Full mitochondrial and nuclear genome comparison confirms that Onchocerca sp. “Siisa” is Onchocerca ochengi

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Abstract

Onchocerca ochengi is a nodule-forming filarial nematode parasite of cattle. It is the closest known relative of the human parasite Onchocerca volvulus, with which it shares the black fly vector Simulium damnosum. Onchocerca sp. “Siisa” was described in black flies and in cattle and, based on limited mitochondrial sequence information, appeared to be about equally phylogenetically distant from O. ochengi and O. volvulus. Based on molecular genetic markers and apparent interbreeding, we later proposed that O. sp. “Siisa” belongs to the species O. ochengi. However, we did not demonstrate directly that the hybrids were fertile, and we were still unable to resolve the phylogenetic relationship of O. ochengi, O. sp. “Siisa,” and O. volvulus, leaving some concerns with the conclusion mentioned above. Here, we present fully assembled, manually curated mitochondrial genomes of O. ochengi and O. sp. “Siisa,” and we compare multiple individuals of these two taxa with respect to their whole mitochondrial and nuclear genomes. Based on the mitochondrial genomes, O. ochengi and O. sp. “Siisa” are phylogenetically much closer to each other than to O. volvulus. The differences between them are well within the range of what is expected for within-species variation. The nuclear genome comparison provided no indication of genetic separation of O. ochengi and O. sp. “Siisa.” From this, in combination with the earlier literature, we conclude that O. ochengi and O. sp. “Siisa” should be considered one species.

Keywords Onchocerca ochengi · Onchocerca sp. “Siisa” · Mitochondrial genome · Filarial nematode

Introduction

The filarial nematode genus Onchocerca consists of about 30 described species that parasitize predominantly ungulates, but there are also species living in other hosts (Krueger et al. 2007; McFrederick et al. 2013). Onchocerca ochengi is a nodule-forming filarial nematode parasite of cattle and the closest known relative of Onchocerca volvulus, the causative agent of human onchocerciasis (Trees et al. 2000). It was hypothesized that O. ochengi and O. volvulus arose from a common ancestor as recently as 10,000 years ago, probably upon domestication of cattle (Krueger et al. 2007). Both O. ochengi and O. volvulus share the same black fly vector Simulium damnosum and they are very similar with respect to their biology and at the molecular and the antigenic levels. O. ochengi has therefore been established as an attractive animal model to study aspects of onchocerciasis (Makepeace and Tanya 2016; Trees et al. 2000). It must be noted, however, that unlike O. volvulus, O. ochengi does not cause any obvious pathology, probably because the co-existence with its host is much older and, as a consequence, more benign (Trees et al. 2000). Although not formally
Onchocerca these worms as clades as O. ochengi this species present in Cameroon (we referred to the two O. ochengi concluded that intermediate (Eisenbarth et al. 2013) and definitive host indi-
ference among the larvae (Eisenbarth et al. 2013), adults, or
peared to assume an “intermediate” phylogenetic position be-
tween O. ochengi and O. volvulus. These authors referred to these worms as Onchocerca sp. “Siisa,” leaving the exact
taxonomic status to be determined by future studies. Later, based on partial sequences of the mitochondrial 12S, 16S,
and cytochrome oxidase subunit 1 (cox1) genes, we showed that Onchocerca sp. “Siisa” was also present in black flies in
Cameroon and that cattle are the, or one of the, definitive hosts of Onchocerca sp. “Siisa” (Eisenbarth et al. 2013). Later, we
concluded that Onchocerca sp. “Siisa” belongs to the species O. ochengi and is one of two major mitochondrial clades of
this species present in Cameroon (we referred to the two clades as O. ochengi variant ochengi and O. ochengi variant
Siisa) (Hildebrandt et al. 2014). This claim was based on the
following observations. (1) We found no morphological differ-
ence among the larvae (Eisenbarth et al. 2013), adults, or
nodules (Hildebrandt et al. 2014) between the two types (no-
tice, however, that in one later study (Eisenbarth et al. 2016),
the small number of O. sp. “Siisa” appeared marginally
but weakly significantly shorter than the O. ochengi from
the same sampling sites). (2) Both variants co-occur in the same
intermediate (Eisenbarth et al. 2013) and definitive host indi-
viduals (Eisenbarth et al. 2013; Hildebrandt et al. 2014). (3)
Within a definitive host individual carrying both types, we
failed to detect any assorted mating (Hildebrandt et al. 2014).
(4) Based on the limited nuclear sequence information,
there appeared to be no genetic differentiation of the nuclear
genomes between the two mitochondrial clades (Hildebrandt
et al. 2014). (5) Based on the analysis of a limited number of
single copy nuclear loci of individual adult males and females
and of individual microfilariae, we found that variant-mixed
pairs readily produced progeny, the fertility of which was,
however, not demonstrated directly (Hildebrandt et al. 2014).

Furthermore, like Krueger et al. (2007), we were unable to
resolve the mitochondrial phylogenetic relationships of O. volvulus, O. ochengi, and Onchocerca sp. “Siisa,” and
O. ochengi and Onchocerca sp. “Siisa” appeared about equally
distant from each other as either one of them from O. volvulus (Eisenbarth et al. 2013; Hildebrandt et al. 2014).
O. ochengi and O. volvulus, in turn, are well-established as being separate species, even having different numbers of chro-
mosomes (Doyle et al. 2016; Post 2005).

To clarify this issue, we decided to compare the entire mi-
 tochondrial and nuclear genomes of the two taxa. We present
here two complete, manually finished and annotated mito-
ochondrial genomes derived from one individual of each of
O. ochengi and Onchocerca sp. “Siisa,” and we compare the
whole mitochondrial genome sequences based on short read
sequencing of an additional nine individuals representing both
variants with these sequences. We show that (1) the separation
into two mitochondrial clades is reflected throughout the ge-
nome and (2) that when considering the entire mitochondrial
sequences, O. ochengi and Onchocerca sp. “Siisa” are clearly
more similar to each other than either of them is to O. volvulus
and the difference between the two is well within the range
expected for within-species variability. Further, based on
whole nuclear genome data, we show that there is no indica-
tion of nuclear genetic differentiation between the two mito-
ochondrial clades, strongly suggesting that they interbreed and
do not represent reproductively isolated populations.

Materials and methods

Isolation of adult Onchocerca sp. worms from skin
nodules

The skin nodules containing adult Onchocerca worms were
collected in the Ngaoundéré abattoir, Adamawa Region,
Cameroon, as described (Wahl et al. 1994) between October
2013 and March 2014. The nodules were stored in 80% eth-
anol and shipped to Max Planck Institute (MPI) for
Developmental Biology in Tübingen, Germany, for analyses.

DNA extractions, PCR, and library preparation

Adult worms were isolated from the nodule tissue by collage-
nase digestion as described (Kläger et al. 1996). Briefly, nod-
ules were incubated at 37 °C overnight in 0.2% collagenase
with phosphate buffered saline (PBS) solution and then
washed several times with PBS. DNA was extracted from
single worms using the Epicenter DNA extraction kit
(Epicenter, USA) according to the manufacturer’s instruc-
tions. The DNA was quantified using Qubit fluorimeter mea-
surement (Invitrogen Life Technologies, USA). Before library
preparation, partial cox1, 12S, and 16S were PCR amplified
using the primer pairs and conditions reported earlier
(Eisenbarth et al. 2013) and sequenced using one of the PCR
primers. The sequencing reactions were done using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied
Biosystems) according to the manufacturer’s protocol, and
the reactions were submitted to the in-house sequencing facil-
ity at the MPI for Developmental Biology at Tübingen for
electrophoresis and base calling. The partial cox1, 12S, and
16S were compared with the nucleotide database entries using
BLAST at the National Center for Biotechnology Information (NCBI) to determine if the worms were *O. ochengi* and/or *O. sp. “Sisaa”*. DNA libraries were prepared from 50 ng of genomic DNA using the Low Input DNA library preparation kit (Rubicon Genomics, USA) according to the manufacturer’s instructions. The libraries were quantified using Qubit and Bioanalyzer (Agilent Technologies, USA) and then normalized to 2.5 nM. The samples were sequenced as 400 bp paired ends in one multiplexed lane using HiSeq2000 platform (Illumina Inc., USA) at MPI for Developmental Biology in-house genome facility.

**Assembly and analysis of the *O. ochengi* and the *O. sp. “Sisaa”* reference mt genomes**

For the two samples M1 (male *sp. “Sisaa”) and M3 (male *O. ochengi*), the mitochondrial DNA (mtDNA) sequences were extracted from the whole genome sequencing data (Jex et al. 2010). The extracted sequences were assembled manually and aligned to the complete mitochondrial genome sequence of *O. volvulus* (AF015193) (Keddie et al. 1998) using MUSCLE (Edgar 2004) and assembled using SeqBuilder (DNASTAR, Inc). Regions that could not be unambiguously assembled based on the short-read sequences were PCR amplified and sequenced by conventional Sanger sequencing as described above. The assembled mitochondrial genomes were aligned using MUSCLE (Edgar 2004). The protein-coding and tRNA genes of the *O. ochengi* mtDNA were annotated based on the published *O. volvulus* mitochondrial genes (Keddie et al. 1998). The codon usages in the 12 protein-coding genes were examined using the invertebrate mitochondrial genetic code as a reference, and the amino acid frequencies were compared with the nucleotide composition of the respective codon families (Singer and Hickey 2000). The transfer RNA (tRNA) genes were identified by ARWEN v1.2 (Laslett and Canback 2008) using the metazoan mitochondrial tRNA data set as source. Percent identities, sequence length, and transition to transversion ratio of nucleotide substitutions for these two isolates were calculated using MEGA6 (Tamura et al. 2013).

**Analysis of whole genome sequencing data**

Raw reads were aligned to the *O. ochengi* reference genome (http://www.nematodes.org/genomes/onchocerca_ochengi/) and variants were called as described in Rödelsperger et al. (2014). While all three male samples showed at least 90-fold genome-wide coverage, only very few reads of the female samples could be aligned to the reference genome. Further investigation of a subset of unaligned reads by BLASTN searches against the NCBI nucleotide data base revealed large-scale contamination with bovine sequences. For this reason, only data for the highly covered mitochondrial genome was suited for further analysis of female samples.

To screen for evidence for recent admixture between the three male samples, we visualized the genotypes of variable sites and the frequencies of variants in 10-kb windows along selected contigs. The raw reads were deposited at the European Nucleotide Archive under the study accession PRJEB23566.

**Analysis of sequence polymorphisms**

For all samples, the mtDNA sequence was extracted from the whole genome sequences (Jex et al. 2010). The nucleotide sequences to be compared (either the entire mitochondrial genome or only the protein-coding sequences, as specified in the text) were aligned using the MUSCLE algorithm (Edgar 2004). The nucleotide alignment was checked for translational reading frame shifts by translation and then visual inspection. Pairwise nucleotide diversity was calculated between the isolates using MEGA6 (Tamura et al. 2013). For some analyses, as specified in the text and the tables, *O. volvulus* (AF015193) and *Onchocerca flexuosa* (HQ214004) were included for comparison.

**Phylogenetic analyses**

For the phylogenetic analysis, we considered the mitochondrial ribosomal DNA sequences plus the nucleotide sequences of the 11 of the 12 mt protein-coding genes that were available for all samples considered (nad4L is not included). In addition to the 11 new samples from this study, we also included *O. volvulus* (AF015193), *O. flexuosa* (HQ214004), *Onchocerca gutturosa* (PRJEB7568, unpublished, provided by M. Blaxter, University of Edinburgh), and *O. ochengi* (unpublished but downloadable from http://www.nematodes.org/genomes/onchocerca_ochengi/; see also https://www.biorxiv.org/content/early/2017/12/20/236539) and *Dirofilaria immitis* (AJ537512) as outgroup. The sequences were concatenated (Suppl. file 1) and aligned using MUSCLE (Edgar 2004). The molecular phylogenetic analysis was done using MEGA6 (Tamura et al. 2013). The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). Initial trees for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using a JTT model. The final phylogenetic tree was reconstructed using the maximum likelihood method with 1000 bootstrap replications (Tamura et al. 2013).
Results and discussion

O. ochengi variant ochengi and Onchocerca sp. “Siisa” reference mitochondrial genomes

We conducted short read whole genome sequencing experiments with 11 adult single Onchocerca worms (3 males, M1–M3, and 8 females, F1–F8). While the males yielded good coverage, the female samples were very heavily contaminated with bovine (host) sequences indicating that collagenase digestion did not remove all host tissue and resulting in a small proportion of Onchocerca-derived reads. Nevertheless, the available information was sufficient for extraction of the mitochondrial sequences. For two males, one O. ochengi and one Onchocerca sp. “Siisa” (based on their cox1, 12S, and 16S sequences; c.f. Eisenbarth et al. 2013), we manually assembled and annotated the full mitochondrial genomes including the AT-rich noncoding region. Regions that could not be unambiguously assembled based on the short-read data were PCR amplified and sequenced using conventional Sanger sequencing. The resulting reference genomes were deposited in GenBank under the accession numbers KX181289 (O. ochengi) and KX181290 (Onchocerca sp. “Siisa”). The O. ochengi mitochondrial genome is 13,744 bp long (Fig. 1). Compared to O. volvulus (AF015193) (Keddie et al. 1998) and O. flexuosa (HQ214004) (McNulty et al. 2012), the O. ochengi mitochondrial genome is slightly smaller due to shorter intergenic regions. The mitochondrial gene content and order (Fig. 1) is the same as in O. volvulus (Keddie et al. 1998) with 12 protein-coding genes, 22 tRNA genes, and coding units for the small (12S, rrnS) and the large (16S, rrnL) ribosomal subunit RNAs. Like all other nematode species whose mitochondrial genomes were sequenced (except for Trichinella spiralis; Lavrov and Brown 2001), O. ochengi lacks the protein-coding gene atp8. The AT-rich (83.02%) noncoding region is 318 bp and is located between the termination codons and the start codon of nad4L.

Graphical representation. All genes are transcribed clockwise. Protein-coding and rRNA genes are indicated with the standard nomenclature. The tRNA genes are indicated with the one-letter code of their corresponding amino acids. There are two tRNA genes for leucine: L1 for codons CUN and L2 for UUR; and two tRNA genes for serine: S1 for codons UCN and S2 for AGN. “NCR” refers to the noncoding region.

Fig. 1 The mitochondrial genome of O. ochengi (KX181289). a Graphical representation. All genes are transcribed clockwise. Protein-coding and rRNA genes are indicated with the standard nomenclature. The tRNA genes are indicated with the one-letter code of their corresponding amino acids. There are two tRNA genes for leucine: L1 for codons CUN and L2 for UUR; and two tRNA genes for serine: S1 for codons UCN and S2 for AGN. “NCR” refers to the noncoding region. b Positions of the different genes. The first nucleotide of the start codon of the cox1 gene was set as one. For protein-coding genes, the initiation and the termination codons are indicated, and for tRNA genes, the anticodons are indicated. Columns SNPs and % difference refer to the comparison of the O. ochengi (KX181289) and the O. sp. “Siisa” (KX181290) genomes. Two SNPs are in small intergenic regions. Notice, five tRNA sequences overlap with the start of protein-coding genes by 1–3 nucleotides (tRNA(L: CUN)-cox3, tRNA(K)-nad4L, tRNA(Y)-nad1, tRNA(S:AGN)-nad2, tRNA(T)-nad4), tRNA(H) overlaps with the end codon by two nucleotides. Two tRNAs (tRNA(Y) and tRNA(H)) are part of the termination codon with rRNAs and rRNAs by 7 and 3 nucleotides, respectively. Three pairs of tRNAs share 1–7 nucleotides overlapping with each other (tRNA(L:UUR)-tRNA(N), tRNA(C)-tRNA(S:UCN), and tRNA(E)-tRNA(S:AGN))
the cox3 and the tRNA(A) genes. The 19 short intergenic regions vary in length from 1 to 46 bp.

The mitochondrial genome of *O. ochengi* is very A-T rich (overall 73.22%) and there is a bias for the nucleotide T being on the coding strand. Of G-C base pairs, the G is preferentially on the coding strand. The nucleotide composition of the coding strand is as follows: A = 2607 (18.97%), T = 7456 (54.25%), G = 2765 (20.12%), and C = 916 (6.66%). All codons are used except for Ala (GCC), Pro (CCC), Ser (TCC), and Thr (ACC). Codons composed of A and T nucleotides are predominantly used, reflecting the very strong bias toward A+T in the mitochondrial genome of *O. ochengi* (Suppl. Tab. 1).

The *Onchocerca* sp. “Siisa” mitochondrial genome (KX181290) is identical with KX181289 in length and structure, but the two reference genomes differ at 157 single nucleotide positions (1.15% difference). Differences are present in all protein-coding and rRNA genes, in three of the tRNA genes, and in intergenic regions (Fig. 1b).

**Comparison of the mitochondrial protein-encoding genes of additional individuals and species**

Next, we conducted pairwise nucleotide sequence comparisons of the protein-coding sequences (in total 10,407 bp) between the 11 *Onchocerca* individuals from this study and the published sequences of *O. volvulus* (AF015193) (Keddie et al. 1998) and *O. flexuosa* (HQ214004) (McNulty et al. 2012) (Table 1). This comparison was limited to protein-coding genes because they could be unambiguously aligned for all samples included. The pairwise nucleotide diversities among the *Onchocerca* individuals isolated from cattle for this study fell into two nonoverlapping groups ranging from 0.029 to 0.211% and from 1.24 to 1.36%. These two groups confirm the existence of the two mitochondrial clades within our sample and represent within- and between-clade comparisons, respectively. By virtue of mitochondrial sequence, we consider male 1 and female 5 *Onchocerca* sp. “Siisa” and all the others *O. ochengi*.

The pairwise nucleotide diversities between *O. volvulus* and either *O. ochengi* or *Onchocerca* sp. “Siisa” were very similar to each other and clearly larger than between the latter two (the largest difference between *O. ochengi* and *Onchocerca* sp. “Siisa” individuals was 1.36%, while the smallest difference between *O. volvulus* and any *O. ochengi* or *Onchocerca* sp. “Siisa” individual was 3.28%). *O. flexuosa* with differences to *O. ochengi*, *Onchocerca* sp. “Siisa,” and *O. volvulus* of around 10.3% is clearly more phylogenetically distant.

To further evaluate the phylogenetic relationship of *O. ochengi*, *Onchocerca* sp. “Siisa,” and *O. volvulus*, we reconstructed a maximum likelihood (ML) phylogenetic tree (Fig. 2). We included the same samples as in the nucleotide comparison (Table 1) plus the *O. ochengi* mitochondrial genome sequence available from the Blaxter laboratory (http://www.nematodes.org.genomes/onchocerca_ochengi/; see also https://www.biorxiv.org/content/early/2017/12/20/236539), for which also a nuclear genome is available, and the sequence of *O. gutturosa* (PRJEB7568, unpublished, provided by M. Blaxter, University of Edinburgh) plus *D. immitis* (AJ537512) as outgroup sequences. The phylogenetic tree is based on the 11 mitochondrial protein-coding genes for which the sequence was available for all samples included and the ribosomal sequences (Suppl. file 1). This analysis confirms the existence of two separate groups corresponding to *O. ochengi*, and *Onchocerca* sp. “Siisa”, which, however, clearly group together in comparison with *O. volvulus*.

| Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|--------|---|---|---|---|---|---|---|---|---|----|----|----|
| F1     | 0.096 |
| F2     | 0.048 |
| F3     | 0.048 |
| F4     | 0.076 |
| F5     | 1.29 |
| F6     | 0.086 |
| F7     | 0.067 |
| F8     | 0.105 |
| M1     | 1.28 |
| M2     | 0.211 |
| M3     | 0.086 |
| O. volvulus | 3.29 |
| O. flexuosa | 10.28 |

Numbers 1–11 are the individuals isolated for this study (F female, M male). Number 12 is *O. volvulus* (AF015193). Number 13 is *O. flexuosa* (HQ214004). The numbers are pairwise nucleotide differences in percent. For values < 1, three decimal positions, and for values > 1, two decimal positions are listed. M1 and M3 are the individuals the reference sequences were derived from.
Taken together, our phylogenetic analyses based on the entire mitochondrial genomes clearly show that *Onchocerca* sp. “Siisa” (Krueger et al. 2007) is more closely related to *O. ochengi* than to *O. volvulus* and does not assume an “intermediate” position between these two taxa or even group with *O. volvulus* as it may have appeared based on the limited sequence information considered in earlier studies (Eisenbarth et al. 2013; Krueger et al. 2007).

It was found that the mitochondrial pairwise nucleotide differences between individuals of closely related nematode species (species within the same genus) are typically around 10 to 20% while differences within a species average below 1% and surpass 2% only in exceptional cases, which can go up to 6% (Blouin 2002). The differences we observed between *O. ochengi* and *Onchocerca* sp. “Siisa” are therefore well within the range expected for within-species variation.

**Comparison of the nuclear genomes of representatives of the mitochondrial variants “ochengi” and “Siisa”**

The mitochondrial genome is usually only maternally inherited and does not undergo meiotic recombination (Mishra and Chan 2014; Sato and Sato 2011). Therefore, the sequence comparison between the mitochondrial genomes of two individuals provides information about the time elapsed since the last common female ancestor to which both individuals are connected entirely through females but it does not...
reflect if the two matrilineages did or still do interbreed and therefore belong to the same species or not. We therefore compared the nuclear genomes of the three males, which had good sequence coverage.

The frequency of variants ranged between 0.001 and 0.009 per site for the nine largest contigs (Fig. 3a). With regard to the *O. ochengi* reference genome, all three individuals showed varying but correlated distance profiles, suggesting that the three males isolated at the same time and place for our study are more similar to each other than either one of them is to the reference individual, which is of mitochondrial type *ochengi* (Fig. 2) but was collected at a different time and place, however, also in Cameroon. Hence, the degree of mitochondrial sequence variability is not predictive for the extent of nuclear sequence variability. To further screen for evidence of recent admixture between isolates that were classified as “Siisa” and “ochengi,” we searched for genomic regions that would give rise to different phylogenetic relationships between the isolates (Fig. 3b). While the genotypes on contig nOo.2.0.Scaf00013 clearly separates the Siisa male M1 from the ochengi males M2 and M3, two other contigs show different genealogies indicating toward recent recombination between the genetic lineages.

**Conclusions**

Of course, we cannot exclude that somewhere in Africa a very recent speciation event occurred and the mitochondrial sequences of this newly formed species is more similar to one of the two mitochondrial clades of *O. ochengi* described here than to the other. However, for the moment, taken all our results together and also considering the results of Hildebrandt et al. (2014), at least for our study area, there is no indication for any form of reproductive isolation between what was described as *O. ochengi* and as *Onchocerca* sp. “Siisa” based on mitochondrial DNA sequence. Therefore, there is no reason to postulate that they represent different subpopulations or even species, and therefore, *Onchocerca* sp. “Siisa” should be considered *O. ochengi*. It is interesting that, so far, all but one individual *O. ochengi* we isolated in

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**Fig. 3** Analysis of the nuclear genome. **a** Variant (from the reference genome) frequency (%) is shown in nonoverlapping 10-kb window across the nine largest *O. ochengi* contigs. **b** The rectangles represent three selected contigs and their genotypes at variable positions in all three male samples. The nucleotides at polymorphic positions are color coded; “other” indicates small insertion/deletions or heterozygous positions. All three contigs show different phylogenetic relationships between the samples indicating toward recent admixture.
Northern Cameroon and genotyped for three previous studies (Eisenbarth et al. 2013, 2016; Hildebrandt et al. 2014) and for this study could be clearly assigned to one of the two mitochondrial clades described. In total, these were 472/473 individuals, isolated as adults from nodules or as larvae from black flies (microfilariae that might have been the progeny of already counted adults are not included in this number). This result indicates that ultimately almost the entire population of O. ochengi in this area is derived from two different females, whose progeny immigrated simultaneously or sequentially or that were the survivors of a dramatic population bottleneck. It will be interesting to see if in other parts of Africa additional mitochondrial clades exist.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors. All animal-derived material was isolated after the animals had been slaughtered in the context of the normal operations of the abattoir at Ngoundéré. The meat was processed to the Creative Commons license, and indicate if changes were made.

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