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# Molecular Identification of Abomasal Trichostrongylids Infesting Zebu Gudali Cattle in Ngaoundéré, Cameroon

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## Abstract

Trichostrongylids are reported to be the most prevalent and highly pathogenic gastro-intestinal nematode parasites in livestock, particularly in young calves and small ruminants. However, conventional diagnostic tools routinely used in Cameroon cannot reliably distinguish the different species within a genus. This limits our current understanding of co-infestations in livestock with multiple closely related species. Here, molecular tools and morphology were combined to characterize the infections of Gudali zebu cattle in Ngaoundéré, Cameroon with trichostrongylids of veterinary importance (*e.g. Haemonchus* spp., *Trichostrongylus* spp. and *Cooperia* spp.). The hypervariable region I of the small subunit 18S rDNA (SSU HVRI) and the Internal Transcribed Spacer II (ITS-2) DNA region of individual trichostrongyloid worms were amplified, sequenced, and compared with available database entries. Consistent with earlier findings the SSU HVRI was invariable within genera in our data set but the ITS-2 was useful for molecular taxonomy. Trichostrongylid species identification based on sequence information is compromised by several, presumably, erroneous database entries. Our findings argue that within this single host species concurrent infestations occur of at least two different species of *Haemonchus* (*H. placei* and *H. similis*), two species of *Cooperia* (*C. punctata* and *C. oncophora* or *C. pectinata*) and one species of *Trichostrongylus (T. axei*). This finding illustrates the complexity of the trichostrongylid population structure in Gudali cattle in Ngaoundéré and has implications for the health and husbandry of the local livestock.

## Keywords

18S rDNA SSU, ITS-2, Trichostrongylids, *Haemonchus* spp., *Trichostrongylus* spp., *Cooperia* spp., Gudali zebu cattle

## Introduction

Gastro-intestinal nematodes are a major threat to livestock economies worldwide. Control of these parasites is dependent upon the use of broad-spectrum anthelminthics. However, development of drug resistance threatens the sustainability of parasite control [1,2]. In Cameroon, gastrointestinal worms are considered a major constraint of bovine and ovine production with trichostrongylids (e.g. *Haemonchus* and *Trichostrongylus*) being the most important parasites [3,4]. The local production systems involving the rearing of sheep and cattle in the same pasture may lead to frequent challenge of both species with parasites normally present in the other species.

Therefore, correct identification of the prevailing species, as well as understanding the epizootiology, population structure and diversity of parasitic worms is particularly important for the study of anthelmintic resistance and associated genes [5] and the establishment of sustainable strategies of parasite control [6].

The family of *Trichostrongylidae* contains members, which live in the abomasum, the small intestine and the large intestine of cattle and small ruminants and includes the genera *Haemonchus*, *Trichostrongylus*, *Cooperia*, *Teladorsagia* and *Marshallagia* [7]. From these five genera, three (*Trichostrongylus*, *Teladorsagia* and *Marshallagia)* can also infect the human host [8]. In this publication, we focus on the genera *Haemonchus*, *Trichostrongylus* and *Cooperia*, which occur in the abomasum of cattle.

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Four species of *Haemonchus*, namely *H. contortus*, *H. placei*, *H. similis* and *H. longistipes*, two species of *Trichostrongylus* (*T. colubriformis* and *T. axei*) and five *Cooperia* spp. (*C. pectinata*, *C. curticei*, *C. oncophora*, *C. spatulata* and *C. punctata*) have been described to infect domestic ruminants worldwide [9-11]. The host preferences and the degree to which the different species still interbreed are a matter of debate. As for Central Africa, the occurrence of *C. pectinata* and *C. punctata* has been described in the abomasum of cattle in Northern Cameroon [4].

Measurement of the male bursa and spicula, and differences in the synlophe length and pattern, the reproductive system and the shape of the posterior end of females are commonly used to assign adult worms to different genera and species within the trichostrongylid family [12-14]. However, interbreeding between the species clearly mitigates the morphometric identification [15-17].

Determining the species on basis of the morphology of their eggs in faecal examinations or after subsequent *in-vitro*-culture of infective larvae is practically impossible for lack of unambiguous diagnostic morphological features, even at high magnification microscopy. Therefore, molecular techniques are needed to supplement the morphological classification and to identify the worm population from faecal samples, *i.e.* without having to slaughter the animal.

PCR amplification of selected genomic DNA fragments followed by sequencing has proved to be most useful for categorizing closely related nematodes [18,19].

The nuclear 18S rDNA (SSU) is highly conserved among eukaryotic organisms. It codes the RNA of the small subunit of the ribosomes and is about 1700 base pairs in length. Within the SSU four hyper-variable regions (HVR-I to -IV) were described, which tend to differ between species and genera but are frequently fairly constant within one species. Therefore, the SSU HVRs are popular for taxonomy and phylogenetic studies [20-22].

Much in contrast to these slowly evolving genes, the internal transcribed spacers (ITS-1 and ITS-2), which separate the coding units for the three ribosomal RNAs on the nuclear chromosomal DNA are not part of the functional ribosome and are therefore subject to rather frequent mutation. Consequentially, these highly variable regions frequently differ also between very closely related species or sub-species [23-26].

In order to gain more insight into the abomasal trichostrongylid populations in cattle in Cameroon we isolated individual worms of the genera *Haemonchus*, *Trichostrongylus* and *Cooperia* from zebu cattle in the Adamawa region, and analysed their SSU HVR-I and ITS-2 sequences.

## Materials and Methods

### Sample collection and parasite identification

Twelve abomasa from female adult zebu Gudali cattle were collected from the municipal abattoir of Ngaoundéré between February and March 2015. Another abomasum came from a *post-mortem* analysis of an 18-months-old calf which died of unknown aetiology at the Vina du Sud in 2016. The predominant local cattle are zebu Gudali short horn (*Bos indicus*). After slaughter of each animal, the two ends of the abomasa were sealed separately and the abomasa were immediately transported in plastic bags to the veterinary research laboratory at the Institute of Agricultural Research for Development (IRAD), Wakwa, Ngaoundéré for examination.

Each abomasum was sliced open and its content washed with tap water. The mucosa was carefully examined and washed to remove any adhering worms. The collected washing product was passed through sieves meshes of 200 and 100 µm, respectively. Collected nematodes were separated under the dissecting microscope into groups according to their length and transferred into clean petri dishes containing phosphate buffered saline (PBS), and later identified to genus and/or species as described by Hansen and Perry [27], Yin, et al. [28]. Representatives of each recognized species were pooled and stored at -20 °C either in RNA-later or 95% ethanol for further analyses.

### Single worm lysis and genotyping

A total of 208 adult worms were randomly selected from 13 cattle and were morphologically assigned to the three genera *Haemonchus* (120), *Trichostrongylus* (40) and *Cooperia* (48). The worms were individually placed in PCR tubes and prepared for PCR analysis as described by Hildebrandt, et al. [29]. Briefly, single worms were transferred into 0.2 ml PCR tubes containing 10 µl $H_2O$ and three times freeze-thawed using dry ice to cool, with vigorous vortexing in between. 10 µl of 2 × lysis buffer (20 mMTris-HCL pH 8.3, 100 mM KCl, 5 mM $MgCl_2$, 0.9% NP-40, 0.9% Tween 20, 0.02% Gelatine, 240 µg/ml Proteinase K) were added and mixed. The suspension was incubated at 65 °C for 8 hours, followed by 95 °C for 15 minutes to inactivate the proteinase K. If samples were not processed immediately, they were stored at -20 °C. The HRV-I and ITS-2 loci were PCR amplified as described by Eberhardt, et al. [22] and Chaudhry, et al. [17], respectively, with minor modifications. Briefly, PCR was performed in 25 µl final volume composed of 2 µl of DNA template, 2.5 µl of 10x ThermoPol reaction buffer (New England BioLabs), 0.5 µl bovine serum albumin (10 mg/ml), 0.5 µl of dNTPs mix (2 mM), 0.5 µl of 10 pmol/µl of each primer, 0.3 µl of 0.06 U Taq DNA polymerase (New England BioLabs) and 18.2 µl of nuclease free water. The primers used and the cycling conditions are given in Table 1. The reactions were performed in an automated thermocycler (Biometra T professional gradient Thermocycler, 2013 model). 5 µl of each PCR product were loaded on agarose gels stained with ethidium bromide to confirm the presence of a PCR product prior to sequencing. The sequencing reactions were performed using the BDTv3.1 kit (Applied Biosystems) following the manufacturer's instructions in 10 µl of final volume which consisted of 0.5 µl PCR product, 2 µl of 5x buffer, 0.3 µl of BDT, 0.5 µl of 10 pmol/µl of primer and 6.7 µl water. ITS-2 fragments were sequenced from both ends using the PCR primers, whereas the regions around the SSU HVR-I was sequenced using the internal sequencing

**Table 1:** Primers and PCR programs.

| | Target portion | Primers | Reference | PCR Program |
|---|---|---|---|---|
| RH5401 | SSU HRV I | AAAGATTAAGCCATGCATG | Eberhardt, et al. 2007 [22] | 95 °C for 2 min<br>95 °C for 30 sec<br>52° °C for 30 sec 40X<br>72 °C for 2 min<br>72 °C for 10 min |
| RH5402 | SSU HRV I | CATTCTTGGCAAATGCTTTCG | | |
| RH5403 seq | SSU HRVI | AGCTGGAATTACCGCGGCTG | | +4 °C |
| NC1F | ITS-2 | ACGTCTGGTTCAGGGTTGTT | Chaudhry, et al. 2015 [17] | 95 °C for 5 min<br>95 °C for 1 min<br>57 °C for 1 min 35X<br>72 °C for 1 min<br>72 °C for 5 min<br>+4 °C |
| NC2R | ITS-2 | TTAGTTTCTTTTCCTCCGCT | | |

primer RH4503. The samples were submitted to the in-house genome centre at the Max Planck Institute for Developmental Biology for electrophoretic analysis and base calling.

## Genotype analysis

Each chromatogram returned from the sequencing facility was visually evaluated in order to detect ambiguous positions and a defined fragment of the sequence was retrieved. For the SSU HVR-I we considered a fragment corresponding to position numbers 57-516 in the GenBank entry L04152, and for the ITS-2 the entire fragment, which is variable in length (in GenBank entry JF680983 *H. contortus* 231 bp, positions 614-844) because these fragments could be reliably determined using the PCR and sequencing primers specified above. This resulted in three different SSU HVR-I sequences and 44 different ITS-2 sequences. If a sequence contained ambiguous positions, this sequence was considered different from sequences with unambiguously one of the two possible nucleotides at this position. Each ITS-2 sequence was used as query in a BLASTn search against the non-redundant nucleotide databases. The search was performed at the National Centre for Biotechnology Information NCBI (https://blast.ncbi.nlm.nih.gov) on April 4th 2018. For each sequence, the most similar sequence in the databases was retrieved. If multiple sequences were equally similar, one entry was selected unless equally similar sequences were supposed to be derived from different species, in which case one entry for each species was selected (there were two such cases; first, KX829170 [*Haemonchus contortus*] and X78812 [*Haemonchus placei*], over the region considered, are 100% identical with each other and with our worm number 30 and second, KY741868 [*Cooperia pectinata*] and KT215383 [*Cooperia oncophora*], over the region considered, are 100% identical with each other and differ at one position from our worms in cluster 14.

## Phylogenetic analysis

All alignments and phylogenetic analysis were done using the MEGA 7.0 software package [30]. Alignments were done using muscle with default settings. Phylogenetic trees were reconstructed using various models, as specified in the figure legends and in the text and evaluated by 1000 bootstrap repetitions.

# Results and Discussion

All the 13 abomasa examined were co-infected with multiple trichostrongylid species. An approximate total of 10,000 adult worms were recovered: 50% of them belonged to the genus of *Trichostrongylus*, 45% *Haemonchus*, 3% *Cooperia* and other trichostrongylids were 2%.

## SSU HVR-I

The small subunit 18S rDNA (SSU) sequence was successfully amplified from 120 adult *Haemonchus* spp. originating from all 13 cattle. They were all 100% identical with the sequences EU086374, DQ503465 and L04152 describing *H. contortus*, *Haemonchus sp*. and *H. similis*, respectively, which do not differ in their sequences [25,31,32].

All 40 worms morphologically classified as *Trichostrongylus* spp. had the same SSU HVR-I sequence which has previously been reported [33] to belong to *T. colubriformis* (AJ920350). There was no *T. axei* sequence available from the databases for comparison.

None of 48 *Cooperia* spp. gave rise to a PCR product, suggesting the used primers may not be appropriate for this genus. The primers we used were identical with the primers SSU18A (RH5401) and SSU26R (RH5402) described in Dorris, et al. [34] and used in a number of nematode molecular taxonomic studies [19,21,35,36], because they work for many different, however not all, nematode genera. *Cooperia* appears to belong to the latter.

These results confirm that SSU HVR-I is a reliable molecular marker which permits one to assign to the correct genus the most important abomasal trichostrongylids in zebu cattle in our study area. However, earlier authors found for the genus *Haemonchus*, and for other nematodes, that sometimes closely related species do not differ in their SSU HVR-I [32,35,36]. Therefore, additional sequencing of other loci, like the ITS-2 is desirable.

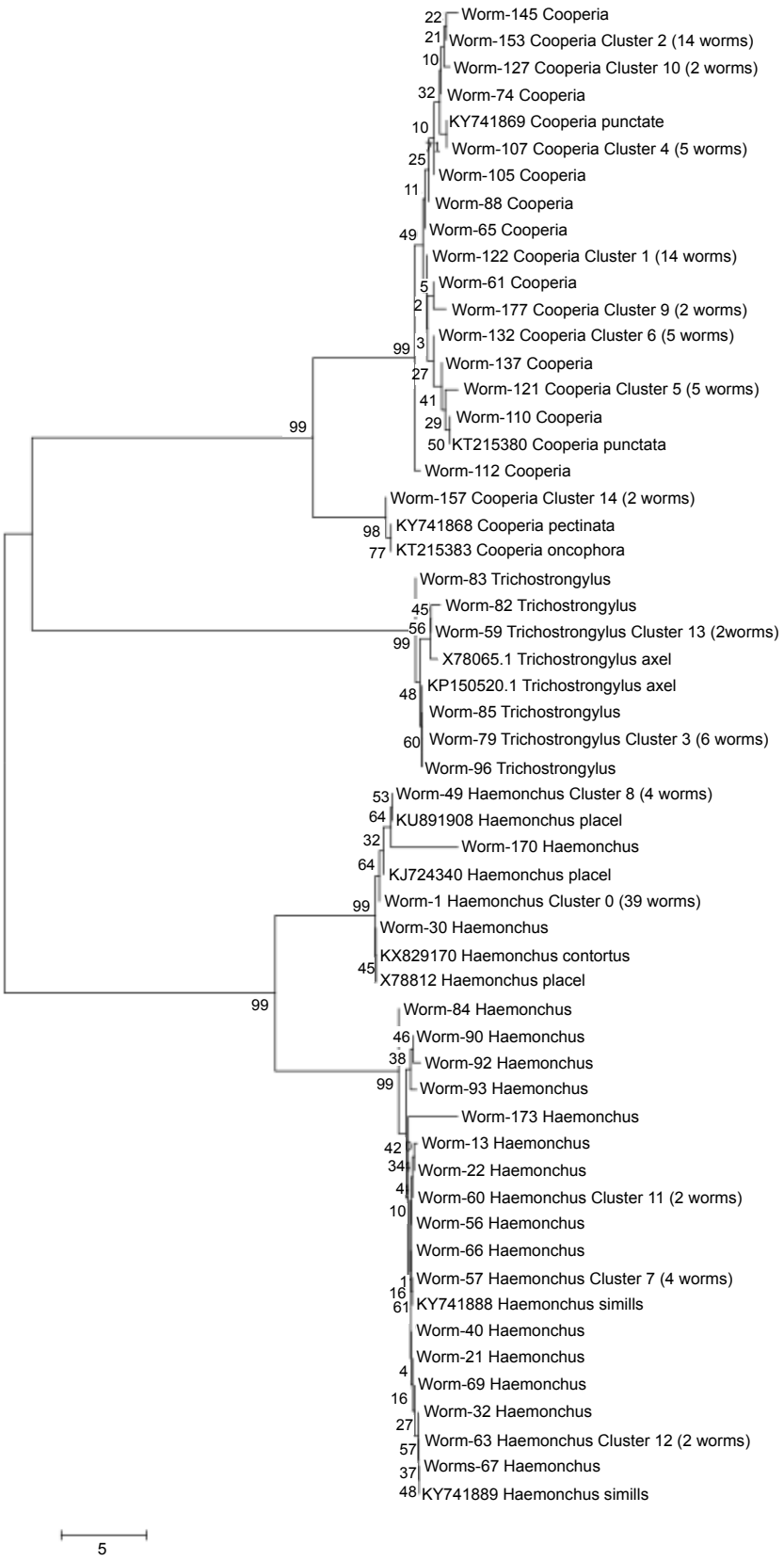


**Figure 1:** Neighbour-joining tree of the ITS-2 sequences from the worms isolated in this study and their best BLAST hits. The bootstrap values of 1000 repetitions are given. Nomenclature: Sequences retrieved from the databases are indicated by their accession numbers followed by the species as listed in the corresponding database entry; sequences obtained in this study are labelled with their worm number followed by the genus, the worm had been assigned to, based on morphology. If a sequence was found in multiple worms this is referred to as a cluster, which is defined by one randomly selected worm with this sequence, the cluster number and in () the number of worms with this sequence.

## ITS-2

We identified in total 44 different sequences, each of which was found in between 1 and 39 individual worms. First, we reconstructed phylogenetic trees with these 44 sequences plus their closest relatives in the databases (Figure 1) (best BLAST hits, see Materials and methods). The sequences fall into three large groups with very high bootstrap support. These groups correspond perfectly to the three genera (Figure 1).

Within the *Trichostrongylus* group there is no strongly supported sub-division and all sequences have one of two sequences derived from *T. axei* as their best BLAST hit.

The *Haemonchus* group is divided into two excellently supported subgroups. In one group, all sequences have one of two sequences derived from *H. similis* as their best BLAST hit. The other group is formed by sequences with best BLAST hits attributed to *H. placei* or *H. contortus*. Notice that the single alleged *H. contortus* sequence (KX829170) identified as

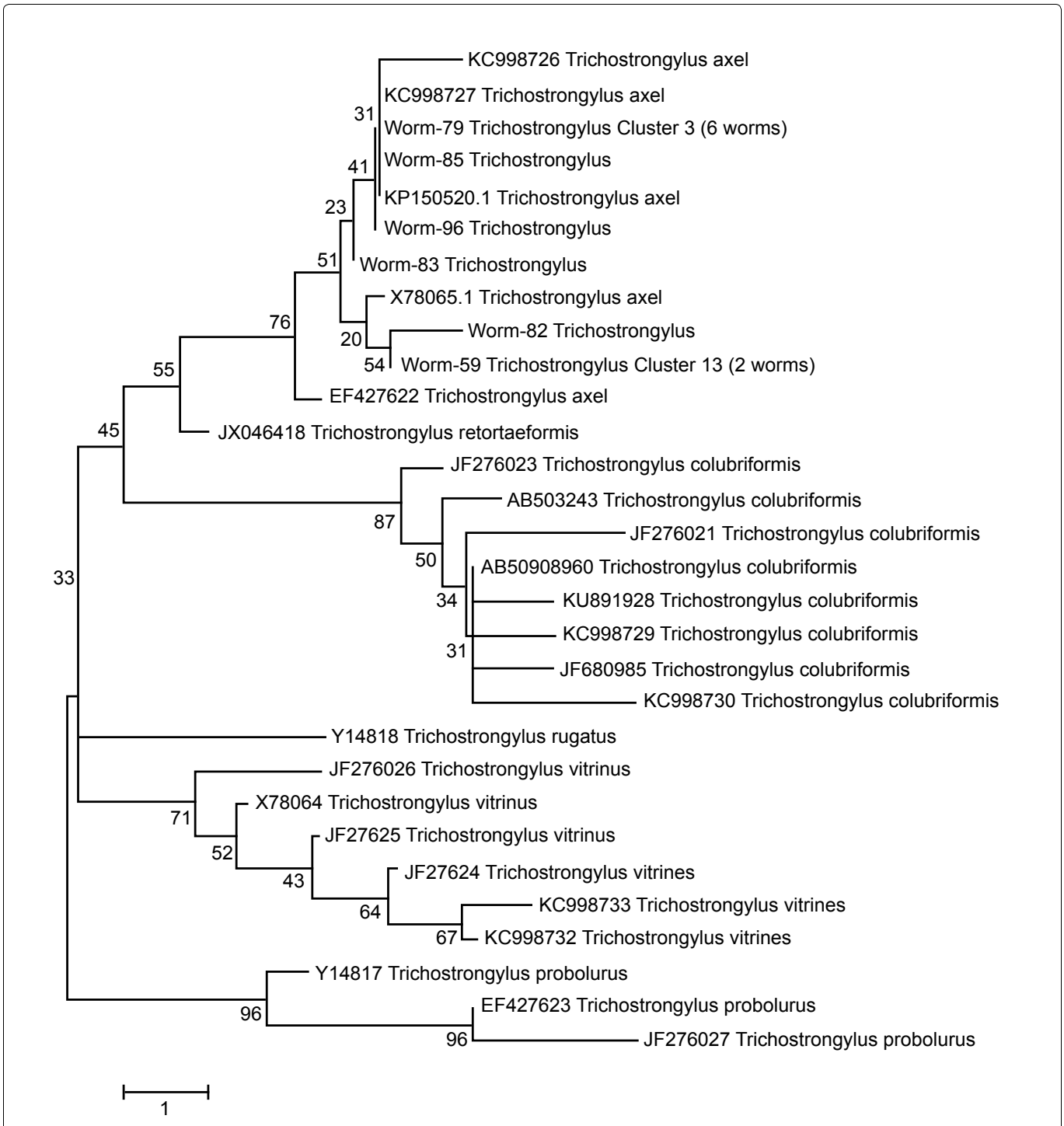


**Figure 2:** Neighbour joining tree of the *Trichostrongylus* ITS-2 sequences from the worms isolated in this study and selected database entries. The bootstrap values of 1000 repetitions are given. The nomenclature is as in Figure 1.

best BLAST hit is identical with a sequence (X78812) described as derived from *H. placei*.

Also, the *Cooperia* group is divided into two most highly supported subgroups. In one group, all sequences have one of two sequences derived from *C. punctata* as their best BLAST hit. The second group contains only one sequence isolated in this study (but found in two different worms) along with its best BLAST hit which consists of two identical sequences supposedly derived from *O. pectinata* (KY741868) and *O. oncophora* (KT215383), respectively.

In order to better compare our data with the information in the databases, for each genus we performed phylogenetic analyses with additional sequences. For several species of *Trichostrongylus*, *Haemonchus* and *Cooperia* various ITS-2 sequences with supposed within-species variation of up to three percent had been deposited in the databases. This reflects probably both, a truly existing within-species sequence variability and an occasional misidentification of the species. We conducted BLAST searches in order to identify all database entries for full length ITS-2 sequences for all species of the three genera under investigation that had been reported to occur in ruminants. Only sequences without ambiguity codes were considered. If for a given species less than six entries were identified, all of them were taken, if more than five entries were found, five to nine entries representing the full sequence variation were selected.

Figure 2 shows the relationships of all *Trichostrongylus* sequences analysed. All sequences from our study fall into one fairly well-supported group together with all *T. axei* database entries. No other sequences are part of this group. This strongly indicates that all *Trichostrongylus* worms isolated for this study belong to the species *T. axei*. This result is in agreement with earlier results based on morphological species determination, which found that *T. colubriformis* and *T. axei* were the dominant species found in Cameroon in sheep/goats and cattle, respectively [3].

Figure 3 shows the relationships of all *Haemonchus* sequences analysed. Their sequences fall into three very well-supported groups. One group contains all *H. longistipes* sequences and no sequences from our study. The second group contains a portion of our new sequences and all *H. similis* sequences from the databases. The third group combines the database entries for *H. placei* and *H. contortus* and all of our other sequences that do not fall into the *H. similis* group. Within the group there is a well-supported subgroup containing all our sequences, all database entries for *H. placei* and one for *H. contortus*, which is identical with *H. placei* sequences as described above. *H. contortus* and *H. placei* are known to be very closely related and to interbreed occasionally [37]. It is therefore not unexpected that they cannot be perfectly separated solely based on the short ITS-2 sequence. Nevertheless, the phylogenetic tree shown suggests that the species identification for KX829170 is wrong. If we accept this, our analysis strongly argues that the *Haemonchus* worms we found in cattle are in part *H. similis* and in part *H. placei*.

Figure 4 shows the relationships of all *Cooperia* sequences analysed. This tree is less well resolved than the *Trichostrongylus* and the *Haemonchus* trees. Nevertheless, all but one of our sequences fall into a well-supported group, which also contains all database entries for *C. punctata* and the two entries for *C. spatulata*. In the tree shown, all our sequences appear closer to *C. punctata* entries than to *C. spatulata*, however, this is only supported by low bootstrap values. The remaining of our sequences forms a perfectly supported group together with two identical sequences retrieved from the databases, one however, supposed to be derived from *C. pectinata* and the other from *C. oncophora*. From this we conclude that the vast majority of *Cooperia* worms in our sample belong to the species *C. punctata* but a second species, *C. pectinata* or *C. oncophora*, is present at low frequency. We favour the hypothesis that these worms were *C. pectinata* for the following reasons. i) All the other sequences derived from *C. oncophora* fall in other, not well resolved, places of the tree making it very likely that the species assignment in sequence KT215383 is erroneous; ii) *C. oncophora* has been described as an economically important nematode in the temperate regions of the world [38] while *C. pectinata* is known to occur in Northern Cameroon [4]. It might, however, be possible that *C. oncophora* was recently introduced to Cameroon together with the European cattle imported for the national breeding program.

## Conclusion

Young trichostrongylid larvae generated from larval culture of faeces of live animals cannot be reliably distinguished morphologically. The present study used adult abomasal trichostrongylids, obtained post-mortem, whose genus can be determined based on morphology to evaluate the power of the SSU HVR-I and the ITS-2 sequences for molecular taxonomy.

We show that PCR amplification and sequencing of the HVR-I-containing SSU fragment using standard nematode primers is a reliable approach for identifying the genera *Haemonchus* and *Trichostrongylus*, but fails for *Cooperia* spp. However, this DNA fragment does not provide the information for taxonomic classification beyond the genus. The ITS-2 sequence has more discriminative power. However, when comparing a sequence with the databases, caution is required since most likely the species assignment in some database entries is wrong. Therefore, one should not rely on the best BLAST hit alone for species identification. Based on their ITS-2 sequences and on our comparison with many database entries we conclude that in cattle in our study area i) *T. axei* is the predominant if not only *Trichostrongylus* species; ii) There are two prevailing species of *Haemonchus*, namely *H. placei* and *H. similis*, and iii) The large majority of *Cooperia* worms are *C. punctata* but at least one additional species, presumably *C. pectinata*, is present.

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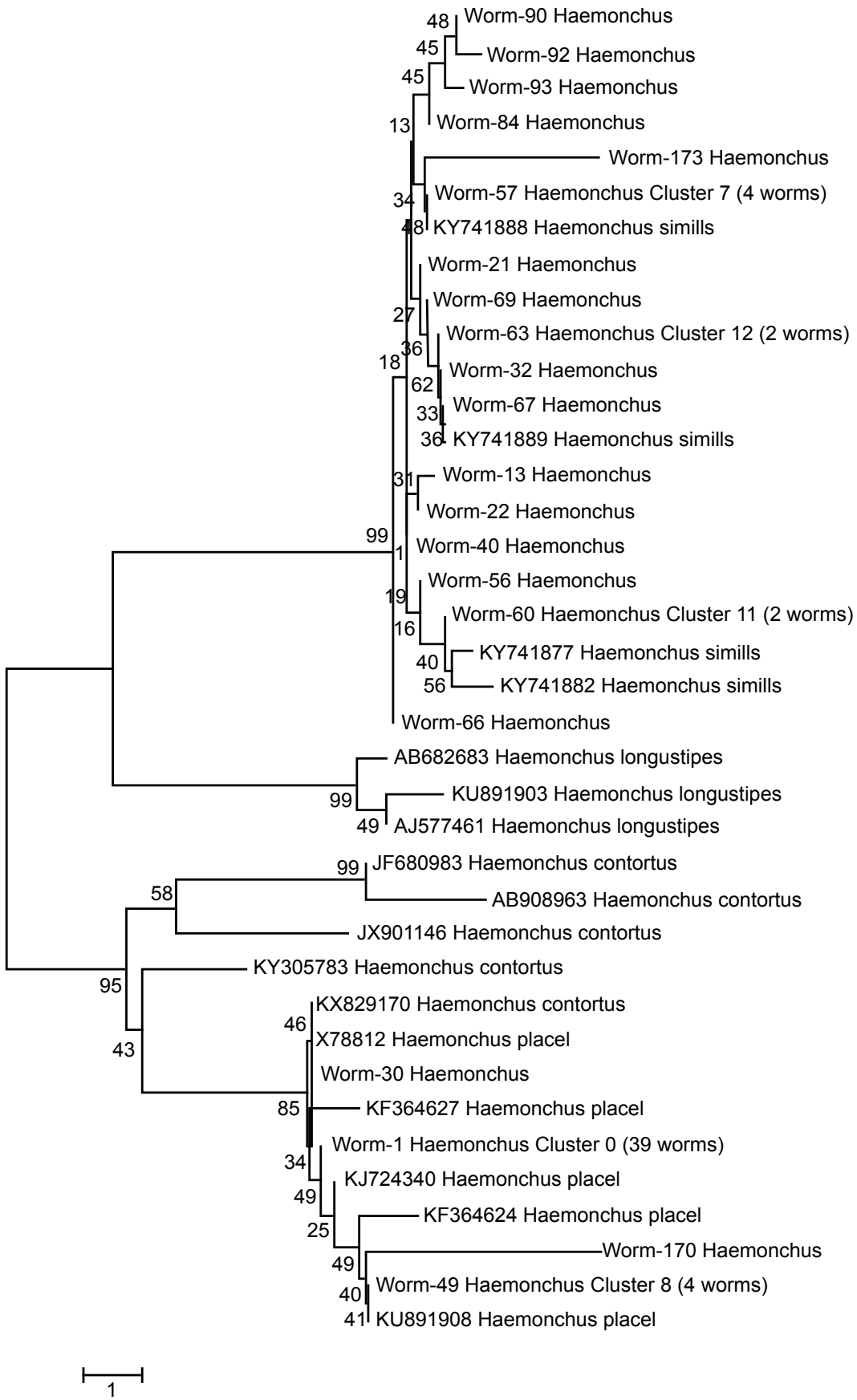


**Figure 3:** Neighbour joining tree of the *Haemonchus* ITS-2 sequences from the worms isolated in this study and selected database entries. The bootstrap values of 1000 repetitions are given. The nomenclature is as in Figure 1.

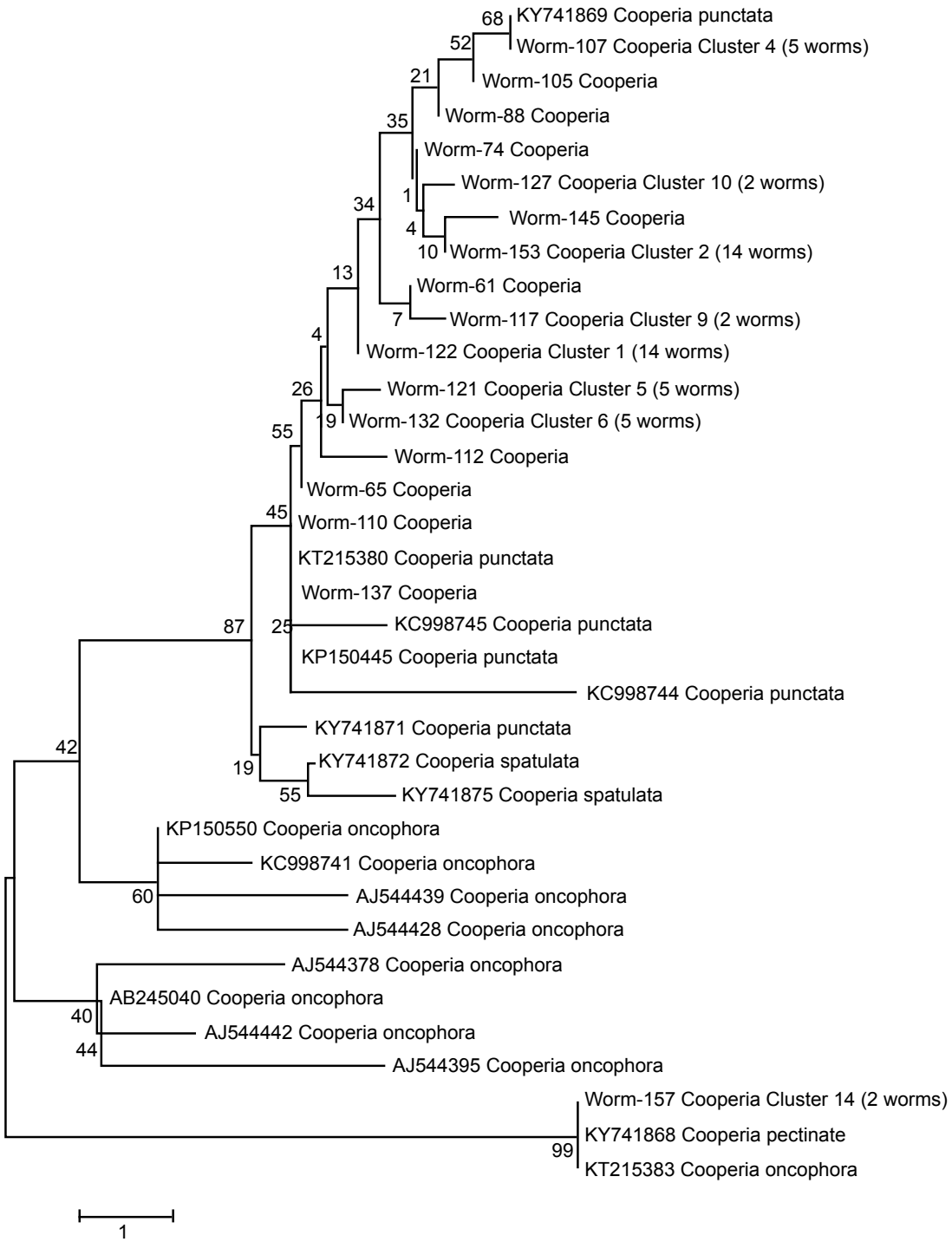


**Figure 4:** Neighbour joining tree of the *Cooperia* ITS-2 sequences from the worms isolated in this study and selected database entries. The bootstrap values of 1000 repetitions are given. The nomenclature is as in Figure 1.

support.

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## Conflict of Interest

None.

## Ethics Statement

This study was approved by the Institute of Agricultural Research for Development and the University of Ngaoundéré. Abomasa were collected at the slaughterhouse of Ngaoundéré by veterinarians and well-trained personnel

after the animals had been slaughtered as part of the routine operations of the abattoir. The meat of the animals was processed for human consumption.

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