total of 160 worms. All of them had the same *SSU* sequence as the worms we had found in sheep in Germany (Table 1). From the cultures derived from the calves we analyzed a total of 224 individuals. The *SSU* of 198 of them was as described by Dorris et al. (2002) and the *SSU* of 26 of them was as described by Eberhardt et al. (2007) (Table 1). Thus, both sequences also exist in at least one location in Africa.

From the veterinary hospital at the University of Pennsylvania we received mixtures of dead infective nematode larvae isolated from fecal samples from two calves. From these samples we PCR amplified the *SSU* and cloned the PCR product (see Section 2). We found a total of 107 clones that contained inserts derived from *Strongyloides* (80 from one calf and 27 from the other). Eighty-one (72 and 9, respectively) of these clones showed the sequence described by Dorris et al. (2002) and 26 (8 and 18, respectively) the one described by Eberhardt et al. (2007). These results demonstrate that both types also exist in at least one location in North America.

3.3. The bearers of the two different SSU sequences form separate populations and therefore belong to different species

According to a biological species concept, two sympatric populations that are reproductively (genetically) isolated from each other belong to different species (*cf.* Mayr, 1970; Sudhaus and Rehfeld, 1992). For the following reasons we are convinced that the bearers of the two different *SSU* sequences fulfill this condition and we propose to consider them different species:

(i) Sequence divergence: The two *SSU* sequences differ in four out of the 468 bp that we analyzed

Table 1
Distribution of individual *Strongyloides* with the two different *SSU* sequences in fecal cultures from lambs and calves

	Males			Females			L3is		
	Total	*Spa*	*Svi*	Total	*Spa*	*Svi*	Total	*Spa*	*Svi*
Lamb Germany	1	1	0	161	161	0	129	129	0
Lamb Mali	8	8	0	30	30	0	122	122	0
Lamb total	9	9	0	191	191	0	251	251	0
Calf Germany	9	1	8	103	7	96	441[b]	2[b]	438[b]
Calf Mali	7	6	1	6	6	0	211	14	197
Calf total	16	7	9	109	13	96	652[b]	16[b]	635[b]
Calf mixed[a]	14	6	8	68	11	57	261	16	245

Spa: *S. papillosus*, *Svi*: *S. vituli*.
[a] Numbers from animals with confirmed mixed infections only. These numbers are included in the rows above.
[b] One L3i appeared to be a hybrid (see text). Therefore, the total is one greater than the sum of *Spa* + *Svi*.

Table 2
Infection of rabbits with *Strongyloides* derived from calves

Rabbit	Original material		Isolated from rabbit *Svi*/*Spa*		
	L3is used for infection	Estimated proportion of *Svi*[a]	Males	Females	L3is
1	1000	>90% ($n = 55$)[b]	0/26	0/22	15/36
2	150	ND	0/0	0/3	3/52
3	350	87.5% ($n = 112$)	0/0	0/19	0/62
4	200	>95% ($n = 99$)[b]	0/3	0/118	0/232
1–4	1700		0/29	0/162	18/382

Spa: *S. papillosus*, *Svi*: *S. vituli*.
[a] The species of the indicated number (n) of L3is from the same culture as the ones used for infection was determined by *SSU* genotyping.
[b] No *S. papillosus* larvae were found.

(Fig. 1). Given the high degree of conservation of the *SSU* in the genus *Strongyloides* (Dorris et al., 2002) and in other nematodes (*cf.* Blaxter et al., 1998; Eyualem and Blaxter, 2003; Floyd et al., 2002; Mayer et al., 2007), it is most unlikely that the observed difference exists within one and the same species. Such a difference rather argues for a considerable time of genetic separation (see Section 4).

(ii) Reproductive isolation in spite of sympatry: We have analyzed the *SSU* sequence of a total of 343 *Strongyloides* isolated from calves with confirmed mixed infections. Of those, 310 were as described by Dorris et al. (2002) and 33 were as described by Eberhardt et al. (2007) (Table 1), indicating that the two species form distinct, apparently reproductively isolated sympatric populations.

For the reminder of this publication we treat the two forms as separate species. We retain the name *S. papillosus* for the species in sheep. For the form that we found exclusively in cattle we reinstate the species *S. vituli* Brumpt, 1921 (see Section 4).

3.4. The two species differ in their host preference

Among 451 *Strongyloides* isolated from a total of 33 lambs we did not find a single *S. vituli* and of the 776 worms isolated from a total of 24 calves only 36 (4.6%) were *S. papillosus*. Interestingly the proportion of the latter was much higher among the free-living animals (16%, $n = 125$) than among the L3is of the direct cycle (2.5%, $n = 652$) (Table 1; $p = 3.78e{-}11$ [binomial test, the R project for statistical computing, http://www.R-project.org]). When only worms isolated from calves with confirmed mixed infections are considered, the numbers are 20.7% ($n = 82$) and 6.1% ($n = 261$), respectively (Table 1; $p = 8.41e{-}6$). This might be an indication that calves are suboptimal hosts for this species (see Section 4).

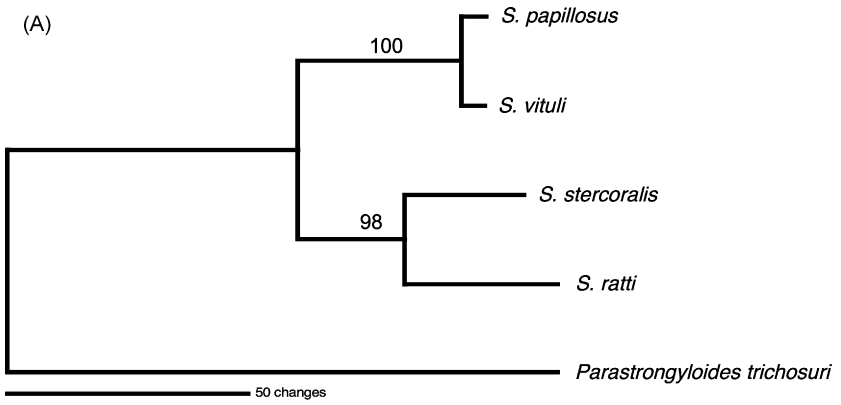
The two species also showed a difference in host preferences when we attempted to raise them in rabbits. We infected four rabbits with L3is isolated from calves with mixed infections. Although among the larvae used for infection *S. vituli* predominated strongly, in the resulting infections in rabbits we found predominantly (in two cases) or exclusively (in two cases) *S. papillosus* (Table 2). Even in the cases where *S. vituli* were found they disappeared quickly during the infection (data not shown).

3.5. Phylogenetic relationship of the two species

To determine the phylogenetic position of *S. vituli* within the genus *Strongyloides*, we cloned parts of three genes, *rpl-10*, *rpl-26* and *rpl-27*, that code for ribosomal proteins and used these sequences, together with the *SSU* sequence for phylogenetic analysis (Fig. 2). The two species from ruminants group together and are clearly separated from the two well-studied species, *S. ratti* and *S. stercoralis* (Viney, 2006).

3.6. Distinguishing the two species

We have failed to find any morphological differences that would enable reliable distinction between the two species (see Section 4). The most straightforward way of identifying the species is to PCR amplify the *SSU* and analyze it by sequencing or restriction analysis with *Psi* I according to the protocols described in Section 2. Digestion of the fragment produced by PCR with the primers RH5401 and RH5402 results in products of 391 bp, 298 bp, 113 bp and 63 bp in *S. papillosus* and of 504 bp, 298 bp and 63 bp in *S. vituli* (Fig. 1B).



(B)

	1	2	3	4	5
1 *Spa*	-	0.00815	0.05567	0.05974	0.13605
2 *Svi*	12	-	0.05838	0.05499	0.13605
3 *Sst*	82	86	-	0.03870	0.13469
4 *Sra*	88	81	57	-	0.14218
5 *Ptr*	200	200	198	209	-

Fig. 2. Phylogenetic relationship of *S. papillosus*, *S. vituli*, *S. ratti* and *S. stercoralis*. (A) Maximum parsimony tree based on partial sequences of the *SSU*, *rpl-10*, *rpl-26* and *rpl-27* (total 1475 characters). *P. trichosuri* served as out-group for the analysis. The numbers are bootstrap values based on 1000 bootstraps. (B) Pair wise distances between taxa. Below diagonal: Total character differences. Above diagonal: Mean character differences. *Spa*: *S. papillosus*, *Svi*: *S. vituli*, *Sst*: *S. stercoralis*, *Sra*: *S. ratti*, *Ptr*: *P. trichosuri*.

Table 3
Interbreeding experiments

Female × male	Experiment 1		Experiment 2			
	Lin × Lin	Mali^{Spa} × Lin	Tub^{Spa} × Lin	Tub^{Svi} × Lin	Lin × Tub^{Svi}	Tub^{female}
Number of crosses	14	14	2	26	3	19[a]
Number of females with progeny	11	10	1	18	0	0

Lin: Reference isolate for *S. papillosus* (Eberhardt et al., 2007). Mali: isolated from calf from Mali and passed through a rabbit once. Tub: isolated from calf from Tübingen, *Spa*: *S. papillosus*, *Svi*: *S. vituli*.

[a] The majority of these worms were *S. vituli* (see Section 2).

3.7. Hybridization experiments

Free-living adults of *S. papillosus* and the relatively closely related *Strongyloides ransomi* (the *Strongyloides* of swine) have been shown to be capable of producing live larvae upon inter-species mating with each other (Triantaphyllou and Moncol, 1977). To determine if this is also the case for the two closely related species described in this report we performed interbreeding tests as described in Section 2 (Table 3). Females of both species gave rise to larvae when crossed with males from the *S. papillosus* reference isolate Lin and genotyping several of the resulting larvae at the *SSU* locus confirmed that true hybridization did occur. Females that did not have access to males did not produce any progeny indicating that males are required. Since free-living males were rare in our cultures derived from calves we were not able to set up crosses using such males in sufficient numbers to be conclusive. The three males we could cross were identified as *S. vituli*, and none of them sired progeny. These results indicate that, at least under experimental conditions, the two species can form live progeny. However, the fact that we found only a single hybrid in nature is strong indication that the resulting individuals do not contribute genetically to the original populations, as it is expected for hybrids between different species (see Section 4).

4. Discussion

4.1. The Strongyloides in sheep and the predominant Strongyloides in cattle are different

Here we demonstrate that the *Strongyloides* of sheep is genetically different from the predominant *Strongyloides* of cattle and we argue that what is generally

referred to as *S. papillosus* are in fact at least two distinct, relatively closely related, species. We base our claim mainly on two observations, namely sequence differences in the *SSU* and the absence of hybrids. The *SSU* has been used extensively in nematodes for taxonomic and phylogenetic analyses at different taxonomic levels either by itself (Blaxter et al., 1998; Dorris et al., 2002; Eyualem and Blaxter, 2003; Floyd et al., 2002; Holterman et al., 2006) or in combination with additional sequences (Mayer et al., 2007). A detailed evaluation of the *SSU* as a "molecular operational taxonomic unit (MOTU)" can be found in Floyd et al. (2002). Obviously, a MOTU based on *SSU* sequences does not necessarily equal a biological species (*cf.* Floyd et al., 2002). However, several authors have compared the *SSU* of related but well-established species in the genus *Strongyloides* (Dorris et al., 2002) and other nematode genera (Floyd et al., 2002; Mayer et al., 2007). Except for rare polymorphisms among different copies of the *SSU* loci within genomes, none of these authors found within-species differences in the region of the *SSU* analyzed in this publication. On the other hand, all these authors found cases where different species could not be distinguished based on this sequence interval because it is identical. Therefore, classification based on the *SSU* rather wrongfully combines true species than inappropriately splits one and it is most unlikely that the four nucleotides difference we observed between *S. papillosus* and *S. vituli* could exist within one and the same nematode species. If so, we would further expect to find intermediates differing by one, two, or three nucleotides among the tested specimens.

The existence of calves with mixed infections illustrates that these host-animals are exposed to both species. This is also consistent with the rearing regimes at the sampling sites. In Mali sheep and cattle are kept in combined herds. In southern Germany the two species are kept separately but frequently they are housed in close proximity and they sometimes use the same pastures sequentially. Nevertheless, among all the worms isolated from the wild, we found only a single individual that appeared to be a hybrid. We do not know if this was indeed a true hybrid or an artifact due to mishandling a worm such that accidentally two larvae were put into one PCR tube. If it was a hybrid, it could also have been the progeny of free-living parents that happened to be close to the anus of a calf and was therefore accidentally picked up during the sampling process. Indeed, we found that the free-living adults of the two species can give rise to hybrid larvae. Experimental inter-species hybridization was also described by Triantaphyllou and Moncol (1977) for *S. papillosus* derived from sheep and *S. ransomi* from swine. Like Triantaphyllou and Moncol (1977), we do not know if the resulting larvae would have been capable of infecting a host. However, although no formal proof, the virtual absence of hybrids from our samples indicates that normally either no inter-species mating occurs or that the resulting larvae are not successful in contributing genetically to the original populations in sheep and cattle. The formation of inter-species hybrids upon experimental mating has been observed for various animal species (for examples see Mayr, 1970; Sudhaus and Rehfeld, 1992), including nematode species in the genera *Caenorhabditis* (Baird, 2002) and *Pristionchus* (Herrmann et al., 2006b). The existence of such hybrids, even if they are occasionally fertile, is no reason to conclude that the parental populations belong to the same species, as long as the natural populations remain genetically separated (*cf.* Mayr, 1970; Sudhaus and Rehfeld, 1992).

Taken all our results together, we conclude that the *Strongyloides* in sheep and the predominant *Strongyloides* of cattle do not form a single population, but two independent populations that are reproductively isolated, although they co-exist sympatrically. This has implications for the population genetics of these parasites, for example for the chances of the appearance and the dynamics of spreading drug resistances.

4.2. Nomenclature

Our results support the opinion of Brumpt (1921) and we propose that this author should be followed for nomenclature. Accordingly, the names should be: (a) *S. papillosus* (Wedl, 1856) for the species that was found in sheep and, at very low infection levels, in cattle. This species has the *SSU* sequence described by Eberhardt et al. (2007). (b) *S. vituli* Brumpt, 1921 for the species that was found in cattle. This species has the *SSU* sequence described by Dorris et al. (2002). We think that this nomenclature is justified for the following reasons: (i) the *S. papillosus* (then called *Trichosoma papillosum*) described in the original publication was isolated from sheep (Wedl, 1856); (ii) in this and an earlier study (Eberhardt et al., 2007) we found no evidence for more than one species of *Strongyloides* in this host; (iii) to our knowledge, the only author who described a *Strongyloides* isolated from cattle as a new species is Brumpt (1921) who named it *S. vituli*. However, we would like to point out that (Gasperi, 1912) described *Strongyloides* isolated from cattle as a

separate subspecies of *Strongyloides longus* (*S. longus bovis*). Contrary to Brumpt (1921), this author provided a detailed morphological description. *S. longus* was later synonymized with *S. papillosus* (Schwartz and Alicata, 1930).

4.3. Distinguishing the two species

We present protocols to distinguish the two species at the single individual level using molecular tools. We have been unable to determine morphological difference between the two species that would allow to reliably assign an individual to one of the two species. Eggs and L3is are the most easily accessible developmental stages and therefore frequently used for identifications (van Wyk et al., 2004). We measured the total body length and pharynx length (data not shown). As it had been described before (Basir, 1950), these measures varied with time and culture conditions. Morphometric differences have been described as poor characteristics for identifying the species a *Strongyloides* individual belongs to (Augustine, 1940). Similar difficulties have also been described for other nematodes (Eyualem and Blaxter, 2003; Herrmann et al., 2006a,b; Mayer et al., 2007). Therefore, the only way of identification we can offer, which does not invoke the host as the distinguishing criterion is by the methods of molecular biology.

Two species of *Strongyloides* occurring in sheep and cattle is a minimal estimate. We cannot exclude that there are even more species that we fail to distinguish with the methodology employed in this study. As mentioned in Section 4.1 several authors found cases where accepted nematode species did not differ in the portion of the *SSU* analyzed here.

4.4. The two species have different host preferences

While we never found *S. vituli* in sheep, *S. papillosus* did occur in cattle in low numbers. However, it appeared to develop preferentially through the heterogonic cycle when living in this host. This might be an indication that calves are not well suited as hosts for this species. Several authors had noticed that raising *S. papillosus* in rabbits, an experimental and presumably suboptimal host, lead to a high proportion of heterogonic development (Brumpt, 1921; Matoff, 1936; Sandground, 1926; Triantaphyllou and Moncol, 1977).

All host-animals analyzed for this study were considered healthy by their keepers. It remains to be confirmed that the findings described above also hold true for heavily infected lambs and calves that suffer from acute strongyloidiasis.

4.5. Strongyloides papillosus but not S. vituli can be efficiently raised in rabbits

Already Zaffagnini (1973) mentioned that he was incapable of transferring a calf strain of *S. papillosus* to rabbits and he quoted personal communications from other researchers who attempted to do the same to no avail. We successfully infected rabbits with *Strongyloides* larvae isolated from calves four times (Table 2). In all cases *S. vituli* dominated strongly in the original sample. However, the larvae that were later isolated from the rabbits were, either in their vast majority or exclusively, *S. papillosus*. This indicates that only *S. papillosus* established itself in the rabbit efficiently. This has implications for the interpretation of studies where *Strongyloides* was isolated from cattle and propagated in rabbits. It may well be that the species studied was not the predominant *Strongyloides* species present in the original sample.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2008.07.019.

References

Augustine, D.L., 1940. Experimental studies on the validity of species in the genus *Strongyloides*. Am J. Trop Med. Hyg. 32, 24–32.

Baird, S.E., 2002. Haldane's rule by sexual transformation in *Caenorhabditis*. Genetics 161, 1349–1353.

Basir, M.A., 1950. The morphology and development of the sheep nematode, *Strongyloides papillosus* (Wedl, 1856). Can J. Res. [D] 28, 173–196.

Blaxter, M.L., De Ley, P., Garey, J.R., Liu, L.X., Scheldeman, P., Vierstraete, A., Vanfleteren, J.R., Mackey, L.Y., Dorris, M., Frisse, L.M., Vida, J.T., Thomas, W.K., 1998. A molecular evolutionary framework for the phylum Nematoda. Nature 392, 71–75.

Bonfoh, B., Zinsstag, J., Ankers, P., Pangui, L.J., Pfister, K., 1995. Epidemiology of gastrointestinal nematodes in small ruminants in the plateau areas in Togo. Rev. Elev. Med. Vet. Pays Trop. 48, 321–326.

Brumpt, E., 1921. Recherches sur le determinisme des sexes et de l'evolution des Anguillules parasites (*Strongyloides*). Comptes rendu hebdomadaires des séances et mémoires de la Société de Biologie et de ses filiales 85, 149–152.

Dorris, M., Viney, M.E., Blaxter, M.L., 2002. Molecular phylogenetic analysis of the genus *Strongyloides* and related nematodes. Int. J. Parasitol. 32, 1507–1517.

Eberhardt, A.G., Mayer, W.E., Streit, A., 2007. The free-living generation of the nematode *Strongyloides papillosus* undergoes sexual reproduction. Int. J. Parasitol. 37, 989–1000.

Eyualem, A., Blaxter, M., 2003. Comparison of biological, molecular, and morphological methods of species identification in a set of cultured *Panagrolaimus* isolates. J. Nematol. 35, 119–128.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Floyd, R., Abebe, E., Papert, A., Blaxter, M., 2002. Molecular barcodes for soil nematode identification. Mol. Ecol. 11, 839–850.

Gasperi, F., 1912. L' uncinaria radiata e l'anguilla intestinalis bovis. Natura 3, 111–120.

Grant, W.N., Stasiuk, S., Newton-Howes, J., Ralston, M., Bisset, S.A., Heath, D.D., Shoemaker, C.B., 2006. *Parastrongyloides trichosuri*, a nematode parasite of mammals that is uniquely suited to genetic analysis. Int. J. Parasitol. 36, 453–466.

Herrmann, M., Mayer, W.E., Sommer, R.J., 2006a. Nematodes of the genus *Pristionchus* are closely associated with scarab beetles and the Colorado potato beetle in Western Europe. Zoology (Jena) 109, 96–108.

Herrmann, M., Mayer, W.E., Sommer, R.J., 2006b. Sex, bugs and Haldane's rule: the nematode genus *Pristionchus* in the United States. Front Zool. 3, 14.

Holterman, M., van der Wurff, A., van den Elsen, S., van Megen, H., Bongers, T., Holovachov, O., Bakker, J., Helder, J., 2006. Phylum-wide analysis of *SSU* rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown Clades. Mol. Biol. Evol. 23, 1792–1800.

Jäger, M., Gauly, M., Bauer, C., Failing, K., Erhardt, G., Zahner, H., 2005. Endoparasites in calves of beef cattle herds: management systems dependent and genetic influences. Vet. Parasitol. 131, 173–191.

Lentze, T., Hofer, D., Gottstein, B., Gaillard, C., Busato, A., 1999. Häufigkeiten und Bedeutung von Endoparasiten bei Kälbern aus Schweizer Mutterkuhbetrieben. Dtsch Tierärztl Wochenschr 106, 275–281.

Matoff, K., 1936. Beobachtungen über die larvale Entwicklung von *Strongyloides papillosus* (Wedl, 1856) und Infektionsversuche mit filariformen Larven. Parasitol. Res. 8, 474–491.

Mayer, W.E., Herrmann, M., Sommer, R.J., 2007. Phylogeny of the nematode genus *Pristionchus* and implications for biodiversity, biogeography and the evolution of hermaphroditism. BMC Evol. Biol. 7, 104.

Mayr, E., 1970. Populations, Species, and Evolution. The Belknap Press of Harvard University Press, Cambridge.

Nakamura, Y., Motokawa, M., 2000. Hypolipemia associated with the wasting condition of rabbits infected with *Strongyloides papillosus*. Vet. Parasitol. 88, 147–151.

Nakamura, Y., Tsuji, N., Taira, N., 1994a. Wasting condition under normal cardiac rhythms in rabbits following *Strongyloides papillosus* infection. J. Vet. Med. Sci. 56, 1005–1007.

Nakamura, Y., Tsuji, N., Taira, N., Hirose, H., 1994b. Parasitic females of *Strongyloides papillosus* as a pathogenetic stage for sudden cardiac death in infected lambs. J. Vet. Med. Sci. 56, 723–727.

Pienaar, J.G., Basson, P.A., du Plessis, J.L., Collins, H.M., Naude, T.W., Boyazoglu, P.A., Boomker, J., Reyers, F., Pienaar, W.L., 1999. Experimental studies with *Strongyloides papillosus* in goats. Onderstepoort J. Vet. Res. 66, 191–235.

Sambrook, J., Russel, D.W., 2001. Molecular Cloning—A Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press, New York.

Sandground, J.H., 1926. Biological studies on the life-cycle in the genus *Strongyloides* Grassi, 1879. Am J. Hyg. 6, 337–388.

Schwartz, B., Alicata, J.E., 1930. Species of the nematode genus *Strongyloides* parasitic in domestic swine. J. Agric. Res. 40, 11–23.

Speare, R., 1989. Identification of species of *Strongyloides*. In: Grove, D.I. (Ed.), Strongyloidiasis: A Major Roundworm Infection of Man. Taylor & Francis, London, pp. 11–83.

Streit, A., 2008. Reproduction in *Strongyloides* (Nematoda): a life between sex and parthenogenesis. Parasitology 135, 285–294.

Sudhaus, W., Rehfeld, K., 1992. Einführung in die Phylogenetik und Systematik. Gustav Fischer Verlag, Stuttgart, Jena, New York.

Swofford, D.L., 2002. PAUP: Phylogenetic Analysis Using Parsimony, and Other Methods, Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

Taira, N., Ura, S., 1991. Sudden death in calves associated with *Strongyloides papillosus* infection. Vet. Parasitol. 39, 313–319.

Triantaphyllou, A.C., Moncol, D.J., 1977. Cytology, reproduction, and sex determination of *Strongyloides ransomi* and *S. papillosus*. J. Parasitol. 63, 961–973.

van Wyk, J.A., Cabaret, J., Michael, L.M., 2004. Morphological identification of nematode larvae of small ruminants and cattle simplified. Vet. Parasitol. 119, 277–306.

Viney, M.E., 2006. The biology and genomics of *Strongyloides*. Med. Microbiol. Immun. 195, 49–54.

Viney, M.E., Lok, J.B., 2007. *Strongyloides* spp. (May 23, 2007), WormBook. In: The *C. elegans* Research Community (Ed.), WormBook, doi/10.1895/wormbook.1.141.1, http://www.wormbook.org.

Wedl, C., 1856. Über einige Nematoden. In Sitzungsberichte der kaiserliche Akademie der Wissenschaften. Mathematisch-naturwissenschaftlichen Classe (Wien, Kaiserliche Akademie der Wissenschaften), pp. 122–134.

Wymann, M.N., Bonfoh, B., Traore, K., Tembely, S., Zinsstag, J., 2007. Species diversity and acquisition of gastrointestinal parasites in calves aged 0–13 months in periurban livestock production in Mali. Vet. Parasitol. 143, 67–73.

Zaffagnini, F., 1973. Parthenogenesis in the parasitic and free-living forms of *Strongyloides papillosus* (Nematoda, Rhabdiasoidea). Chromosoma 40, 443–450.