Population Genetic Studies in the Parasitic Nematode *Onchocerca ochengi*

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Reproductive biology of *Onchocerca ochengi*, a nodule forming filarial nematode in zebu cattle

Julia C. Hildebrandt, Albert Eisenbarth, Alfons Renz, Adrian Streit

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Abbreviations

PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
DNA	Deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
L_1-L_4	Larval stages one to four
bp	base pair(s)
coxI	Cytochrome c oxidase subunit I
nd5	NADH dehydrogenase subunit 5
NCBI	National Center for Biotechnology Information
MUSCLE	Multiple Sequence Comparison by log-Expectation

Zusammenfassung

Die zu den Nematoden gehörende Filarienart *Onchocerca ochengi* ist ein Knoten bildender Rinderparasit und sehr nah verwandt mit dem Parasiten des Menschen *Onchocerca volvulus*, Verursacher der Flussblindheit. Beide Arten sind im tropischen und subtropischen Afrika endemisch und teilen die gleichen Vektoren, nämlich Kriebelmücken des *Simulium damnosum* Komplex.

Es ist technisch und ethisch einfacher die grundlegende Biologie eines Pathogens in einem Tiermodell, in diesem Fall der Rinder Parasit *O. ochengi*, als im Menschen zu untersuchen. Um bessere Einblicke in die Populationsstruktur und das Fortpflanzungs Verhalten von *O. ochengi* zu erhalten, werden für diese Arbeit nukleäre und mitochondrielle Marker genutzt. Zunächst habe ich die Methoden zur Genotypisierung von einzelnen *O. ochengi* Adulten, Embryonen und Mikrofilarien auf Einzelkopie-Niveau etabliert und Regionen bestimmt, an denen Einzel Nukleotide Polymorphismen auftreten, die als molekulare Marker dienen. Diese Methoden und Marker habe ich genutzt um Eltern-Nachwuchs-Verhältnisse zu bestimmen.

Dabei ergab sich, dass Weibchen oft Nachkommen von mehreren Männchen gleichzeitig haben. Meistens, aber nicht immer, sind diese vermutlichen Väter noch in den Knoten zu finden, wenn ihre Nachkommen bereit sind zu schlüpfen. Das zeigt, dass Männchen, obwohl sie die Knoten verlassen können, dazu tendieren für eine gewisse Zeit bei einem Weibchen zu bleiben.

Ich konnte einen großen Anteil der Mikrofilarien aus einer Hautprobe ihren Eltern zuzuordnen und dabei zeigen, dass verschiedene Weibchen zu unterschiedlichen Anteilen zum Gesamtreservoir der Hautmikrofilarien beitragen.

Des Weiteren konnte ich nachweisen, dass Rinder die Endwirte von *Onchocerca* sp. ‚Siisa' sind. Letztere ist eine Form von *Onchocerca* die bisher nur im Vektor beschrieben wurde und aufgrund von (mütterlich vererbten) mitochondriellen DNS Sequenzen von anderen *Onchocerca* Arten abgegrenzt wurde. Mit Hilfe der bi-parental vererbten Nukleären Markern zeigte ich, dass O. sp. ‚Siisa' frei mit *O. ochengi* kreuzen und daher der gleichen Art angehören.

Summary

The filarial nematode *Onchocerca ochengi* is a nodule forming parasite of cattle and very closely related to the human parasite *Onchocerca volvulus*, the causative agent of river blindness. Both species are endemic in tropical and subtropical parts of Africa and share the same vectors which are blackflies of the *Simulium damnosum* complex.

It is technically and ethically easier to study the basic biology of a pathogen in an animal model, like in this case the bovine parasite *O. ochengi* than in humans. To get better insights in the population structure and reproductive behavior of *O. ochengi*, the studies in this thesis make use of nuclear and mitochondrial molecular genetic markers. First I established methods for genotyping individual *O. ochengi* adults, embryos and microfilariae at single copy loci and I identified regions containing single nucleotide polymorphisms that could serve as molecular markers. I used these markers and method to determine parent offspring relationships.

I showed that females often produce offspring from multiple males simultaneously. Most of the time, but not always, these putative fathers were still found in the nodules at the time their progeny was ready to hatch. This indicates that males, although they can leave nodules, tend to stay with a particular female for extended periods of time. I was able to assign a large fraction of microfilariae isolated from a skin sample to their parents and thereby show that different females contribute variably to the pool of skin microfilariae.

Furthermore I showed that cattle is the vertebrate host of *Onchocerca* sp. 'Siisa', a form of *Onchocerca* previously described only in the vector *O.* sp. 'Siisa' had been separated from other species of *Onchocerca* based on (only maternally inherited) mitochondrial DNA sequences. Using the bi-parentally inherited nuclear markers, I showed that *O.* sp. 'Siisa' interbreeds freely with *O. ochengi* and therefore belongs to this species.

1. Introduction

1.1. General introduction

A parasite is an organism that takes from another organism any benefit and does not give anything back in exchange, without killing this organism in the short run. It is a one-way beneficial relationship.

In the course of evolution parasites co-evolved with their hosts, thereby forcing each other to keep evolving in case of the host to get rid of the parasite or control it at a tolerable level and in case of the parasite to stay unnoticed by or to resist the defensive response of the host. This adaptation takes time or more precisely many generations, and frequently leads to a balanced host – parasite – relationship in which the parasite does not harm the host severely. It was proposed that parasites which infest new host species and therefore had no time for extensive co-evolution tend to harm their hosts more than necessary[1].

The ways parasites developed to manipulate the host are incredibly sophisticated. One example is the lancet liver fluke *Dicrocoelium dendriticum* which incorporated in its lifecycle in addition to the host snail and ant as intermediate hosts[2] The parasite enters the brain of the ant and changes its behavior in a way that the lifecycle of the parasite can be continued[3].

The field of parasites comprises a wide range of organisms, there are ectoparasites many of them belonging to the arthropoda, like fleas or lice for example. They suck the blood of the hosts, some are permanently living on the host and use the host as their habitat like the head louse *Pediculus humanus capitis*[4]. Some use the host only as a food source like the leech *Hirudo medicinalis*[5]. They suck blood from mammals but spend most of their time in ponds. In some species only the females show a parasitic behavior, like several species belonging to the Culicidae Even simple organisms like the protozoan *Plasmodium falciparum*, causative agent of Malaria, are considered as parasites[6]. In humans it is mainly found in the blood or in the liver. Also other protozoan parasites play a major role as human pathogens. For example waterborne outbreaks of diarrhea are caused by *Giardia lamblia*[7], whose main location of reproduction and pathological symptoms is the small intestine of the host. Also the

sleeping sickness, a disease in Africa, is caused by a protozoan parasite namely *Trypanosoma brucei*[8].

Another type of parasitism is brood parasitism, like the Common cuckoo (*Cuculus canorus*) performs. It is not the classical parasitism where the hosts´ bodysources are directly affected. The cuckoo lays its eggs in the nests of other birds. As soon as it hatches the cuckoo fledgling will throw the hosts´ fledglings or eggs out of the nest. The parents which represent the hosts will then feed the imposter as if it was their own offspring, actually even more as the parasite developed ways to mimic the calling of an entire brood of the host[9].

Among all the different types of parasites the kind of parasite one might think of first are intestinal worms like tapeworms for example *Taenia solium* the pork tapeworm which can infect humans when they eat pork meat containing infective stages which was not well cooked. They live in the intestine and absorb the nutrients of the food which the hosts´ digestion enzymes release[10].

Not all parasitic worms infest the digestive tract, some are found in the connective tissue like *Loa loa*[11] migrating through it or *Dracunculus medinensis*[12] which spends some part of the lifecycle connective tissue.

1.2. Nematode parasites

These last two are examples for nematode parasites. Nematodes are multicellular organisms belonging to the Ecdysozoa, they are round in cross section and longitudinally stretched. After they hatch from the egg they develop from the first to the fourth larval stage with three molts in between after a fourth molt they are adult. Some non-parasitic species can go into a dauer stage instead of the third larval stage in which they can survive a long time without food and resist harsh environmental conditions. One example for such a nematode is the free living *Caenorhabditis elegans*, its normal life cycle can be completed in 3 days but the dauer stage can buy them another at least 60 days[13]. In parasitic species this dauer stage corresponds to the infective stage in which they can remain until they find a host[14]. An example of a parasitic species is *Strongyloides ratti*, the infective larvae can live for more than 24 days[15].

One order among the nematodes are the Spirurida, to them belongs the superfamily filarioidea. The latter are characterized by a threadlike shape; males have a coiled tail and are smaller than the females; the first stage larvae (microfilariae) are in the skin or blood of the host, they have an intermediate sanguivorous insect host and they are parasites of vertebrates[16]. One representative of that group forms the main object of this thesis; it is the filarial nematode *Onchocerca ochengi*.

1.3. *Onchocerca ochengi* lifecycle

Onchocerca species undergo two host switches during their lifecycle between a vertebrate host and an insect intermediate host. Adults are only found in the vertebrate host which is usually an ungulate. Among the very few known exceptions to this are *O. volvulus* infecting humans[17] and *O. lupi* which parasitizes in dogs[18]. Blackflies of the genus *Simulium* act as intermediate hosts and vectors which transport the infective larvae to a new vertebrate host.

O. ochengi and *O. volvulus* share the same vector which is the blackfly *Simulium damnosum*[19]. During its bloodmeal the blackfly takes up microfilariae (first larval stage L_1). The microfilariae migrate to the muscle of the thorax of the fly where they molt to the second larval stage (L_2). After a few days and another molt the third larval stage (L_3), which is the infective stage, migrates to the head of the fly[20]. At this stage the infective larvae of *O. ochengi* and *O. volvulus* are difficult to distinguish[19]. At a subsequent bloodmeal of the blackfly the L_3 get into the new vertebrate host, in case of *O. ochengi* cattle. After two more molts the larvae develop to adult worms. Females can reach a length of 30 cm while males are only about 5 cm long. The females induce the formation of a nodule made of host tissue consisting mainly of collagen. Inside that they stay sedentary for the rest of their life which can last 10 to 15 years[21]. In one nodule is usually one female and zero to three males in exceptions even more, in average one male per female[22]. If multiple males are present, usually the female carries embryos sired by different males (see chapter 3.1 of this thesis). The males mate with the females in which then embryos develop in eggs. One female harbors about 75 000 to 100 000 healthy embryos per reproductive cycle[23]. When the embryos are fully developed they hatch as L_1 in the female and leave it actively. They migrate to the skin of the host where they wait to be taken up by a blackfly during its bloodmeal. In one milligram of skin are in average 2 to 9 microfilariae[24] for comparison: in one mg of skin in humans are 30 to 93 *O. volvulus* microfilariae[25]. The longevity for microfilariae of *O. volvulus* in the skin was calculated to be up to 2,5 years[26], for microfilariae of *O. ochengi* there was a longevity of 89 days in average estimated[23].

1.4. Endosymbionts of *Onchocerca* species

In 1977 Kozek et al[27] detected intracellular bacteria in the lateral chords of adult *O. volvulus* as well as in all developmental stages. Since then several filarial species were tested positive for intracellular bacteria. These bacteria were found to be closely related to *Wolbachia* in arthropods[28]. *Wolbachia* are known to be reproductive parasites in many arthropods, altering several aspects of the sexuality of their hosts, for example feminizing them[29]. In the filarial nematodes *Wolbachia* seem to be important symbionts as the worms lose their fertility and even die when treated with antibiotics which kill the intracellular bacteria[30]. The role the *Wolbachia* play in this symbiontic relationship remains still unclear. However, they make a good target for treatment of filariasis without damaging the vertebrate host

Another effect the *Wolbachia* have on the hosts´ immune system is the inflammatory reaction that might be triggered by the Lipopolysaccharides originating from the Wolbachia which are released when the worms die[31].

1.5.1. Onchocerca volvulus – causative agent of river blindness

In central Africa and parts of South America river blindness is of major importance for public health in endemic areas. As the name suggests endemic areas are close to rivers. Infected people become blind after a few years which leads locally to high incidences of blindness among adults[32-33]. That drives many people to vacate endemic areas and move to places away from rivers which are mostly less fertile areas[34].

1.5.2 Clinical manifestation

The microfilariae of *O. volvulus* migrate into the skin and eyes of infected people and eventually die there. Once they are dead the immune system detects them and responds with an inflammatory reaction. When this process happens in the eyes it can lead to lesions in the cornea, which turns opaque after some years, or to destruction of the posterior eye, nervus opticus, retina and choroid[35]. The symptoms in the skin are characterized by for example rash, dermatitis, depigmentation, hyperpigmentation or atrophy in varying intensities[21].

1.5.3. Treatment

Nodules can be removed surgically if they are in a tissue, which is easily accessible, for example over a hip bone or on the head, so the nodule is easy to palpate. If the nodule is in a deeper tissue, it might stay unnoticed[36].

Diethylcarbamazine was used against microfilariae but the side effects were so severe that it is no longer in use. The intensity of the side effect depends on the amount of microfilariae that get killed during treatment. Symptoms of side effect include rash, itching, muscle pain, fever. If microfilariae are present in the eyes, itching and swelling occurs also there. This phenomenon is called after the man who first described it the Mazzotti effect[37].

The macrocyclic lactone ivermectin kills also skin microfilariae and developing stages in the females but without initiating the Mazzotti effect[38]. Its better tolerability in patients is the reason why it replaced Diethylcarbamazine. Both drugs show no macrofilaricidal activity. Thus, treatment has to be repeated as soon as the adults resume reproduction. Best results are achieved when applied every 6 months[39].

A relatively new macrofilarial treatment is Doxycycline. It is an antibiotic which destroys the *Wolbachia* endobacterial symbionts. *Onchocerca* worms without their symbionts become sterile and die. Doxycycline has to be taken every day over a period of six weeks, which makes it difficult and expensive for mass treatment[40].

In areas where *Loa loa* is coendemic with *O. volvulus*, ivermectin cannot be applied because of severe side effects like encephalopathy which can occur[41]. As Doxycycline has no effect on microfilariae of *Loa loa*, it represents an alternative treatment[42].

1.6. Eradication attempts

1.6.1. Vector control

Vector control does not help decreasing the number of nodules in patients but it prevents transmission and therefore new infections. In the forties of the 20th century first attempts were started to reduce the vector from endemic areas by eliminating the vegetation from rivers, which serve as breeding sites for the vector. In 1974 the WHO started the Onchocerciasis Control Programme (OCP) which was active in Benin,

Burkina Faso, Côte d'Ivoire, Ghana, Guinea Bissau, Guinea, Mali, Niger, Senegal, Sierra Leone and Togo until 2002. Insecticides were sprayed from aircrafts over rivers[43]. That kills the larvae of the vector *Simulium damnosum* which breed in rivers. Until 1987 this was the only method against onchocerciasis. Since the introduction of ivermectin, the vector control plays a minor role[43].

1.6.2. Ivermectin mass treatment

In endemic areas ivermectin is used for mass treatment of the population. Applied at least once per year it kills the microfilariae but not the adults. For a certain time (until new microfilariae are produced) the transmission of the disease is interrupted. As the adult worms survive the treatment and have a lifespan of 10 to 15 years, the treatment has to be continued at least during that time[44]. The African Programme for Onchocerciasis Control (APOC) was established in 1995 and is still active until 2015 distributing ivermectin to the population in 19 countries (Angola, Burundi, Cameroon, Central African Republic, Chad, Congo, Democratic Republic of Congo, Ethiopia, Equatorial Guinea, Gabon, Kenya, Liberia, Malawi, Mozambique, Nigeria, Rwanda, Sudan, Tanzania and Uganda)[45-46]. Recent data from Cameroon show that transmission still continues, though at a low level (personal communication A. Renz).

1.7.1. Speciation by host switch, importance of phylogenetic analyses

Speciation can occur driven by several events. One example is a mating incompatibility which might occur through a mutation[47]. Another event could be separation of one population into two after a geological change like in the case of marine threespine sticklebacks, which invaded freshwater lakes after the retreat of the Pleistocene glacier[48]. A host switch can also lead to speciation, forming also a physical separation of the population. *Onchocerca* species parasitize in ungulates, but there are two exceptions which are evolutionary probably young and might have been formed by a host switch. *Onchocerca lupi* was described in 1967 in a wolf in Georgia[49]. In 1991 it was first reported in a dog in California[50] and in 2011 in a human in Turkey[51]. Since 2011 several reports were published about various cases of zoonotic infections from *O. lupi* in humans in Turkey, Tunesia, Iran and the US. Even in places where

O. lupi was not known to be endemic infections occured[51]. For the first cases it was presumed that it was an aberrant infection of a bovine *Onchocerca* species. But not for all cases the mitochondrial sequences were tested. Since sequencing mitochondrial genes is becoming a routine analysis, it is easier to identify organisms and tell if several independent infections are actually caused by the same agent and if that agent is in fact a bovine *Onchocerca* species or something else at least if there is already a similar sequence published. This shows the importance of sequencing certain genes and making them publicly available.

O. volvulus most likely evolved by a host switch from a common ancestor with *O. ochengi*. There might have been also a chromosome fusion involved, which makes mating difficult. *O. ochengi* has 4 autosomes and an X and a Y chromosome, *O. volvulus* has only 3 autosomes and XY[52].

1.7.2. *O. ochengi – O. volvulus*

In comparison to infections with *O. volvulus* in humans, infected cattle with *O. ochengi* show no clinical manifestations in the skin or in the eyes. There could be several reasons, one is the fact that the microfilarial density in humans is higher than in cattle. Another reason why cattle might not develop pathological effects in their eyes is that they do not live long enough; in humans this process takes years. It is possible that cattle are better adapted to *O. ochengi* and vice versa and therefore they do not harm each other. It should be mentioned in this context that there appear to be two different strains of *O. volvulus*, which differ in their pathogenicity. A forest strain which causes no blindness[53] and a savannah strain which is prone to cause blindness[54]. One of the reasons for the difference in pathology is that the forest strain is less invasive to the eyes[55]. The strains differ genetically and can be distinguished by a diagnostic 150 bp repeat[56]. So far no genetic reason was found that explains the pathogenetic difference.

1.7.3. Onchocerca ochengi as a model for *O. volvulus*

Onchocerca ochengi is phylogenetically the closest known relative of *O. volvulus*. The two species have probably evolved from a common ancestor only about 10 000 years ago when man started to domesticate cattle in Africa[57]. They still share common

alleles (see chapter 4 of this thesis). Both have the same insect vector, the blackfly *Simulium damnosum* s. l. The larvae that are found in the vector are morphologically difficult to distinguish from each other[58]. The adult females of both species live sedentary in nodules in the skin of their host. *O. ochengi* is used as a model to study the biology of these filariae in a way which is not possible in humans for *O. volvulus*[59].

1.8. Onchocerca ochengi 'Siisa'

Before molecular methods were available, species identification was based solely on morphology. Depending on the larval stage, some species look indistinguishably similar. If the morphological criterion is the length of larvae, there are often overlapping sizes of different species. Another misleading conclusion can be drawn from the host where the sample was taken from. Sometimes worms of the same species show different morphological features in different host animals which makes the identification difficult if found for the first time in that host[60]. Since molecular genetic comparisons of certain sequences, the mitochondrial for example, are possible it is much easier to distinguish different samples, even though they might appear morphologically almost identical. However, there exists some degree of sequence variability among different individuals of the same species. Therefore, also based on sequence alone it is frequently not possible to unambiguously decide if to individuals belong to the same or to two closely related species.

In 2007 Krüger et al. reported the finding of an infective larva in a *Simulium* fly from Uganda, which appeared intermediate between *O. ochengi* and *O. volvulus* based on mitochondrial markers. It did not group with any other *Onchocerca* species[17]. As it was found close to the river Siisa and they could not assign it to a known species, the authors called this worm *Onchocerca species* 'Siisa'. Since it was discovered in the insect vector, the vertebrate host remained unknown. They also showed that for the O-150 repeat marker, the classical marker to identify the forest and savannah strain of *O. volvulus*, *O.* sp. 'Siisa' clusters with *O. volvulus* and not with *O. ochengi*.

Here, in collaboration with others, I show that a) *O.* sp. 'Siisa' also occurs in Cameroon; b) the host of *O.* sp. 'Siisa' is cattle and c) *O.* sp. 'Siisa' is a mitochondrial clade of *O. ochengi* (see chapters 3.2 and 3.3 of this thesis).

2. Aim of this thesis

The aim of this thesis was to investigate the reproductive behavior of *Onchocerca ochengi* based on molecular genetic analyses. In particular I intended to answer the following questions. How many males contribute genetically to the offspring of one female? If multiple males are present in one nodule, how many of them reproduce with the female? Do males stay after mating in a particular nodule or leave it and search for a new female? From which nodule/female do the skin microfilariae originate? Do many or only a few adults contribute to the pool of skin microfilariae?

In addition I wanted to clarify the taxonomic status of the nodule forming *Onchocerca* in cattle at our sampling site in Cameroon. To this end I intended to use nuclear and mitochondrial molecular genetic markers to determine if all worms in a particular host individual that based on morphological criteria are considered *O. ochengi,* indeed form one freely intermixing population or if there is indication for multiple, genetically separate populations.

3. Results and Discussion

3.1. Single worm genotyping demonstrates that *Onchocerca ochengi* females simultaneously produce progeny sired by different males

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Synopsis

It had been observed that in average there is one male per female in the nodule of the filarial nematode *Onchocerca ochengi* and the nodules are relatively far apart of each other. Therefore it was speculated that *O. ochengi* males stay with a particular female and defend it against intruders, thereby preventing the risk of not finding another female after leaving a nodule. This strategy would render the reproductive strategy essentially monogamous. However, the reproductive behavior had never been investigated at a molecular genetic level. In part this was due to the technical difficulties on molecular genotyping of individual *O. ochengi* worms.

For this publication we developed a protocol for the reliable PCR amplification of single copy loci from different developmental stages of *O. ochengi* including embryos and microfilariae. We identified five genomic fragments containing single nucleotide polymorphisms, referred to as molecular markers. We dissected 32 *O. ochengi* nodules and we genotyped the female worms and the 67 adult male worms, present in these nodules as well as a fraction of the progeny from within the uteri of the females, In 18 of the 32 females progeny derived from multiple males were found. In 5 nodules the males present were not sufficient to explain the genotypes of the entire progeny. We conclude that *O. ochengi* females frequently produce progeny sired by different males simultaneously and that most but not all males are still present in the nodules when their offspring is ready to hatch.

Own contribution

I performed all experiments described in this manuscript except of the excision of the

O. ochengi nodules from the cattle skins. I analyzed the data together with the co-authors and I participated in writing the manuscript.

3.2. Molecular evidence of 'Siisa form', a new genotype related to *onchocerca ochengi* in cattle from North Cameroon

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Synopsis

Simulium damnosum s.l. serves as vector for several *Onchocerca* species. To identify the latter correctly we genotyped 12S, 16S and *coxI* mitochondrial loci of L3s, which were obtained from blackflies in North Cameroon and of adult worms isolated from nodules of a Zebu cattle from Ngaoundéré, Cameroon. In both hosts we found two different clades of mitochondrial haplotypes. One of them contained the sequence of a worm earlier described as *Onchocerca* sp. 'Siisa', which had been isolated only from the vector. Therefore its vertebrate host was unknown. Both haplotypes are about equally distant from each other as is either one of them from *O. volvulus*. Both haplotypes occurred together in the same individual blackflies and zebu. *O. volvulus* was also found in this study but only in the vector and not in cattle. These results showed that *Onchocerca* sp. 'Siisa' also occurs in Cameroon and that at least one of its vertebrate hosts are zebus.

Own contribution

I performed all the genotyping of the worms isolated from cattle.

3.3. Reproductive biology of *Onchocerca ochengi*, a nodule forming filarial nematode in zebu cattle

Julia C. Hildebrandt, Albert Eisenbarth, Alfons Renz, Adrian Streit

Veterinary Parasitology (2014) in press

Synopsis

To analyze the *O. ochengi* population infesting a single host all the nodules of two Zebu cattle from Ngaoundéré, Cameroon, were excised and the adults and a portion of the offspring found within the nodules were genotyped at six nuclear and two mitochondrial loci. Furthermore samples of the skin were taken and the microfilariae found in there were analyzed in the same way. We found a high number of alleles at the nuclear and at the mitochondrial loci, which allowed the determination of the parents not only for the offspring isolated from the uteri of their mothers but also for a high proportion of the skin microfilariae. This also allowed us to address the question if representatives of the two mitochondrial clades described above interbreed. We found no indication for assorted mating and inter-clade pairs were not less likely to produce progeny together than intra-clade pairs. Further, the analysis of nuclear allele frequencies and distribution did not probide any indication of multiple genetically separate populations. Taken together, these strongly suggest that both mitochondrial clades belong to the same species, namely *O. ochengi*.

The analysis of the skin microfilariae revealed that the contributions of individual parents to the pool of skin microfilariae is highly variable at least at a giben time and place.

Own contribution

I performed all the experiments in this manuscript except for the excision of the *O. ochengi* nodules fom the cattle skins. I analyzed the data together with the co-authors and I participated in writing the manuscript.

4. Comparison between alleles of *O. ochengi* and *O. volvulus*

To compare the genetic complexity between *O. ochengi* and *O. volvulus*, I was provided with samples of human origin: *O. volvulus* nodules from patients in Bolo, Cameroon, taken by Dr. Peter Enyong in 1991 and brought to Tübingen by Dr. Alfons Renz. The nodules were all in one 50 ml Greiner tube in 100% Ethanol, there is no information about from how many different hosts the nodules originated. The sample comprised a total of 22 nodules containing 25 males and 27 females. All the adult worms and a portion of the offspring -if present- were genotyped at six nuclear loci, corresponding to the six nuclear loci described in for *O. ochengi* in chapter 3.3[61] and at the mitochondrial 12S and 16S loci. In all cases *O. volvulus* was sufficiently similar to *O. ochengi* that the same PCR primers could be used. At all six nuclear loci I found alleles that were shared between *O. ochengi* and *O volvulus* but at *ytP159*, *161*, *162*, *164* and *169* there existed also alleles not present in our *O. ochengi* samples.

Figures 1 to 6 show haplotypes networks for the six nuclear markers comparing the alleles found in *O. ochengi* and the ones found in *O. volvulus*. For each marker the different sequences of each allele were aligned with the method MUSCLE using the MEGA5.2 software. Saved as .nex format they could be applied to the TCSv1.21 software. This program calculates a haplotypes network representing the similarity between the sequences. Every nucleotide difference is illustrated as a knot. Pictures 1A to 6 A depict the resulting haplotypes networks of the TCSv1.21 calculations. Light grey dots are haplotypes from *O. volvulus*; dark grey dots are from *O. ochengi*. Dots which are half dark and half light represent alleles shared by both species. Pictures 1B to 6B show the frequencies of the *O. ochengi* alleles in the host individual that is the major focus of chapter 3.3. over all the adults for the respective marker in form of a pie chart (data from Figure 4 of Hildebrandt et al. (chapter 3.3.), males and females combined, see also Figure 7 column “cattle B”). The allele numbers of shared alleles are indicated in A and B.

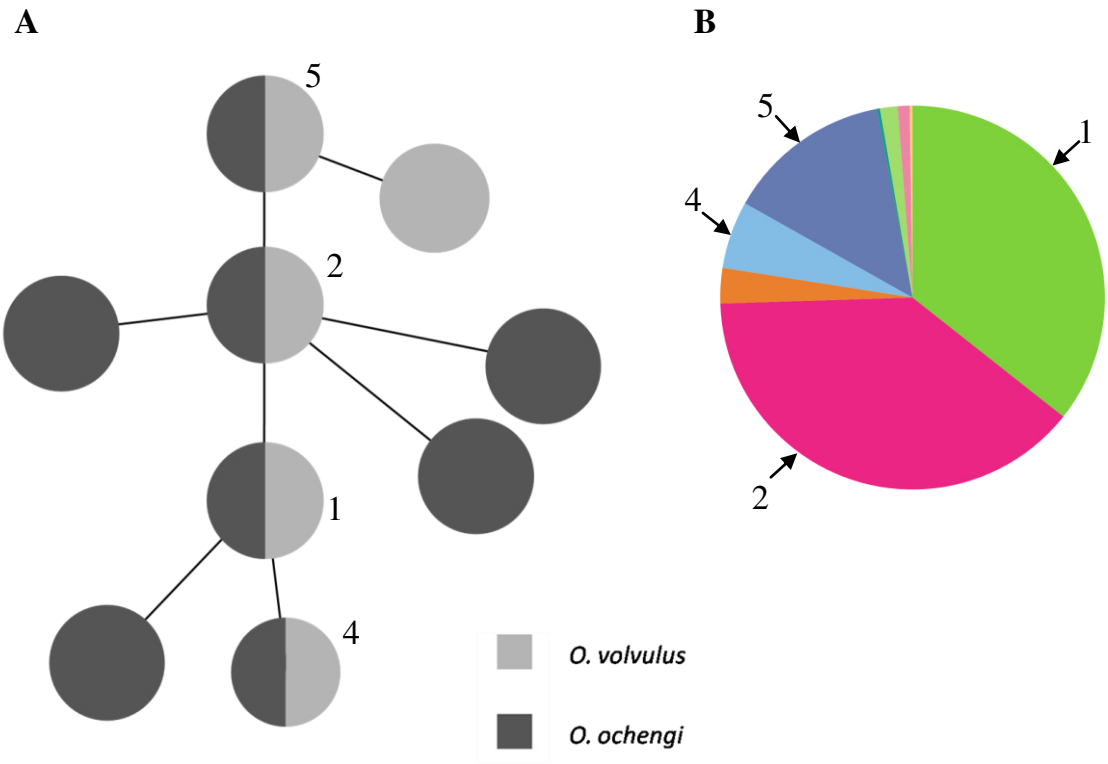

Figure 1: Haplotype network of marker *ytP159*.

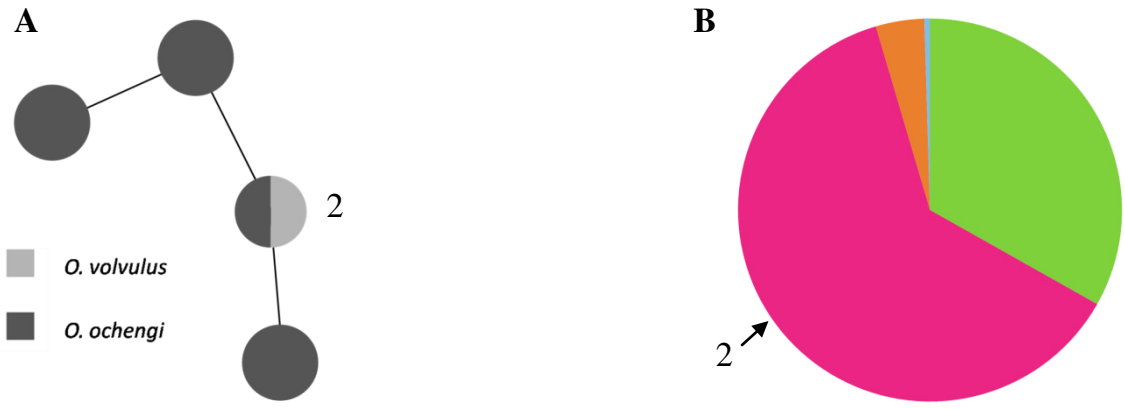

Figure 2: Haplotype network of marker *ytP160*.

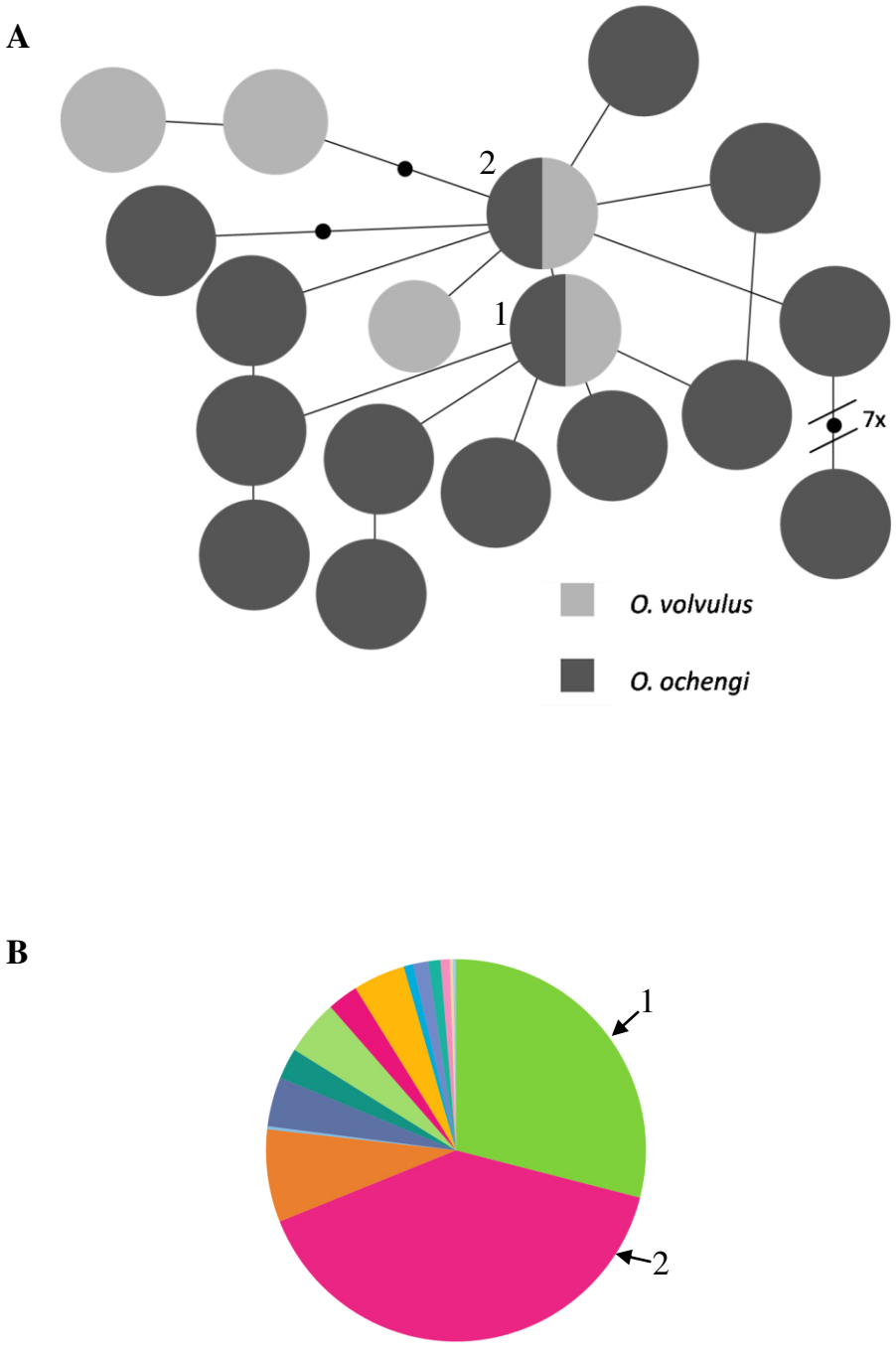

Figure 3: Haplotype network of marker *ytP161*.

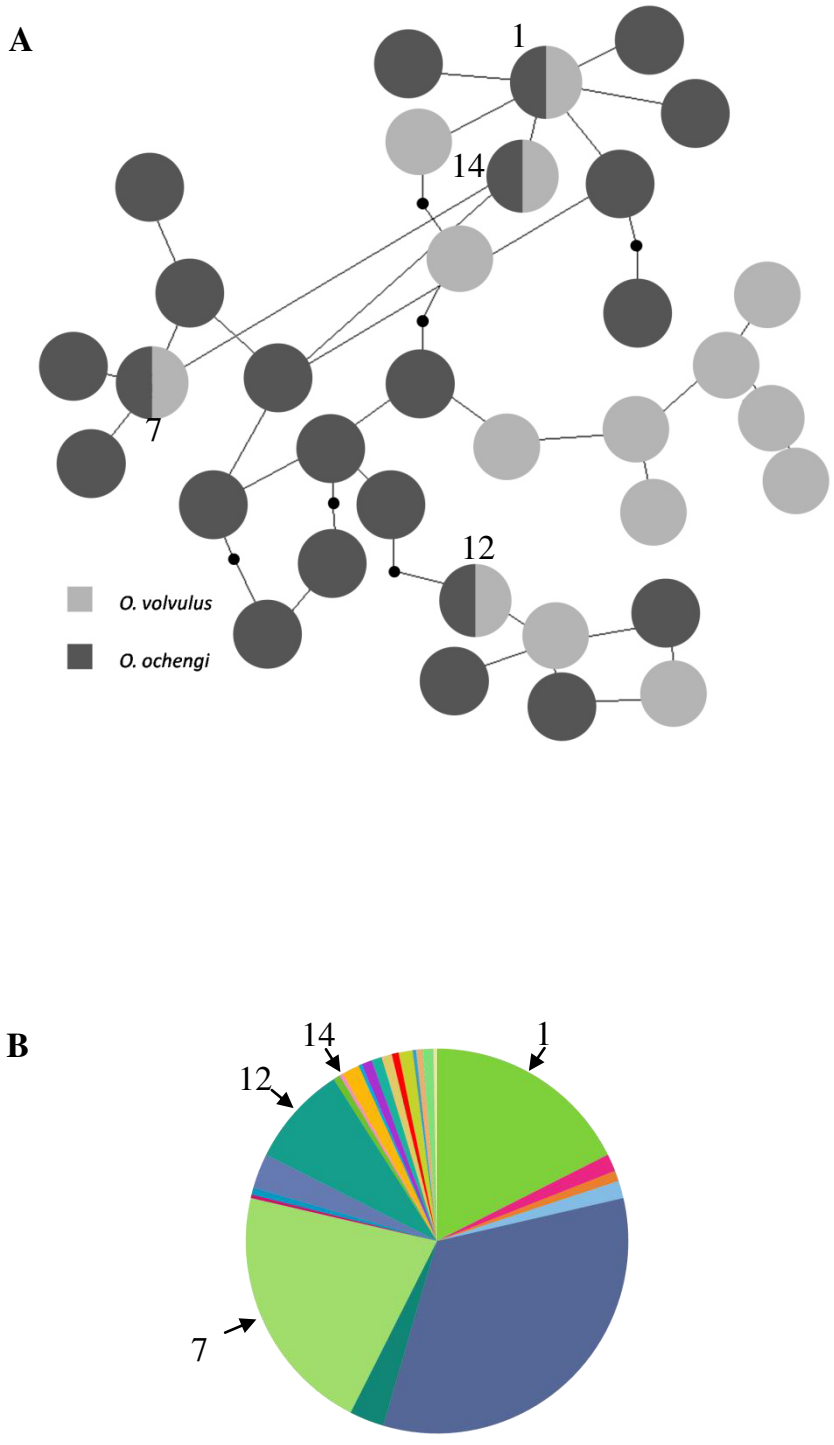

Figure 4: Haplotype network of marker *ytP162*.

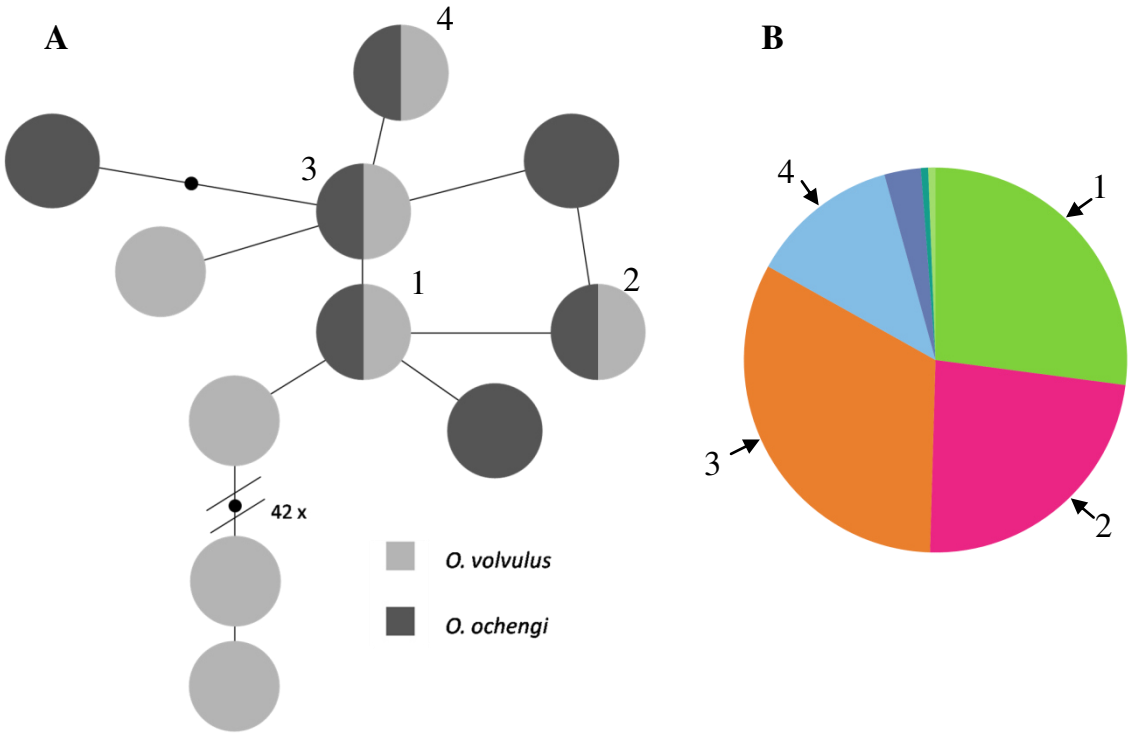

Figure 5: Haplotype network of marker *ytP164*.

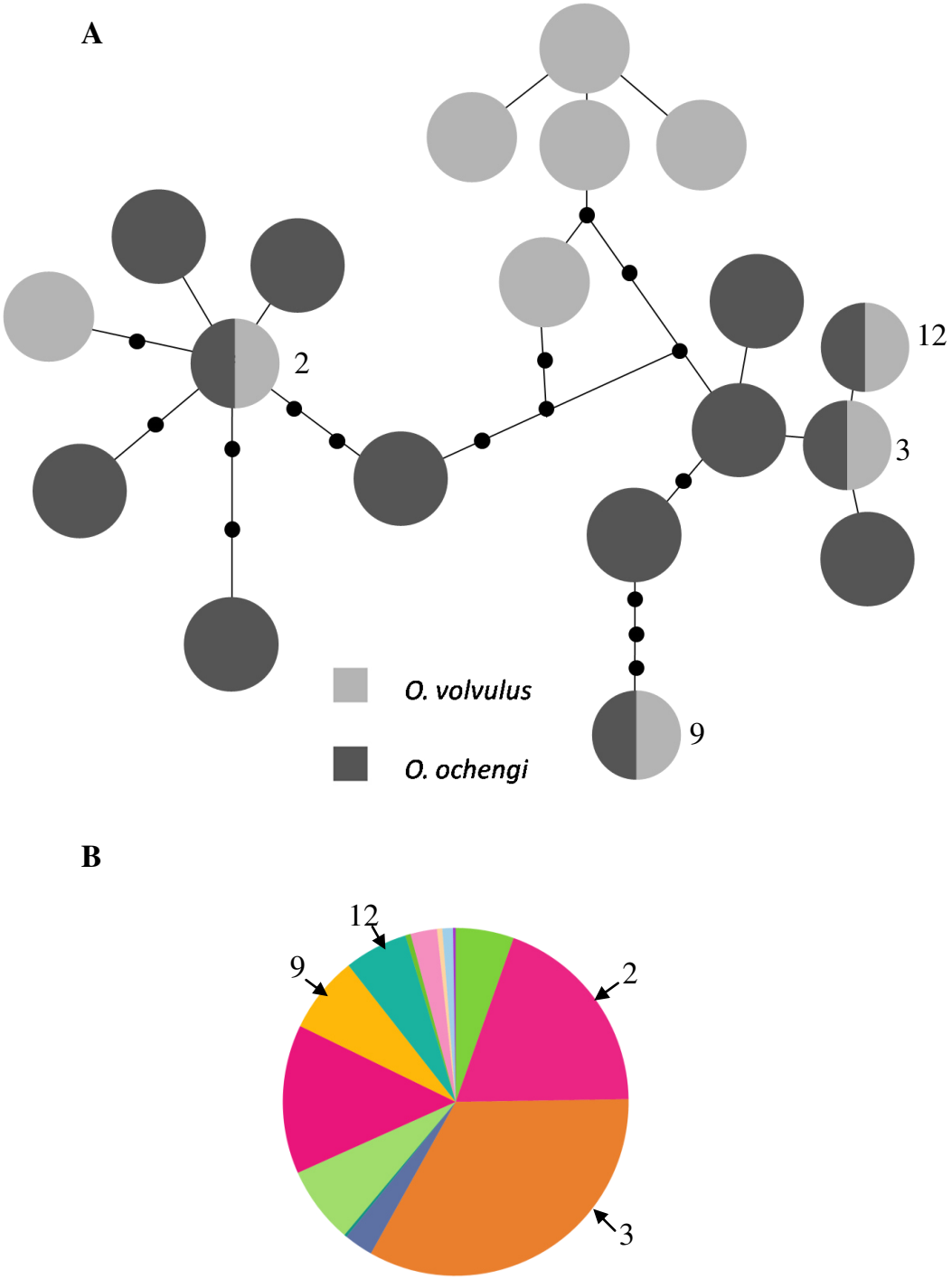

Figure 6: Haplotype network of marker *ytP169*.

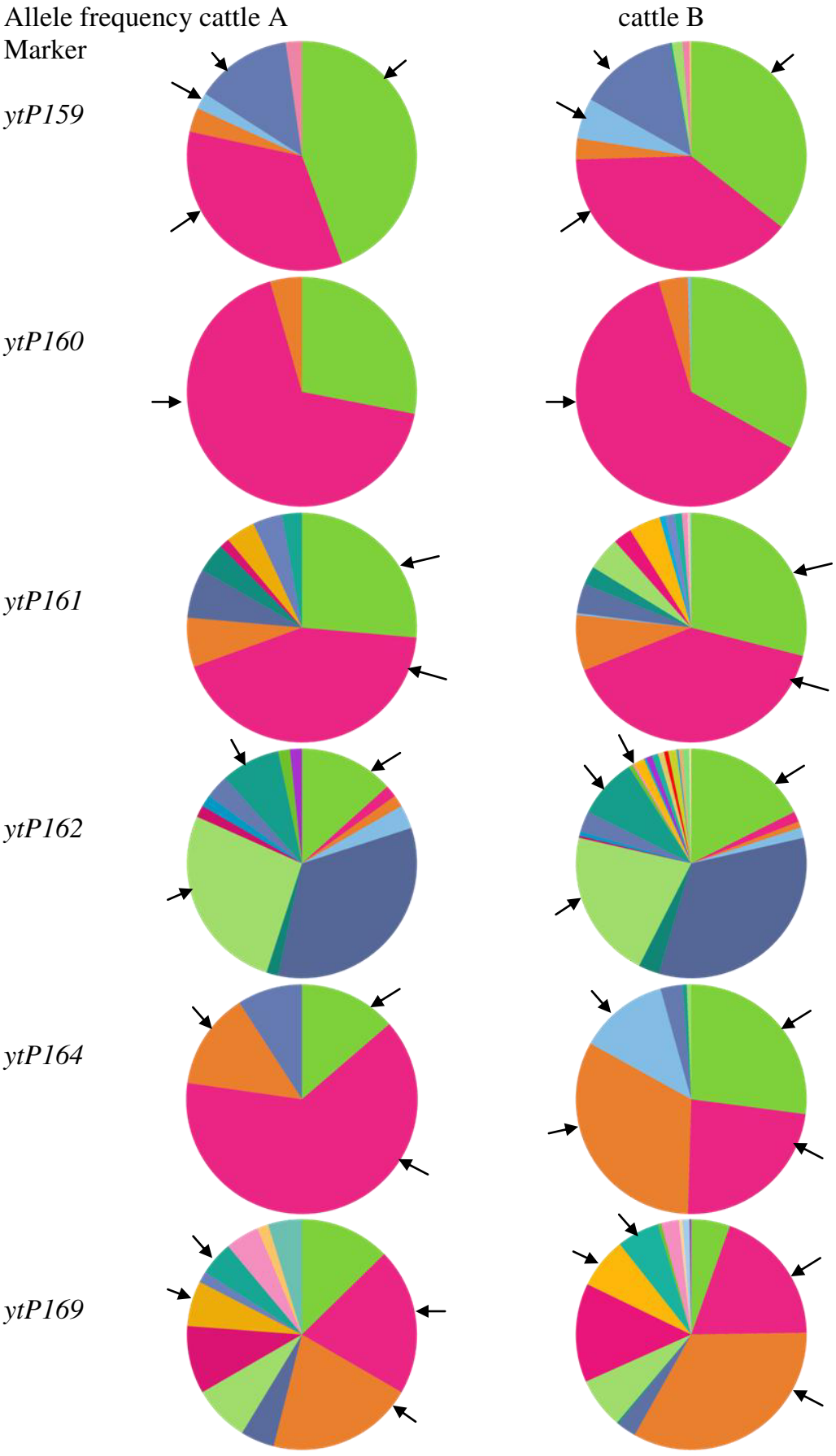

Figure 7: Allele frequencies of the markers *ytP159-162*, *164* and *169* of cattle A and B

Figure 7 shows the allele frequencies of all markers for both cattle that were analyzed in chapter 3.3. They are referred to as cattle A and B, with B being the one chapter 3.3 mainly focuses on. Each color represents one allele and the size of the piece indicates the frequency of the respective allele. Overall, the *O. ochengi* populations in both host animals have largely the same alleles. Notice that the number of individuals analyzed is larger in B than in A. The alleles shared between *O. ochengi* and *O. volvulus* tend to be present at fairly high frequencies in *O. ochengi*.

One explanation for why the two species still share alleles could be that there is still gene flow occurring between them. If this were the case, it would be of direct medical releance because also resistances against anthelminthic drogues arising in *O. ochengi* in cattle could be transferred to *O. volvulus* and threaten the current control measures. Alternatively, it could be that these alleles are still maintained from the common ancestor which means that those alleles are older than the species[62]. In this context it is interesting to note that the maximum number of shared alleles I found was four. This would be consistent with a scenario where one male and one female, both heterozygous for different alleles at a particular locus made the host switch. This second alternative also illustrates that nuclear sequences alone are not appropriate to identify the species a particular individual belongs to.

It has to be taken into account that the *O. ochengi* samples were from Ngaoundéré where the cattle was slaughtered in 2011 and the *O. volvulus* samples were from 1991 from Bolo (Kumba). That is a difference of 20 years and roughly 900 kilometers. The 20 year time difference is probably not very significant as the generation time is at least a year. It is rather unlikely that either the cattle or the fly would travel between these two locations. Therefore, no direct comparison can be made as the genetic structure of the *O. ochengi* population from the Bolo area is not known. A good experiment to compare both populations in a proper way would be to take samples from both species from the same location or at least the same area and sample at the same time.

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Appendix

Lebenslauf / Curriculum vitae

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In Stuttgart (Baden-Württemberg)

Seit November 2010	Dissertation
	Unter Anleitung von Adrian Streit
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	Mit dem Thema
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2009-2010	Diplomarbeit
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	Mit dem Thema
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2005-2009	Studium der Biologie
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Parasitol Res (2012) 111:2217–2221
DOI 10.1007/s00436-012-2983-x

SHORT COMMUNICATION

Single worm genotyping demonstrates that *Onchocerca ochengi* females simultaneously produce progeny sired by different males

Julia C. Hildebrandt · Albert Eisenbarth · Alfons Renz · Adrian Streit

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Abstract *Onchocerca ochengi* is a filarial nematode parasite of African cattle and most closely related to *Onchocerca volvulus*, the causing agent of river blindness. *O. ochengi* females induce the formation of a nodule in the dermis of the host, in which they remain sedentary in very close association with the host's tissue. Males, which do not adhere to the host's tissue, are also found within the nodules at an average number of about one male per nodule. Young *O. ochengi* females tend to avoid the immediate proximity of existing nodules. Therefore, *O. ochengi* nodules are dispersed in the ventral inguinal skin at considerable distances from each other. It has been speculated that males avoid the risk of leaving a female once they have found one and remain in the nodule as territorial males rendering the reproductive strategy of *O. ochengi* essentially monogamous. We developed a protocol that allows reliable PCR amplification of single copy loci from different developmental stages of *O. ochengi* including embryos and microfilariae. From 32 *O. ochengi* nodules, we genotyped the female worms and the 67 adult male worms, found in these nodules, together with a fraction of the progeny from within the uteri of females. In 18 of 32 gravid females progeny derived from multiple males were found. In five nodules, the males isolated from the same nodule as the female were not sufficient to explain the genotypes of the entire progeny. We conclude that frequently *O. ochengi* females simultaneously produce progeny sired by different males and that most but not all males are still present in the nodule when their offspring is ready to hatch.

Electronic supplementary material The online version of this article (doi:10.1007/s00436-012-2983-x) contains supplementary material, which is available to authorized users.

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Introduction

The filarial nematode *Onchocerca ochengi* is a parasite of cattle in tropical and subtropical regions of Africa. It is most closely related to *Onchocerca volvulus*, the causative agent of human onchocerciasis. *O. ochengi* and *O. volvulus* share the black fly *Simulium damnosum s.l.* as a vector (Renz et al. 1994; Wahl et al. 1994). Due to the close phylogenetic relationship and the many parallels in the biology of these two worms, *O. ochengi* serves as an animal model for *O. volvulus* (Renz et al. 1995). In spite of ongoing efforts to combat onchocerciasis, i.e., by pan-African mass-treatments of endemic areas (APOC, WHO, http://www.who.int/blindness/partnerships/APOC/en/), *O. volvulus* continues to be a threat to the health of millions of people, and new therapies and control measures are required (Hoerauf et al. 2011). Development of resistance against ivermectin, the only one drug presently used in mass treatment, is likely to occur, and the spread of resistance will depend on the population biology dynamics and mating behavior of the *Onchocerca* worms.

O. ochengi females induce the formation of nodules in the dermis of the host, in which they remain sedentary in very close association with the host's tissue. They remain reproductive for many years, presumably as long as their hosts live (5 to 10 years; Determann et al. 1997; Wahl et al. 1994).

Young *O. ochengi* females tend to avoid the immediate proximity of existing nodules. Therefore, *O. ochengi* nodules are dispersed in the ventral inguinal skin at considerable distances from each other. Sometimes, in very heavily infested cattle (>100 nodules), groups of 5 to 15 nodules can be found close to each other in the udder and teats. Nevertheless, each nodule remains separated from other nodules, like the grapes of wine. This is in contrast to females of *O. volvulus*, which tend to group together and form clumps of nodules, consisting of female worms of different ages (Wahl et al. 1994; Schulz-Key 1988).

Upon mating, the embryos develop and finally hatch in the uteri of the female. The microfilariae (first-stage larvae) migrate to the peripheral skin and wait to be taken up by a black fly during its blood meal. In the vector, the larvae develop to the third stage and during a later blood meal the fly transfers the infective third stage larvae to a new host. After reaching adulthood, females induce the formation of a nodule and males search for a mate.

The spacing of *O. ochengi* nodules, sometimes more than 10 to 50 cm, poses a certain challenge for males to find multiple mates. Males are much smaller than females and do not adhere to the host's tissue. They are found within the nodules at an average number of one male per nodule (Renz et al. 1994), and the situation with exactly two males is less frequent than expected by chance, indicating a territorial defense of single males (AR, unpublished observations). Further, it has been observed that the males and the females in a particular nodule often are of similar age. Based on these observations, it has been speculated that *O. ochengi* males become territorial once they have found a female and avoid the risk of leaving the nodule to search for additional mates. This would make the reproductive strategy of *O. ochengi* essentially monogamous (Renz et al. 2010).

O. ochengi microfilariae are notoriously difficult for DNA preparation for molecular genotyping. The so far most reliable protocol described involves cutting the microfilariae with a laser dissecting microscope (Post et al. 2009) but this procedure is very cost- and labor-intensive. Starting from the protocol we routinely use for *Strongyloides* spp. (Eberhardt et al. 2007; Nemetschke et al. 2010), we systematically varied all parameters and devised a protocol that allows reliable PCR amplification of single locus genomic sequences from individual *O. ochengi* microfilariae (Protocol 1).

Protocol 1. Preparation of single *O. ochengi* microfilariae for PCR amplification of single locus genomic DNA

Equipment and reagents

- *O. ochengi* worms in PBS
- 2x lysis buffer (20 mMTris-HCl pH 8.3, 100 mMKCl, 5 mM $MgCl_2$, 0,9 % NP-40, 0,9 % Tween 20, 0,02 % Gelatine, 240 µg/ml Proteinase K [add just before use])
- Mouth pipette
- PCR machine
- PCR grade water

Method

1. Transfer a single worm into a PCR tube with 20 µl H_2O
2. Close the tube and freeze, thaw and vortex vigorously. Repeat three times.
3. Add 20 µl of 2x lysis buffer and mix by finger tapping.
4. Incubate at 65°C for 8 hours in a PCR machine.
5. Incubate at 95°C for 15 minutes to inactivate the proteinase K.
6. Add 10 µl of water and use up to 5 µl as template for PCR amplification.

We isolated five molecular markers (*ytP159, ytP161, ytP62*, *ytP164*, *ytP169*, Table 1, Suppl. Table 1) based on Expressed Sequence Tags available from the National Center for Biotechnology Information following the strategy described earlier by our laboratory for *Strongyloides* sp. (Eberhardt et al. 2007; Nemetschke et al. 2010). "Molecular markers" is the term we use for fragments of genomic DNA that can be PCR-amplified with defined primers and contain one to several single nucleotide polymorphisms (SNPs).

We dissected 48 individual *O. ochengi* nodules from the skins of naturally infected cows that had been collected post mortem from Zebu cattle at the abattoir in Ngaoundéré, Cameroon. Of those nodules, eight contained no males and the females did not have progeny in their uteri. One nodule contained a gravid female, but no male. In seven nodules, the females were without progeny although males were found. Thirty-two nodules contained females with developing embryos in the uterus and at least one male. Of the last category, we genotyped all adults we found and a fraction of the progeny for multiple markers (Tables 2 and 3). In order to avoid selecting microfilariae that might have migrated to the nodule, we analyzed only embryonic progeny that were still in the eggshell or microfilariae directly from the uteri.

First, we asked if all progeny isolated from a particular female stem from the same partner, present or not in the nodule. In 18 of the 32 gravid females, progeny of multiple males were identified, indicating repeated inseminations by two or more males.

Next, we asked if the genotypes of the microfilariae were consistent with the hypothesis that their fathers were present in the nodule. In 27 of the 32 nodules, this was the case. In 3 of the 12 nodules containing one male, the present male was not the father of all progeny. Also in two of the nine nodules, containing three males, there was at least one father per nodule missing. As mentioned above, we also found a nodule with a gravid female but no male.

From our data, we conclude that reproduction in *O. ochengi* is not predominantly monogamous, though most fathers tend to stay with their gravid females at least for as long as it takes for their progeny to reach the microfilarial stage. Nevertheless, at least some males appear to leave the

Table 1 Molecular markers used

Marker	Primers[a]	Length in base pairs[b]	Number of different alleles found[c]
ytP159	fw: TGCGTTTTCTGATCGTATTT rev: CCCTTTTGAATCAATGATGA	446	8
	seq: TGCGTTTTCTGATCGTATTT		
ytP161	fw: TATCTCCTCTTTCGGTGTCA rev: ATTCTGCTGAAGCTTTCCTT	405	14
	seq: TATCTCCTCTTTCGGTGTCA		
ytP162	fw: AGGCACATGTTTTGGTAGTGG rev: AGTTTGCCGGTCATTGATTC	629	25
	seq1: CCTATAGAACTTCTCTTGAG		
	seq2: CTCAAGAGAAGTTCTATAGG		
ytP164	fw: GCATCTTCGCTATCCTTTGC rev: CGAATGGAAACAGCAGCAG	448	7
	seq: AGACTTATCCGTGGTT		
ytP169	fw: CGACATTTGCTATGGGAAGC rev: CACCATCGCAGCTGTGTACT	372	15
	seq: CGACATTTGCTATGGGAA		

[a] *fw* forward primer; *rev* reverse primer; *seq* primer used for sequencing. For *ytP162* two sequencing primers pointing from the same position into opposite directions were used

[b] Overall length of the PCR product including the primers

[c] Each marker contains multiple SNPs. One particular combination of bases at the variable positions within a marker is referred to as an allele. Details are given in Supplementary Table 1. PCR reactions were done with 5 μl of worm lysate (see Protocol 1) in a total volume of 25 μl of ThermoPol Buffer (New England Biolabs) supplemented with 0.2 mg/ml bovine serum albumin, 0.5 mM $MgCl_2$, 0.2 μM primer (each), 120 μM dNTPs (each) and 1.25 U of Taq DNA polymerase (New England Biolabs). An initial denaturation step of 95 °C for 3′ was followed by 35 cycles of 95 °C for 30″, 58 °C for 30″, 72 °C for 1′ and a final extension step of 72 °C for 7′. 0.3 μl of the resulting product were used for sequencing using the BDTv3.1 kit (Applied Biosystems) following the manufacturer's instructions. The samples were submitted to the in house sequencing facility for analysis

Table 2 Results for the individual nodules with males and progeny

Nodule number[a]	Number of males found	Number of progeny genotyped	Minimal number of fathers	Males in nodule sufficient to explain progeny	Minimal number of fathers not found
AI	1	14	1	Yes	0
AH	2	15	2	Yes	0
A19	3	8	2	No	1
A22	4	4	2	Yes	0
A35	2	11	2	Yes	0
B1	1	9	2	No	1
B3	1	11	1	Yes	0
B8	3	7	2	Yes	0
B9	3	6	2	Yes	0
B10	1	12	1	Yes	0
B11	1	3	2	No	1
B13	4	12	3	Yes	0
B15	3	7	1	Yes	0
B16	1	13	2	No	1
B20	3	6	1	Yes	0
B21	3	21	2	Yes	0
B23	2	27	2	Yes	0
B24	4	11	2	Yes	0
B25	3	14	2	No	1
B26	3	15	1	Yes	0
B30	1	22	1	Yes	0
B31	2	15	2	Yes	0
B32	2	16	1	Yes	0
B33	1	19	1	Yes	0
B34	2	18	2	Yes	0
B35	1	13	1	Yes	0
B36	3	15	3	Yes	0
B37	1	17	1	Yes	0
B38	1	20	1	Yes	0
B39	2	21	2	Yes	0
B40	1	10	1	Yes	0
B44	2	26	1	Yes	0

[a]Nodules A19, A22, A35 were isolated on 19.01.2011 from one animal, nodules B1-44 were isolated on 13.01.2011 from one animal; Nodules AI, AH were isolated in the context of an earlier study and recovered from the freezer. All nodules contained only a single female worm

nodule after siring progeny. It is possible that different males follow different strategies. Some males may be territorial and by remaining in the nodule they may father a large portion of the progeny of the corresponding female. Others may be roamers and try to mate with multiple females thereby "stealing" a portion of the progeny from the territorial males. Mixed strategies like this have been described for various organisms from different phyla (Gross 1996).

Table 3 Results from Table 2 summarized

Nodules (females) with	Number	Number of nodules consistent with one father	Number of nodules consistent with all fathers in nodule	Minimal number of males not found
1 male	12	9	9	3
2 males	8	2	8	0
3 males	9	3	7	2
4 males	3	0	3	0
Total	32	14	27	5

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

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Supplementary table 1: Details to the markers used

Marker	Primers	Sequence[a]	Alleles[b]
ytP159	fw: TGCGTTTTCTGATCGTATTT rev: CCCTTTTGAATCAATGATGA seq: TGCGTTTTCTGATCGTATTT	TGCGTTTTCTGATCGTATTTCGGAA TTCAAAAAATTAGATGTAGCTGTT ATGGCGTGTTCAACTGACTCGCATT TCTCGCATCTTGCATGGGTAAATAC CGACCGAAAAATGGGTGGACTTGG TCAGATGAATATACCAATTCTTGCT GATACCAATCATGCAATCAGCAAG GCATATGGTGTGCTCAAGGAAGAT GAAGGAATTGCTTATCGGTACGTA TTCTTTGATATGAGTAAGATGTGAA GCCATCGAAGGCA**R**CGAGCGATTT GAA**R**ATATGTGGCATCAACTT**Y**AT GACTTTTTTAGAGTTATTGTTCTTC AGTTCTTGCGAATACTT**Y**CTCTTCT TTGTTGTGTT**R**TGATTGAAATGGTT GAAATCAGATTGTCATAGTTTATtG AAAACAAT**R**TTTGAACTTATTTCAG TGGA**Y**TATTCATCATTGATTCAAAA GGG	1 GACTAAC 2 GACTAGC 3 GACTGAC 4 AACTAAC 5 GACTAGT 6 GGCTAGC 7 GACCAGC 8 GATTAGC
ytP161	fw: TATCTCCTCTTTCGGTGTCA rev: ATTCTGCTGAAGCTTTCCTT seq: TATCTCCTCTTTCGGTGTCA	TATCTCCTCTTTCGGTGTCAACTTC ACTTTTTATGACTTATCTTGCGGCA GATGGCACAACAAAGCAACAATTG CAAGATGTTCTTGGAGGAAGTAAT TACATATTGAAATTTTTTTAATTCG AAATACTGAAAAAG**S**AATAATCAC GCA**M**ATTACCTCAAAGTTG**R**AA**K**T TTGGACATCAAGAATGCT**R**TAACT GTAAGGATAGATTTCATAAATG**W**T AAAATAATCGTTTCTAAATTA**R**CAT AAAATCAATTTTTCAG**M**TGCAAG**Y** G**K**AAGCGAATTTCGATTACACTTT GCTA**R**GCTACTGGTAGAGATGGCA AATGTGGAAAACGA**W**AATTATAC GTTAAATTTAGCAAATCGCCTTTAC GTAGAGCAAAACTTTCCGACAAAG GAAAGCTTCAGCAGAAT	1 CAATATAACTAA 2 CAATATACCTAA 3 CAATATACCTGA 4 CAATAAACCTAA 5 CAATATAATTAT 6 CAATGTACCTAA 7 CAATATAACTGA 8 CAAGATGCCTAA 9 CAATATAACGAA 10 CAGTATAACTAA 11 CCATATACCTAA 12 GAATATAACTGA 13 CAATATGCCTAA 14 CAATGTAACTAA
ytP162	fw: AGGCACATGTTTTGGTAGTGG G rev: AGTTTGCCGGTCATTGATTC seq1: CCTATAGAACTTCTCTTGAG seq2: CTCAAGAGAAGTTCTATAGG	AGGCACATGTTTTGGTAGTGGAAA GTACGATATATGATTTG**R**TACTAA **R**ACTTGCCCCGACGAGCTGTAAAA TGAAGGTATGTTTCAACTATCCGAT TGCTGACCGTAATATAAAATTTGC ATCATTCTTTCGTTTTTATTTCCGAC AAATTTCTTGCCTCTATTCAAGAGG ATTCTGATGTCGCATTTTTGGAAGG AAACATGAGAAAAAGTC**Y**A**M**GTA CAACAAATTTTTCTATTGACTTTTT GATTG**S**GAAAATATAATACGC**W**AA A**M**TA**Y**TGGCTGTATTCCAAAGCTT TAC**K**AAAATTTTGTAAATATAATC GCAAAAA**R**TATGCCGCAAAGAAAA TCTACAGAATCTCGATYTTTCGCTT ATTTTACAGGGTCTCAAGAGAAGT TCTATAGGGAAATGTAAAAGAAAC ATGAAGCAAAAACCGAAGGTTAGA GAATTATTCC**R**CAAAAAGCAATTA TTAATGATTTCTGAG**R****K**CGCTATTT GATACATTTAGACCGTTTTTTGTAT CAAAGA**Y**AATAACAGTATCCTTGC TGAGTTTATCTTGACACAGTGTATT TGCTATTAAAATT**M**TGATA**Y**TTTTC AGGATGTTGCTCTTAGAATATCTGA	1 GGCACTATGAAATTAT 2 GGCACTCTGAAATTAT 3 AGTACTATTGAGTCCC 4 AGCACTATGAAATTAT 5 AGCACTATTGAATTAC 6 AGCACTATTAGGTTAC 7 GGCACTATGAAATTCC 8 AGCACTATGAAGTCAT 9 AGCAGTACGAAATTAT 10 GGCACTACGAAATTAT 11 AGTCCTATTGAGTCCC 12 AGCACTATTGAGTCCC 13 AATACTATTGAGTCCC 14 GGCACTATGAAATTAC 15 GGCACTATGAAAGTCC 16 AGCACTATTAAATTAC 17 AGCAGTATGAAATTCC 18 GGCACTATTGAATTAC 19 AGCACTATTGAATTCC 20 GGTACTATTGAGTCCC 21 AGCACTATTGGGTTAC 22 GGCACTATGAGATTCC 23 AGCAGTATGAAATTCC 24 GGCACAATGAAATTAT

		ATCAATGACCGGCAAACT	25 AG CACTATGAAATTCC
ytP164	fw: GCATCTTCGCTATCCTTTGC rev: CGAATGGAAACAGCAGCAG seq: AGACTTATCCGTGGTT	GCATCTTCGCTATCCTTTGCTGCAC AAAGTCCAACTGCGACTGCTTCCT AAATCATAAAA**W**TCAATCAATTTA AGTAATTCGCTTTAACAAAA**R**TAA TTTAAAATAATTTTTTAATAAAGAA TATAGAAGATTTAAAAGAAAAACC CGAAAT**R**AAGGAAGATTTTTGATT GGTATTTTGGATGAATTGTCATAAA AAGTTTTTCATGAATTAATTAACTA TTAATTCAA**Y**ACATACAAATTATCC AA**Y**AATTATTGCAAATAAACATTA ATTAATTACACGATACATATTTTG**K** TAGTCATACGAACACATCAAATGT TGCTAAACTTATTCGATTTATAATT ACAAAAACAAAAAAAAGAAAAAT TTATCACCTGTCCGGTATATAAATG GCAAAAACCACGGATAAGTCTTTC TTTGTAAAGATTTCCTGCTGCTGTT TCCATTCG	1 TAGTTG 2 TAATTG 3 TAGTTT 4 AAGTTT 5 TGGCTT 6 TAGTCG 7 TAATTT
ytP169	fw: CGACATTTGCTATGGGAAGC rev: CACCATCGCAGCTGTGTACT seq: CGACATTTGCTATGGGAA	CGACATTTGCTATGGGAAGCATTA AAAATGTAACTGTCAAAGGTCAGG TCGCATGCAGCGATCGATCGCAAA AAGATGTTGAAATACA**R**TT**R**TGGG A**R**CGTGATACACGTAAGTTCGACT T**W**TCTCATT**Y**TGATCGAATAC**R**AA GTTCTAT**Y**TTTC**Y**TTTTCCTAT**R**AA **Y**TGATATTTGTATCTGATAATAA**W** T**K**A**R**GTGAATTTTAA**S**CTAAT**Y**K**K**G TGATATAAAGTTTTAAATTTAATTT CTAGTGGATCCGGATGATTTGCTG AATACGACGAAGACCGA**Y**GCTCGT GGAAATTTCAAGATATATGGAGAA GAGAATGAAGTAAACAACATTGAA CCGTATCTAATAATAGTACACAGC TGCGATGGTG	1 AGGACGTCGTATACTGT 2 AGAACACCACTTAGCTT 3 AGAACGTCGTATACTGT 4 AGGTTATTGTAGGCTGT 5 AGAACACCACTTACTGT 6 AGAACACCACTGGGCTT 7 AGAACATCGTAGGCTGT 8 AGAACATCGTATACTGT 9 AGATTATTATAGGCTGT 10 AGGACATCACTTACTGT 11 AGGACACCACTTAGCTT 12 GGAACGTCGTATACTGT 13 AGAACACCACATAGCTT 14 AGAACATCGTATACTGC 15 AAAACACCACTTAGCTT

[a]Position number 1 is the first nucleotide of the fw primer. Ambiguity codes at variable positions are in bold and color coded as follows: A or G - yellow; A or T - blue; C or T - pink; C or G - grey; A or C - red; T or G - green. The non highlighted Y in *ytP162* indicates a variable position too close to the sequencing primer for reliable detection. [b]The bases present at the variable positions are listed in the order of occurrence.

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Molecular evidence of 'Siisa form', a new genotype related to *Onchocerca ochengi* in cattle from North Cameroon☆

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ABSTRACT

Onchocerca ochengi, a filarial nematode parasite from African Zebu cattle is considered to be the closest relative of *Onchocerca volvulus*, the causative agent of river blindness. Both *Onchocerca* species share the vector, black flies of the *Simulium damnosum* complex. Correct identification of their infective third-stage larvae in man-biting vectors is crucial to distinguish the transmission of human or animal parasites. In order to identify different closely related *Onchocerca* species we surveyed the sequences from the three mitochondrial loci 12S rRNA, 16S rRNA and *coxI* in both adult worms isolated from *Onchocerca*-induced nodules in cattle and infective third stage larvae isolated from vector flies from North Cameroon. Two distinct groups of mitochondrial haplotypes were found in cattle as well as in flies. One of them has been formerly mentioned in the literature as *Onchocerca* sp. 'Siisa', a filaria isolated from the vector *S. damnosum sensu lato* in Uganda with hitherto unknown host. Both variants are found sympatric, also in the same nodule of the animal host and in the vector. In the flies we also found the mitochondrial haplotype that had been described for *O. volvulus* which is about equally different from the two previously mentioned ones as they are from each other. These results suggest a higher genetic diversification of *Onchocerca ochengi* than previously reported.

1. Introduction

In areas where cattle-biting *Simulium damnosum* flies and Zebu cattle are present in tropical regions in Africa, infections with the filarial nematode *Onchocerca ochengi* (Bwangamoi, 1969) are prevalent. This parasite is closely related to the causative agent of human onchocerciasis *Onchocerca volvulus*, both in respect of phylogenetic distance (Morales-Hojas et al., 2006) and biology (Wahl et al., 1994). Therefore, it serves as an excellent animal model for exploring the biology, chemotherapy and immunology of *Onchocerca* parasites (Achukwi et al., 2007; Renz et al., 1995; Trees et al., 1998). Both species are amongst the filarial worms transmitted by the black fly vector *S. damnosum sensu lato* also including *O. ramachandrini*, a filaria from warthogs (Bain et al., 1993; Wahl, 1996), *Onchocerca* sp. 'Siisa' which definite host is unknown (Krueger et al., 2007), and other yet undefined filarial species (Duke, 1967; Garms and Voelker, 1969). Correct identification of infective third-stage larvae (L3) of *O. volvulus* and differentiation from other filarial species is paramount for the realistic calculation of Annual Transmission Potentials (Duke, 1968), an important epidemiologic parameter to determine the infection risk of a population in endemic areas (Renz et al., 1987; Wahl et al., 1998).

L3s can be classified morphologically according to their shape and length (Duke, 1967; Eichner and Renz, 1990; Franz and Renz, 1980; McCall and Trees, 1989; Wahl and Schibel, 1998), however not unequivocally due to overlaps in their body length distribution. Moreover, different populations within a species and morphologically highly similar sibling species may remain undetected, in particular when no adult specimens are examined (Denke and Bain, 1978) or no supplementary information is available, e.g. differences in pathology (Duke et al., 1966). More recently, DNA-based techniques have been introduced for *O. volvulus* detection, namely dot blot hybridization assays with specific DNA probes (Fischer et al.,

Abbreviations: BI, Bayesian inference; bp, nucleotide base pair; CI, confidence interval; L3, third stage larva; ML, maximum likelihood; MP, maximum parsimony; RPMI, Roswell Park Memorial Institute medium; rRNA, ribosomal RNA; *s.l.*, *sensu lato*.

☆ *Note*: Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers: KC167330–KC167358.

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1997; Meredith et al., 1991; Wahl and Schibel, 1998). However, due to qualitative and/or quantitative limitations, e.g. background hybridization and hybridization signal failure, respectively, the practicability of this method is constrained. Furthermore, the routinely used 150 bp long diagnostic marker for *O. volvulus* O-150 clusters with another *Onchocerca* species, namely *Onchocerca* sp. 'Siisa' (Krueger et al., 2007), thereby hampering species discrimination. Modern molecular-genetic tools enable us to overcome these drawbacks by comparison of the genetic sequences of defined conservative regions across specimens (Ferri et al., 2009). We used three primer pairs to amplify portions of the mitochondrial DNA, for which sufficient data entries of the *Onchocerca* genus are publicly available from GenBank™, namely the 12S and 16S rRNA regions, and parts of the cytochrome oxidase subunit 1 gene *coxI*.

2. Materials and methods

2.1. Infective stage larvae from the vector S. damnosum s.l.

From December 2009 to March 2012, samples were collected at two locations in Northern Cameroon adjacent to *S. damnosum s.l.* breeding sites, namely at Soramboum near the Vina du Nord river in the Sudan savannah: 7°47′14″ N; 15°0′22″ E, where *S. damnosum sensu stricto* and *S. sirbanum* are most prevalent (Renz and Wenk, 1987 and own unpublished data), and at Galim near the Vina du Sud river on the Guinea-grassland of the Adamaoua plateau: 7°12′2″ N; 13°34′56″ E, where *S. squamosum* is the common vector (own unpublished data). The village population from both areas has been treated annually with the antifilarial drug ivermectin for about 15 (Galim) and 25 years (Soramboum). Fly catchers attracted female *Simulium* flies by exposing their legs and trapped the flies with a sucking tube. Daily catches were brought to the Programme Onchocercoses Field Research Station in Ngaoundéré (www.riverblindness.eu), stored at −15 °C and subsequently dissected for filarial infections. The length of intact L3 stages was measured at 50× magnification by an eye-micrometer attached to the stereomicroscope (Wild M5, Switzerland).

2.2. Adult O. ochengi worms extracted from nodules in cattle

To investigate the genetic heterogeneity of *O. ochengi* in cattle, skin samples with palpable worm-nodules in the dermis were obtained from the local abattoir in Ngaoundéré. Worm-nodules containing male and female adults were excised and stored at −15 °C for later analysis. Adult worms were isolated from the nodule tissue by collagenase digestion modified from Schulz-Key et al. (1977). Briefly, nodules were incubated at 37 °C overnight in 0.125% collagenase in RPMI or PBS solution and transferred in fresh medium afterwards.

2.3. DNA preparation, PCR and sequencing

Isolated L3s' and fragments of adult stages were lysed in 75 μl DirectPCR™ lysis reagent (Viagen Biotech, USA) or reaction buffer (30 mM Tris–HCl, 10 mM EDTA, 1% SDS, pH 8.0), supplemented with 1–2 μl proteinase K (20 μg/μl stock, Genaxxon, Germany). Digestion conditions were 5 h or overnight at 55 °C, followed by an enzyme denaturation step (85 °C, 45 min). For lysis of microfilariae and embryonic stages, the protocol according to Hildebrandt et al. (2012) was used. Two microliters of each extract was added in a total volume of 25 μl PCR reaction containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol forward and reverse primer (see below) and 1 U Taq polymerase (Qiagen, Germany). The thermocycler model GeneAmp PCR System (Perkin Elmer, USA) was used with the following program: an initial denaturation step of 4 min at 94 °C was followed by 40 cycles of denaturation (94 °C, 40 s), annealing (conditions amplicon specific, see below), and elongation (72 °C, 90 s) and followed by a final elongation period of 7 min at 72 °C. The primers and annealing conditions were: for 12S as described by Casiraghi et al. (2004) (fw-primer: 5′-GTTCCAGAATAATCGGCTA-3′, rev-primer: 5′-ATTGACGGATGRTTTGTACC-3′; 62 °C, 30 s; for 16S: fw-primer: 5′-TGGCAGCCTTAGCGTGATG-3′, rev-primer: 5′-CAAGATAAACCGCTCTGTCTCAC-3′, 55 °C, 30 s; and for *coxI*: fw-primer: 5′-TGATYGGYGGTTTTGGWAA-3′, rev-primer: 5′-ATAMGTACGAGTATCAATATC-3′, 52 °C, 45 s) (modified from Casiraghi et al., 2001). The PCR products were purified using the PCR Purification Kit (Qiagen, Germany) and sequenced from both ends with the respective PCR primers using the BigDye™ v3.1 Ready Reaction Terminator Kit (Applied Biosystems, USA) according the manufacturer's protocol. Next, excess fluorescent nucleotides were removed by Sephadex G50 (GE Healthcare, UK) column purification (Tabak and Flavell, 1978) or isopropanol precipitation, and the reactions were analyzed on an ABI3100 automated sequencer (Applied Biosystems, USA) according to the manufacturers' instructions.

2.4. Statistic and phylogenetic analysis

Non-parametric multiple comparison between the different species' L3 lengths was done using the Steel Dwass' test by the statistical software program JMP 10.0 (SAS, USA). For the creation of sequence alignments and neighbor-joining consensus trees, the bioinformatics program Geneious version 5.6.5 (Drummond et al., 2012) was used. Extracts from published mitochondrial sequences from the following taxa were added to the phylogenetic analysis: *Brugia malayi* (Genbank: AF538716, Ghedin et al., 2007), *Dirofilaria immitis* (Genbank: NC_005305, Hu et al., 2003) and *Onchocerca flexuosa* (Genbank: HQ214004, McNulty et al., 2012), where *B. malayi* was set as outgroup, as well as own records of *O. ramachandrini* from Soramboum (GenBank: [12S] KC167340–KC167341, [16S] KC167348–KC167349, [*coxI*] KC167356–KC167357). For maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) trees, PAUP*4.0b10 (Swofford, 2002) and MrBayes 2.0.5 (Huelsenbeck and Ronquist, 2001) were used, respectively, and implemented evolution model analyses were estimated by jModeltest 0.1.1 (Posada, 2008) according to Akaike and Bayesian information criteria, as well as decision theory performance-based selection. For the MP and ML trees, heuristic searches with tree bisection-reconnection (TBR) as the branch-swapping algorithm were used. The starting trees were obtained via stepwise addition with random addition of sequences. Additionally, 100 replicates were performed for MP analysis, and all characters were treated as unordered, given equal weights, with gaps treated as missing data. For the BI tree, posterior probabilities were calculated using 1,100,000 generations, employing four simultaneous tree-building chains, with every 200th tree being saved. A 50% majority rule consensus tree was constructed based on the final 75% of generated trees.

3. Results and discussion

3.1. Genotypes of L3s' found in the S. damnosum s.l. vector

From 2872 L3s isolated of 27,425 dissected flies, we determined the 12S rRNA, 16S rRNA and *coxI* sequences of 78 L3 and two L2 isolated from 43 *S. damnosum s.l.* caught at the two different sites in North Cameroon and compared them with GenBank entries. The larvae fell into three groups. 62 (77.5%) grouped together with entries for *O. ochengi* from Mali and Cameroon (12S-GenBank: AY462914; 16S-GenBank: AY462897 (Morales-Hojas et al., 2006);

Table 1
Phylogenetic sequences of L3, adults and microfilariae of closely related *Onchocerca* spp.

Marker	Genotype	GenBank accession number	Consensus sequence[a]	Single Nucleotide Polymorphisms[b]
12S	*O. ochengi* (n=190)	KC167330 – KC167335	CCTTATTTATTAAATTCATTAARACATT AARAAAAAATTACTTTCTTTTCCAATTT CAAAAAAAAATAAAAAACTAATCCAAA AAAAATTCATAATAGTAACACATGAAA CATAAATTCATAAGCCAAATATATATCT GTTTTTAATGCCAAAACAAVAAAGCATT ACAAAATAAAATAAAAACTAAACAATC	1 AACGCC* * AC* T* CC 2 AACGCC* * C* T* CC 3 GACGCC* * AC* T* CC 4 AACGCC* AAC* T* CC 5 AACGCCAAAC* T* CC 6 AACGTC* * AC* T* CC
	O. sp. 'Siisa' (n=22)	KC167336 – KC167338	ATACATGTGCCAACAAAATTCACCAAA AAAGAGGGCTCTCCAGCAAATCACATR TTCCAAAGAAAAATCTAAAGTCAGTCA ATATTTTTTCGGTTTAAAVAAAACTTTA CTCCCGAATTATTAAATTTTGATTACCT GGGTACTAATCCAGTTCAAAAAACAAA	1 AGCACC* * * T* C* CA 2 AGCACC* * * C* C* CA 3 AGCACC* * ACTCACA
	O. volvulus (n=4)	KC167339	TTTTTGATACCAVAAGAAACT***AAAA AAAATVAAA*AVATTTTAC*AAAAAACA AVATAAAAACAAAMTAAAAACTCATAG CACCACAACAAATAGAGTCAAAAATTA AAACGCA	1 AGTATT* * AT* T* TC
16S	*O. ochengi* (n=84)	KC167342 – KC167343	TGGYRTAAAAGTAGCGTAAGTGATTTGT TTTTTTAATGGTTTCARGTATGAATGAA GTTTTTAGCAGTTTTTTTATTTACTTTTT RTTTGAATTATTTTTTTRATTAAAAATTA TTAGTTAAGGTATTACAAAGATAAGTCT TCGGAAATTTTGTTTTGAATTTTGAAATT	1 CGATAGGGAA 2 CGGTAGGGAA
	O sp. 'Siisa' (n=11)	KC167344 – KC167345	TTTRTTTTTAATTTTTTCTTGGGGATGGA TTTTAAGAAAGTTTTATACTATTRTTATT ATTAAAAATTACTCCGGAGTTAACAGGG TTGTAGACATATAAATAGRTTTTTATAT	1 CGACAGAAGA 2 CGACAGGAGA
	O. volvulus (n=3)	KC167346 – KC167347	TAGTGTGCTGCGCTACATCGATGTTGTA TATTTTTTTTGATAATGGAGAGGTTTTTT TTRTTTTGAGACTGTTCTTCTTGTATAAA AAATTGACTTGATATTAGTTTAGTTCGT CG	1 CAACGAAGGG 2 TAAT GAAGGG
coxI	*O. ochengi* (n=6)	KC167350 – KC167351, KC167358	TTGGATGTTGCCTTTRATRTTRGGGGCT CCTGAGATRGCGTTTCCTCGGRTAAATG CTTTRTCTTTTTGRTTTACTTTTGTGGCT TTRTTGATAGTTTATCAGTCTTTTTTTAT YGGRGGTGGYCCTGGTAGRAGTTGGAC TTTTTATCCTCCTCTTAGGGTTGAAGGY CAACCAGAATTGTCTTTRGATACTATGA TTTTAGGTTTACATACTGTRGGAATTGG	1 AAAGGAAGTGCAATAA 2 AAAGGAAGTGCAATAG 3 AAAAGAAGTGCAATAG 1ff. TGGTAGAGTGAT 2ff. TGGTAGAGTGAT 3ff. TGGTAGAGTGAT
	O. sp 'Siisa' (n=3)	KC167352 – KC167354	TTCTTTGTTGGGTGCTATTAATTTTATGG TAACTACTCAGAATATACGGTCVACTGC TGTGACTTTGGATCAAATTAGTATGTTT GTTTGGACTTCTTATTTGACTTCTTTTTT RTTAGTTTTRTCTGTGCCTGTTTTGGCTG GTTCTTTATTGTTTTTGTTGTTGGATCGT AATTTTAATACTTCTTTTTATGATACTAA GAAGGGGGGTAATCCTTTGTTGTATCAG CATTTGTTTTGATTTTTTGGTCATCCTGA	1 GGAGGGAGCATACGA 2 GGAGGGAGCATACGA 3 GGAGAGAGCATACGA 1ff. CAGCAGGACGAT 2ff. CAGTAGGACGAT 3ff. CAGTAGGACGAT
	O. volvulus (n=2)	KC167355	GGTGTATGTTATTATTTTACCTGTTTTYG GTATTATTAGGGARGCGGTTTTATTTTT GACTGATAAGGATCGTTTRTTTGGTCAG ACTAGGATRACTTTTGCTTCTATTTGRA TTGCTGTTTTAGGYACTTCTGTRTGRGG YCATCATATGTATACGGCTGGTTTG	1 GGGGGAGATGTGTAA 1ff. TAATGAGGTAGC

[a] Ambiguity codes at variable positions are in bold and color coded as follows: A or G – yellow; C or T – pink; A or C – red; insertion or deletion (*) – turquoise.
[b] The bases present at the variable positions are listed in the order of occurrence. The numbers indicate different alleles. ff. – continuance of the allele sequence.

coxI-GenBank: AJ271618 (Casiraghi et al., 2001)). The sequences of 13 (16.3%) worms grouped with entries for *Onchocerca* sp. 'Siisa', a phylogenetically closely related filaria from Uganda which has been speculated to originate from wild animals (12S-GenBank: DQ523738; 16S-GenBank: DQ523749, (Krueger et al., 2007); *coxI*-no entry). Finally, the sequences of five samples (6.3%) grouped together with sequences from *O. volvulus* from Mali, Uganda and Cameroon (12S-GenBank: AY462920; 16S-GenBank: AY462902 (Morales-Hojas et al., 2006); *coxI*-GenBank: AM749285 (Ferri et al., 2009)). Mixed infections of *O. ochengi* and *Onchocerca* sp. 'Siisa' occurred in six flies, whereas only two flies carried *O. ochengi* and *O. volvulus* together. No flies had *O. volvulus* and *Onchocerca* sp. 'Siisa' at the same time.

The sequence divergence between the different groups (Table 1) was 2.11–2.75% (12S), 0.75–2.00% (16S) and 2.00–2.93% (*coxI*) while the intra-group variability of the sequences was between 0.15–0.31% (*coxI*) and 0.21–0.64% (12S) for *O. ochengi*, between 0.15–0.31% (*coxI*) and 0.21–0.85% (12S) for *Onchocerca* sp. 'Siisa', and between 0% (12S, *coxI*) and 0.50% (16S) for *O. volvulus*.

From a total of 86 sequenced L3s the body lengths of 87.2% ($n = 75$) could have been determined. Because it had been reported that the L3 length distributions of *O. ochengi* and *O. volvulus* overlap mainly in the area below 700 μm (Wahl and Schibel, 1998) we biased our molecular analysis toward such larvae (61.3% of all measured L3). As expected, our results confirmed the difficulty to correctly identify a few *O. volvulus* L3 larvae among a larger number of L3s of animal origin. Although there was no misidentification of *O. ramachandrini* (mean length: 888 ± 61, $n = 8$) many of the larvae classified as *O. volvulus* proved to be short *O. ochengi* (mean length: 695 ± 77.5, $n = 52$) and *Onchocerca* sp. 'Siisa' (mean: 665 ± 36.2, $n = 11$). The *O. volvulus* L3s confirmed by sequencing measured 635 ± 66.1 μm ($n = 4$).

3.2. Genotypes found in the Zebu cattle host

To investigate the genetic heterogeneity of *O. ochengi* in Zebu cattle, 33 nodules extracted from three cow skins purchased at the local abattoir in Ngaoundéré were dissected, and the adult

Table 2
Allele frequencies of 12S rRNA from different life cycle stages of *Onchocerca ochengi*, *Onchocerca* sp. 'Siisa' and *O. volvulus* from Northern Cameroon.

Species	Developmental stage[a]	Isolated from	*n*	Allele frequencies[b] [%]					
				1	2	3	4	5	6
O. ochengi	Adult	Zebu cattle	48	46	25	19	8	–	2
	– Female		23	39	35	22	–	–	4
	– Male		19	58	21	16	5	–	–
	L3	*S. damnosum s.l.*	62	52	24	16	3	3	2
	– Galim		41	44	27	17	5	5	2
	– Soramboum		21	67	19	14	–	–	–
	L2	*S. damnosum s.l.*	2	100	–	–	–	–	–
	– Galim		1	100	–	–	–	–	–
	– Soramboum		1	100	–	–	–	–	–
	Microfilaria	Zebu cattle	52	71	2	27	–	–	–
	'brezel' stage[c]	Zebu cattle	26	35	42	23	–	–	–
	Total		**190**	**53.7**	**20.5**	**20.5**	**3.2**	**1.1**	**1.1**
Onchocerca sp. 'Siisa'	Adult	Zebu cattle	9	56	44	–			
	– Female		6	67	33	–			
	– Male		2	50	50	–			
	L3	*S. damnosum s.l.*	13	54	31	15			
	– Galim		11	64	27	9			
	– Soramboum		2	–	50	50			
	Total		**22**	**54.5**	**36.4**	**9.1**			
O. volvulus	L3	*S. damnosum s.l.*	4	100					
	– Soramboum		4	100					

[a] For isolated larvae from the vector the respective fly catching sites are given.
[b] The allele numbers correspond to the sequences listed in Table 1 and represent the order of occurrence when excluding data of first-stage larvae.
[c] The 'brezel' stage is an embryonic stage from the female's uterus.

worms (21 males, 29 females and 7 worms of unidentified gender) were subjected to 12S and 16S rRNA analysis. Again, we could clearly identify two distinct groups of sequences. These groups were identical with the *O. ochengi* and the *Onchocerca* sp. 'Siisa' groups respectively (Table 1), identified in the larval stages we found in *S. damnosum s.l.* from both fly-catching sites (see Section 3.1). Details about the 12S rRNA allele frequencies of each genotype are given in Table 2. In five nodules originating from two of the three skins we found adult specimens of both types. It is noteworthy that the proportion of 'Siisa' in the definite host's adult worm population (15.8%, $n = 57$) is similar to the proportion of 'Siisa' L3 of all *O. ochengi* found in the vector (17.3%, $n = 75$) indicating a comparable transmission success between both genotypes. Furthermore, some microfilariae ($n = 52$) and embryonic stages ($n = 26$) from five nodules of a cow's whole skin could be successfully sequenced, showing only alleles of the *O. ochengi* genotype (Table 2). The majority of the worm nodules examined ($n = 31$), however, had no (77.4%) or only decayed microfilariae with disintegrated DNA (6.5%). The reason for this is unclear but may be attributed to a recent anti-helminthic treatment regimen or to density-dependent self regulation of the parasite.

Taken together, we found proof that *Onchocerca* sp. 'Siisa' occurs in cattle, forms nodules like *O. ochengi* and is transmitted by the same black fly vector, namely female *S. damnosum s.l.* At the moment it is not clear whether *Onchocerca* sp. 'Siisa' is a variant of *O. ochengi* with sympatric distribution or if this variant should be considered a sister species. According to the maternal heredity of mitochondrial DNA, our data cannot conclude whether fertile mating between *Onchocerca* sp. 'Siisa' and *O. ochengi* occurs. Of six female 'Siisa' worms found, three were alone in the nodule, two with at least one adult *O. ochengi* male, and one with only microfilarial stages of *O. ochengi*. As the latter contradicts the maternal transfer of mitochondria to the next generation, this phenomenon can be best explained by infiltration of microfilariae from a worm nodule in close proximity. Whereas one of the two identified male 'Siisa' worms was located in a nodule with a female worm and microfilariae of unknown genotype, the other was found together with one female, two males and microfilariae of *O. ochengi*. The gender of one 'Siisa' genotype worm which was associated with adult *O. ochengi* female and males could not be determined.

The phylogenetic relationship between 'Siisa', *O. ochengi* and *O. volvulus* remains puzzling. In all phylogenetic trees (Fig. 1) they form a monophyletic group showing their close evolutionary association, but only the ML-tree groups 'Siisa' as a sister taxon to *O. volvulus* (Fig. 1b). The MP-tree (Fig. 1a) groups 'Siisa' together with *O. ochengi* with a bootstrap support below the 50% threshold, and the BI-tree (Fig. 1c) does not resolve the event either. Nonetheless, the employed substitution model TIM3 + G in the ML-tree ranked first on all varied selection criteria during the model test. It is tempting to speculate that *Onchocerca* sp. 'Siisa' is in a more direct lineage with *O. volvulus*, although comparison of nuclear DNA would be necessary to corroborate this idea. Should this be the case, two scenarios exist, namely either *Onchocerca* sp. 'Siisa', as a variant of *O. ochengi*, is in direct ancestry to *O. volvulus*, or *O. volvulus* re-switched from its former human host back to the bovine host, hence showing the reversal of a host switch event which possibly occurred some 10,000 years ago during the domestication of cattle by man (Bain, 1981). Krueger et al. (2007) already postulated a higher volatility of host switch events in *Onchocerca* species which our study would support. The 'Siisa' variant could even stem from a hybridization event between *O. ochengi* and *O. volvulus*, although this seems unlikely given the difference in chromosome pair numbers (5 vs. 4, respectively, Post et al., 1989). Investigation of the haplotype of *Onchocerca* sp. 'Siisa' could shed more light to this aspect. In any regard, this is a prime example for the co-evolution of *Onchocerca* – *Simulium* complexes, as already observed with the discovery of

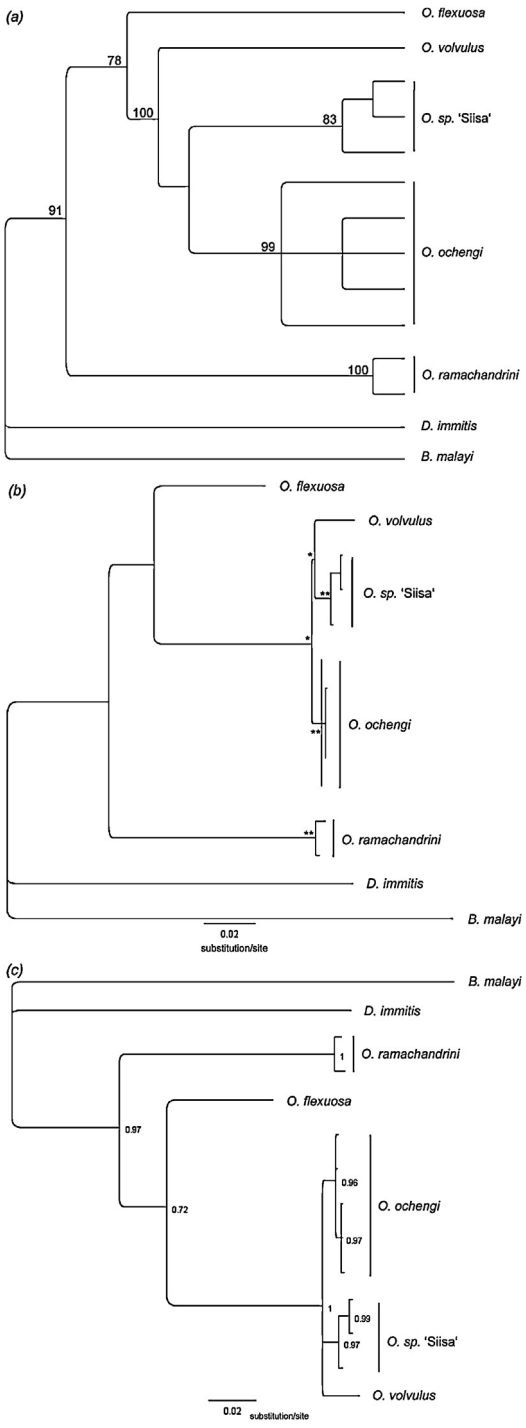

Fig. 1. Phylogeny of *Onchocerca* spp. on the concatenated analysis of three mtDNA sequences (12S and 16S rRNA, *coxI* mtDNA; 1545 bp). (a) Maximum parsimony bootstrap 50% majority-rule consensus tree. (b) Maximum likelihood tree (−ln likelihood = 4179.4), estimated under the TIM3 + G evolution model (nucleotide frequencies: A 0.2407, C 0.0883, G 0.1868, T 0.4842; substitution rate matrix: [AC] 0.2029, [AG] 8.9414, [AT] 1.0000, [CG] 0.2029, [CT] 2.7256, [GT] 1.0000; gamma distribution shape parameter 0.1470). (c) Bayesian inference tree (−ln likelihood = 4178.4), estimated under the GTR + G evolution model (nucleotide frequencies: A 0.2380, C 0.0879, G 0.1893, T 0.4847; substitution rate matrix: [AC] 0.1461, [AG] 11.4273, [AT] 1.5213, [CG] 0.3971, [CT] 3.5493, [GT] 1.0000; gamma distribution shape parameter 0.1440). Analyses have been run on PAUP* 4.0b10 (a, b), and MrBayes 2.0.5 (c). The implemented evolution models have been estimated by jModeltest 0.1.1. Numbers on the branches display values of bootstrap support (a) and Bayesian posterior probabilities (c), respectively, and the asterisks (b) indicate the pair-wise genetic distance (***$P < 0.001$; **$0.01 > P > 0.001$; *$P < 0.05$).

rainforest and savannah strains of *O. volvulus* in West Africa (Duke et al., 1966).

4. Conclusion

This study has identified cattle as at least one of the definitive hosts of *Onchocerca sp.* 'Siisa', a filarial nematode previously only isolated from the vector *S. damnosum s.l.* The mitochondrial genotypes of what is generally considered to be *O. ochengi* form two distinct clades.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Reproductive biology of *Onchocerca ochengi*, a nodule forming filarial nematode in zebu cattle.

Running head: Genetic diversity in *Onchocerca ochengi*

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Abstract

Onchocerca ochengi is a nodule-forming filarial nematode parasite of cattle in tropical Africa and closely related to the human pathogen *O. volvulus*. The adult worms reside in intradermal nodules. While females are sedentary, males may move between nodules. The first stage larvae (microfilariae) disperse in the skin of the host waiting to be taken up by the intermediate host. The density of microfilariae in the skin is largely independent of the number of adult worms present indicating some form of density dependent control. Recently, *Onchocerca* sp. siisa, a form of *Onchocerca* distinguishable from *O. ochengi* by mitochondrial DNA sequences but not by morphology, was described to occur in cattle. This raised the question if *Onchocerca* sp. siisa represents a different mitochondrial clade of *O. ochengi* or a new species. In order to study the reproductive biology and to understand this self-control of the off-spring population we systematically analyzed all *Onchocerca* nodules from the skin of one zebu cow and we examined a sample of microfilariae from a skin biopsy. We identified 87 *O. ochengi* females and 146 males. 56 (64.4%) of the females contained developing embryos. In order to assign the progeny to their respective parents we determined the genotypes at six nuclear and two mitochondrial molecular genetic markers in the adult worms, in a fraction of the progeny present in the uteri of the females and in the skin microfilariae. The 121 skin microfilariae we analyzed originated from at least 17 different mothers, which contributed rather differently to the total. Forty-five larvae (37.2%) were the progeny of a single female. Of the adult worms 16.7% were of the type *Onchocerca* sp. siisa. These worms appeared to interbreed freely with the rest of the *O. ochengi* population and therefore belong to the same species.

Keywords: *Onchocerca ochengi*; *Onchocerca* sp. Siisa; filarial nematode; bovine parasite; genetic diversity; reproductive biology.

1 Introduction

Most representatives of the filarial nematode genus *Onchocerca* are species-specific parasites of various ungulates. Many elicit the formation of nodules (Anderson, 2000). One of the very few non-ungulate hosts with *Onchocerca* parasites is man. *O. volvulus* is the causing agent of river blindness and, in spite of the success of *Onchocerca* control programs, continues to be a threat to millions of people (Hoerauf et al., 2011).

Onchocerca ochengi is a parasite of cattle in tropical Africa, best characterized in zebu (*Bos primigenius indicus*). With an estimated time of evolutionary separation of as little as 10 000 years, it is very closely related to the human pathogen *O. volvulus,* with which it shares the vector, the black fly *Simulium damnosum s.l.* (Krueger et al., 2007; Renz et al., 1994; Wahl et al., 1994). *O. ochengi* is well accessible because it lives in intradermal nodules in the inguinal region of the bovine host that are identifiable by palpation and can be easily removed for examination. This makes *O. ochengi* an attractive model case for studying the biology of nodule forming *Onchocerca* sp. (Renz et al., 1995; Trees et al., 1998).

O. ochengi females induce the collagenous nodules (Figure 1), in which they grow up entangled and in very close contact with the host's tissue. They can reach up to 30 cm in length and they can reproduce for many years—presumably as long as their hosts live (5 to 10 years) (Determann et al., 1997; Wahl et al., 1994). Young *O. ochengi* females appear to avoid the immediate proximity of existing nodules. Thus *O. ochengi* nodules are rather dispersed. Mainly in heavily infested cattle, nodules can sometimes be found close to each other in the udder, teats and umbilicus, but still the individual nodules remain separate. This behavior is different from females of *O. volvulus*, which tend to form clumps of nodules consisting of female worms of different ages (Schulz-Key, 1988; Wahl et al., 1994). Males are much smaller than females (2 to 4 cm). They migrate to the females and are found within the nodules at various numbers, in average about one male per female (Renz et al., 1994). *O. ochengi* embryos develop and hatch in the uteri of their mothers (Figure 1). The first stage larvae (called microfilariae) are released and disperse in the

77 peripheral skin around the nodules where they accumulate and wait to be taken up by a black fly
78 during a blood meal. This accumulation of skin-microfilariae is not strictly linked to the number of
79 reproducing female worms. Rather in adult cattle, the density of skin microfilariae becomes largely
80 independent of the number of adult worms present indicating some form of regulation (Trees et al.,
81 1992). In principle there are three non-mutually exclusive ways of achieving this. First, many (in
82 the extreme case all) females may produce fewer progeny when the density of circulating
83 microfilariae is high. Second, a few (in the extreme case one) dominant females may reproduce at
84 high rates while suppressing the reproduction of other females. Third, microfilariae may have
85 reduced survival in a density-dependent manner.
86 Other than *O. ochengi*, a second nodule-forming species of *Onchocerca*, *O. dukei*, has been
87 described in cattle. Because of the restricted occurrence of its vector, *Simulium bovis,* in Cameroon
88 this species is believed to be limited to the Sudan-savanna, which lies about 150 km to the North of
89 our sampling site (Renz et al., 1994; Wahl et al., 1994; Wahl and Renz, 1991). Further, based on a
90 single observation of two larvae in a *Simulium damnosum s.l.* vector in Uganda, a form of
91 *Onchocerca* morphologically and with respect to its mitochondrial DNA similar to *O. ochengi* was
92 described as *Onchocerca* sp. variant Siisa (Krueger et al., 2007).
93 In two recent studies we reported A) that *O. ochengi* females frequently produce progeny
94 sired by different males simultaneously and that these males most of the time but not always were
95 present in the nodule along with the female (Hildebrandt et al., 2012) and B) that *Onchocerca* sp.
96 variant Siisa occurs in black flies and in nodule forming adults in cattle in Cameroon demonstrating
97 that this variant exists also in West Africa and that the zebu is at least one of its definite hosts
98 (Eisenbarth et al., 2013). In these two studies we analyzed worms isolated in different places and at
99 different times from multiple host individuals but from each host individual only a rather small
100 fraction of the worms present. Further the two studies were limited to the characterization of either
101 nuclear (Hildebrandt et al., 2012) or mitochondrial (Eisenbarth et al., 2013) genetic markers.
102 Therefore, these investigations did not provide any information about what fraction of the adult

103 worms were actually reproductively active and to what extent the different adults contribute to the
104 pool of microfilariae present. Also we could not address the question if the different mitochondrial
105 clades interbreed and therefore belong to one species or if *O. ochengi* and *Onchocerca*. sp. variant
106 Siisa are reproductively isolated from each other and represent different species.
107 Therefore we undertook a detailed analysis of the population of nodule forming *Onchocerca*
108 in one particular host individual with an intermediate parasite load. We isolated all *Onchocerca*
109 nodules we could find in this zebu and we genotyped at multiple nuclear and mitochondrial loci A)
110 the adult worms, B) a fraction of the progeny in the uteri of their mothers (if present), and C)
111 microfilariae from a skin sample. We show that a significant fraction of the adults contribute
112 variably to the pool of circulating microfilariae and we present evidence strongly suggesting that
113 the members of the two mitochondrial clades interbreed freely and therefore belong to the same
114 species.
115
116

2 Materials and Methods

2.1 Parasite material

The skin of a freshly slaughtered 3.5 years old female Zebu cattle was purchased on January 13th 2011 at the abattoir in Ngaoundéré, Cameroon. The skin was spread on the floor and all 88 nodules found were excised. After every nodule excision the position was marked and a picture of the skin taken. Based on these pictures the positions of the individual nodules were marked on the map shown in Figure 2. The nodules were cut open and transferred individually into 1.8 ml Nunc cryofreezing tubes filled with 95% ethanol for storage and transportation at ambient temperature. Later, the nodules were individually transferred into PBS and dissected immediately.

In addition, skin samples (about 25 g each) were taken from the belly, the udder, and the back of the cow and preserved in ethanol. To obtain skin microfilariae, several small chunks (about 1 mm^3 each) were cut out and washed in 500 µl PBS for 5 to 6 hours at 700 rpm in a Thermomixer at room temperature. The samples were digested in 500 µl collagenase of type II 2,5 mg/mL in PBS supplemented with 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at 37°C for 18 to 24 hours at 700 rpm in a Thermomixer. Individual microfilariae were isolated and lysed immediately. Microfilariae were only found in the sample from the udder. For comparison, a second skin (containing 33 nodules) was purchased and processed in the same way on January 19th 2011. Partial analysis of 27 of the nodules from the first skin and of three nodules from the second skin was reported in our previous study (Hildebrandt et al., 2012).

All steps up to the conservation of the material in ethanol were carried out in the Programme Onchocercoses laboratory of the University of Tübingen in Ngaoundéré (www.riverblindness.eu). All subsequent analyses were done at the Max Planck Institute for Developmental Biology in Tübingen, Germany.

2.2 Genotyping

142 The worms were lysed and prepared for genotyping as previously described (Hildebrandt et
143 al., 2012). Worms were genotyped at six nuclear and two mitochondrial markers. For marker details
144 see Supplementary Table 1. Nuclear marker sequences were PCR amplified and sequenced as
145 described in (Hildebrandt et al., 2012) and mitochondrial marker sequences were PCR amplified
146 and sequenced as described in (Eisenbarth et al., 2013).
147 Except for *ytP160* the markers used were the same as in (Hildebrandt et al., 2012) (nuclear
148 markers) and in (Eisenbarth et al., 2013) (mitochondrial markers). Each marker contains several
149 polymorphic positions. For nuclear markers each combination of nucleotides at polymorphic
150 positions within one copy of the marker is referred to as an allele. Because certain combinations of
151 heterozygous and homozygous positions at polymorphic sites can result from more than one
152 combination of alleles, it is not always possible to determine an individual's genotype solely based
153 on the sequencing result. For reproductively active individuals, alleles were determined based on
154 the genotypes of their progeny. Nucleotides present in the same copy of a locus are inherited in a
155 strictly coupled manner. For non-reproducing animals, alleles were determined by cloning the PCR
156 products and the sequencing of multiple clones; however, this was not done for all individuals.
157
158 *2.3 Assigning parents to progeny*.
159 For embryos isolated from the uteri of their mothers a male present in the nodule was
160 accepted as the likely father, if the genotypes at all loci successfully determined were consistent
161 with the embryo being the offspring of this particular male and the mother. For skin microfilariae
162 assignment to a mother was made, if based on nuclear and mitochondrial markers, all females but
163 one could be excluded as mothers.
164
165 *2.4 Phylogenetic analysis of the mitochondrial sequences*
166 For each animal, the 12S and the 16S sequences were concatenated prior to analysis. This
167 resulted in 25 different sequences (haplotypes 1 to 25). The following sequences were also included

in our phylogenetic analysis (GenBank accession numbers): DQ523738, DQ523749 (*O.* sp. Siisa); DQ523740, DQ523751 (*O. dukei*); AY462920, AY462902, KC167339, KC167346, KC167347 (*O. volvulus*); AJ537512 (*Dirofilaria immitis*); AY462914, AY462897 (*O. ochengi*). Alignment and phylogenetic estimation were carried out using MEGA 5 version 5.2 (Tamura et al., 2011) choosing the MUSCLE algorithm for sequence alignment and maximum likelihood using the Tamura-Nei model for estimating the phylogeny. Gaps were included. Node support was estimated by bootstrapping (1000 bootstrap pseudoreplicates). For comparison, other trees, using different models and including or excluding gaps, were also calculated (see legend to Figure 5).

2.5 Comparison of the allele distributions in the different mitochondrial clades

To visualize the relationships of the different alleles at a given locus we calculated a haplotype network using the program TCS ver. 1.21 (Clement et al., 2000). The analysis was performed separately for each nuclear marker using NEXUS files as input and allowing a maximum number of mutational steps of 100. The different mitochondrial clades (see Figure 5) were mapped manually onto the TCS output.

2.6 Animal experimentation and ethics statement

Both cows were slaughtered in the context of the normal operation of the abattoir and were processed for human consumption. No special animal experimentation and ethical clearance was required.

3 Results

3.1 Distribution of the adult worms

A total of 88 nodules were found and dissected from the skin of an individual female zebu. The nodules were located on the ventral side of the host animal and strongly clustered at and around the udder (Figure 2). Two nodules contained decaying females, indicating that either the worms that had induced the nodules were not anymore alive at the time of sampling or that the nodules were insufficiently preserved and had perished during transportation. Two nodules contained two females along with one male, whereas in all other nodules we found a single female and zero to eight males (Figures 2C and 3). The total number of males isolated was 146 (in average 1.7 per nodule).

One non-reproductive female that was found in a nodule without males showed mitochondrial sequences very similar to what had been published for *Onchocerca dukei* (see below "interesting observations"). This individual was not included in any subsequent analyses. Of the remaining 87 females, 56 contained developing embryos, indicating that they were reproductively active (Figures 2B and 3). Consistent with our earlier findings (Hildebrandt et al., 2012), the progeny within a particular female was sometimes derived from multiple males (Figure 3B). Note that the datasets of this publication and of (Hildebrandt et al., 2012) are overlapping but not identical, as described in the Materials and Methods).

3.2 High diversity in nuclear markers

Earlier studies had suggested that a single bite by the vector transfers, in most cases, only one or a few *O. volvulus* larvae (Renz, 1987). There is indication that this is also the case for *O. ochengi* (AR unpublished observation). As a consequence, one would expect that the different parasite individuals in a particular host would have been acquired independently, and therefore be unrelated. Consistent with this, we found high genetic diversity (up to 24 different alleles per locus) among the *Onchocerca* worms within this one host animal (Figure 4). The notion that the worms present in this one host individual represent a large fraction of the genetic variation present in the

entire population is also supported by data derived from a second Zebu slaughtered one week later. In this second animal we found a total of 33 nodules containing 33 females and 20 males. From the six nuclear markers combined, we identified a total of 48 different alleles, only 2 of which were not present in the cow that is the principle subject of this study. The high number of possible allele combinations allowed us to determine likely parents for a large fraction of embryos and larvae.

3.3 Multiple interbreeding clades of mitochondrial haplotypes

We determined portions of the mitochondrial 12S and 16S rDNA sequences, in addition to the nuclear sequences described above. Most of the sequences grouped with one of the mitochondrial clades described by (Eisenbarth et al., 2013) (Figures 5,6A). One clade contains the sequences previously published for *O. ochengi* (referred to as clade or type "Ochengi"), whereas the other one includes the sequence derived from *Onchocerca* sp. variant Siisa (referred to as clade or type "Siisa"). However, three sequences did not fit this pattern. The sequences for two worms (one male, one female) grouped with sequences published for *O. dukei* (Krueger et al., 2007) (haplotypes 24 and 25, Figure 5, see "additional interesting observations"), The sequence for another male (haplotype 7, Figure 5), depending on the model used to reconstruct the phylogenetic relationship and the exact parameter settings, sometimes grouped with the clade "Ochengi" and sometimes appeared to represent its own additional clade.

In the following we address the question of the species status of the two mitochondrial clades described. The three haplotypes 7, 24 and 25 were not included in this analysis. First, we asked if there is evidence for assorted pairing. We considered each combination of a female and a male present in the same nodule a pair and asked if individuals were more likely to form pairs with partners from the same clade. 82.5% of the available females were of the Ochengi and 17.5% of the Siisa type (Fig. 6A). If they had no preference, for males of both clades, one would expect 82.5% of the pairs to be with females of the Ochengi and 17.5% of the Siisa type. We observed (Fig. 6B) 19 (expected 19) pairs of Siisa males with Ochengi females, 4 (expected 4) pairs of Siisa males with

241 Siisa females, 18 (expected 19.6) pairs of Ochengi males with Siisa females, and 94 (expected 92.4)
242 pairs of Ochengi males with Ochengi females. All observed values are very close to the expected
243 ones and there is no indication of assorted pairing.

244 Second, we asked if the inter clade pairs did mate successfully. For 25 (67.6%) out of 37
245 inter clade pairs we could confirm successful mating because we found the resulting progeny in the
246 uteri of the females (Fig. 6B). Of 98 intra clade pairs we could confirm successful mating for 47
247 (48.0%). From this we conclude that inter-clade pairs are not less likely to produce progeny than
248 intra clade pairs. Among the skin microfilariae we genotyped (see next section) we found 46 that,
249 based on their nuclear genotypes, most likely had parents of different mitochondrial type. This
250 shows that the progeny derived from inter clade mating events are viable at least up to the skin
251 microfilarial stage.

252 Third we analyzed the nuclear allele distribution among the two mitochondrial clades. We
253 calculated the relationships of the different alleles using the program TCS ver. 1.21 (Clement et al.,
254 2000) (see Material and Methods for details) and mapped the mitochondrial clades onto the
255 resulting network (Figure 7). Also this analysis did not provide any indication for two separate
256 genetically isolated populations.

257 Although no formal proof, these results very strongly suggest that the two mitochondrial
258 clades, with respect to their nuclear genomes, form one population. There is no reason to postulate
259 that *Onchocerca* sp. Siisa is a new species different from *O. ochengi*. The presence of separable
260 mitochondrial clades probably indicates that the *O. ochengi* population currently found in
261 Cameroon is the product of previously separated but currently connected populations.

262

263 *3.4 Multiple females contribute variably to the pool of circulating microfilariae*

264 As outlined above, 56 females contained embryos and larvae in their uteri indicating that
265 they were reproductively active (Figure 2B). The pool of microfilariae isolated from the skin biopsy
266 from the udder (see Materials and Methods) was genetically diverse (Figure 4). Assuming that we

267 found and genotyped all the parents of the microfilariae we analyzed (see discussion) and taking
268 into consideration the bi-parentally inherited nuclear markers and the maternally inherited (Sato &
269 Sato, 2011 and references therein) mitochondrial markers, we were able to assign 89 of the 121 skin
270 microfilariae to 11 different mothers (Figure 2D). The mothers of the remaining 32 microfilariae
271 could not be unambiguously determined but the larvae must have originated from at least six
272 different additional mothers. The minimal number of mothers required to explain the genotypes of
273 the microfilariae isolated from this one skin biopsy is therefore 17. However, 45 microfilariae
274 (37.2% of the total) were the progeny of a single female. The genotypes of all 45 microfilariae were
275 consistent with the assumption that they were sired by either of the two males present in the
276 mother's nodule. This female was located in the udder but was not the closest reproductively active
277 female to the place where the microfilariae were collected (Figure 2D). The other 10 females
278 contributed 12, 11, 5, 4, 3 (3 animals), and 1 (3 animals) microfilariae respectively. Interestingly,
279 the females that contributed 12 and 11 larvae were located outside of the udder and rather distant
280 from the sampling site. Together, these findings argue against the hypothesis that all reproductive
281 females contribute more or less equally to the population of skin microfilariae. However, based on
282 this study, we cannot know if these contributions remain more or less stable over space and time.
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285 *3.5 Additional interesting observations*
286 Below, we describe a few additional observations we consider interesting. These are single
287 observations and they cannot be used to draw firm conclusions about *Onchocerca* biology.
288 However, we believe that making them public may be worthwhile for others studying *Onchocerca*
289 parasites.
290 First, for one non-reproductive female in a nodule without males, the mitochondrial 12S and
291 16S sequences were close to what had been published for *O. dukei* (Krueger et al., 2007) (haplotype
292 25, Figure 5), a species so far only found in the Sudan savanna of Northern Cameroon,

293 approximately 150 km to the North-East from our study area (Wahl and Renz, 1991). Genotyping
294 of nuclear markers failed. We do not know if this animal was truly *O. dukei* or may be a hybrid
295 between the two species (see also below). This female is not included in any of the analyses.
296 Second, one male was heterozygous at all markers analyzed and always contained one
297 common allele and one allele found in only one (*ytP159*, see below) or no (all other markers) other
298 individuals in our study (Fig. 7 yellow (light grey in print version) label). With respect to its
299 mitochondrial sequences (haplotype 24, Figure 5), it grouped very closely with sequences published
300 for *O. dukei*. Most likely, this individual was a hybrid between an *O. ochengi* father and an *O. dukei*
301 mother. This male did father progeny, which developed at least to the stage we genotyped (late
302 embryo) and for each nuclear marker locus both alleles were represented in the progeny.
303 Interestingly, two males shared rare alleles with this putative hybrid at a single locus each
304 (Fig. 7). One was of the mitochondrial type Siisa and contained, at locus *ytP159*, the same allele as
305 the putative hybrid male (arrow in Fig. 7A) along with a common allele (arrowhead in Fig. 7A). At
306 all other loci it had alleles found multiple times in our sample. The other one was of the
307 mitochondrial type Ochengi and contained at locus *ytP161* an allele very similar to the unique allele
308 of the putative hybrid (arrow in Fig. 7C) along with a common allele (arrowhead in Fig. 7C). At all
309 other loci it had alleles found multiple times in our sample. These findings suggest that there might
310 be limited gene flow between *O. ochengi* and *O. dukei*.
311

4 Discussion

Here, we systematically analyzed the *O. ochengi* population in one particular host animal. With 88 nodules, the worm burden of this cow was in the usual range for *Onchocerca*-susceptible animals of this age and exposed to natural transmission on the Adamaoua plateau near Ngaoundéré (Achukwi et al., 2004; Renz et al., 1995; Trees et al., 1992; Wahl et al., 1994). The number of males we found in each nodule (average of 1.7 per nodule) was somewhat higher than the previously described (Renz et al., 1994). Of the 87 females, 56 (64%) contained developing embryos, indicating that they were reproductively active at the time of sampling. These numbers are in agreement with earlier findings for *O. ochengi* and *O. volvulus*, which had suggested that *Onchocerca* females undergo phases of reproduction interspersed with times of reproductive quiescence (Duke, 1993; Duke et al., 1990; Schulz-Key, 1990).

We found the population of *O. ochengi* in this one host animal to be highly genetically diverse. This suggests that the *O. ochengi* present in one host animal do not tend to be closely related. This was expected based on earlier studies that had demonstrated that the number of infective larvae transmitted in a single bite of *Simulium damnosun* s.l. is very small (Renz, 1987) and that the number of nodules in a particular host grows gradually (Achukwi et al., 2004). While the data presented in Figure 4 are fully suitable to support the claim that many different alleles exist at very different frequencies, they should not be taken as accurate measurements of the allele frequencies. It is likely that some alleles, for which our primers do not work, exist. Also, if the marker sequencing results of non-reproductive individuals could be explained with known alleles, in many cases we did not clone the PCR products and sequence individual clones to confirm these alleles. If only two alleles were possible, they were accepted and included in the analysis, but if multiple allele combinations were possible then the animal was not included in Figures 4 and 7.

In agreement with earlier observations (Hildebrandt et al., 2012), we found that reproductive activity is almost always associated with the physical presence of at least one male in the nodule. It is, however, striking that all four females in nodules with more than four males were not

reproducing. Given that 77.5 % (55/71) of females in nodules with males did contain progeny (Figure 3A) it is very unlikely (0.26% by simple probability calculation) that the four nodules with the highest number of males did not contain progeny just by chance. There are two possible explanations for this. First, it may be that these females were just becoming reproductively active, either for the first time or after a period of reproductive quiescence, and they were therefore particularly attractive for males. Alternatively, too many males may actually be detrimental for the reproduction of females, as has been observed in the model nematode *Caenorhabditis elegans* (Wegewitz et al., 2008).

The fact that a female contains developing embryos does not necessarily mean that it also contributes substantially to the pool of circulating microfilariae. For example, it has been observed that gravid females frequently contain dying progeny (Renz et al., 1995). This may indicate that some factor, for example signals by other females or the actions of a newly arrived male, may at least temporarily prevent certain females from successfully reproducing.

To our knowledge, this is the first study that determined parentage of circulating microfilariae. Our analysis demonstrated that different females contributed differently to the pool of circulating microfilariae at a particular location and time. Our study is a snap shot, looking at one location (the udder) at one particular time point and we cannot conclude anything about the dynamics of this population of worms waiting for a vector.

It must be noted that our assignment of circulating microfilariae to particular parents assumes that we did indeed find and successfully genotype all reproductive adults, but it is likely that some adults were missed. First, we cannot be absolutely sure that we found all nodules in the first place. Furthermore, one would expect that a few males were in the process of migrating between nodules at the time of sampling; such males were certainly missed. We were unable to obtain any nuclear genetic information for eight females; none of these eight females were reproducing at the time of sampling, but this does not exclude the possibility that they had earlier produced microfilariae, and that these microfilariae were still present in the periphery. The reason

364 for the relative high failure rate among non-reproducing females is that for females we used only
365 the most anterior portion of the body, which is devoid of any part of the gonad, which might contain
366 genetically distinct progeny or sperm (see Hildebrandt et al., 2012) As a consequence, only very
367 little DNA was available for females. For reproductive females the genotypes of the mothers could
368 also be derived/confirmed from the genotype of the progeny, which, of course, were not available
369 for non-reproductive females. In spite of these caveats, we are confident that the vast majority of
370 our parental assignments were generally correct for the following reasons: 1) All of the alleles
371 found in the microfilariae (51 nuclear alleles, 13 mitochrondrial haplotypes) were also found in
372 adults. 2) The microfilariae were assigned to mothers solely based on their own and on the mother's
373 genotypes; nevertheless, in the vast majority of cases (86 out of 89), the genotypes of the males
374 found in the nodule with the putative mother were compatible with the genotypes of the progeny 3)
375 There were only 4 microfilariae (likely siblings) for which we found no possible mother in our
376 sample. This indicates that though we are indeed missing some mothers, their number is
377 presumably small.

378 Based on morphology and on mitochondrial sequences, *O.* sp. Siisa had been described as a
379 variant of *Onchocerca* very closely related to *O. ochengi* and *O. volvulus* (Krueger et al., 2007).
380 While it was originally described based on two individuals found in one black fly in East Africa
381 (Krueger et al., 2007), it was later also found in Cameroon (Eisenbarth et al., 2013). This study also
382 demonstrated that *O.* sp. Siisa is a nodule forming parasite of cattle, which, by morphological
383 criteria, would have been classified as *O. ochengi.* Our data strongly suggest that *O. ochengi* and *O.*
384 sp. Siisa interbreed freely and therefore belong to the same species. Since, for technical reasons we
385 could not demonstrate directly that the inter clade progeny formed is indeed fertile we cannot
386 formally exclude an extremely recent genetic isolation of the two clades. However, we consider this
387 most unlikely, mainly for two reasons. First, we could not detect any assorted mating. Should the
388 two mitochondrial clades indeed belong to different species one would have to postulate that the
389 worms themselves cannot tell their own species apart from the sister species. Second, both species

390 resulting from this very recent speciation event would have retained essentially the entire ancestral
391 genetic diversity, indicating that in the process none went through a genetic bottleneck. This is very
392 unlikely.
393 We identified one individual that likely was a hybrid between *O. ochengi* and *O. dukei* and
394 two more individuals that at one locus each carried an allele that might have been of *O. dukei*
395 origin. These findings indicate that there might be occasional gene flow between these two species.
396 Additional studies, preferentially of whole genome sequences of *O. ochengi* and *O. dukei*
397 individuals, will be required to confirm or reject this hypothesis. Although not yet formally
398 published, a reference genome sequence for *O. ochengi* is already publically available (see
399 http://www.nematodes.org/genomes/onchocerca_ochengi/). Given the rapid development of
400 sequencing technologies single worm genome sequencing should be technically possible very soon,
401 at least for adult males, which provide much more genomic DNA than microfilariae and other than
402 adult females do not carry progeny with different genotypes in their bodies.
403 To end on a very speculative note: it is interesting that, based on mitochondrial sequences,
404 *O. volvulus* is phylogenetically as closely (Fig. 5) or even slightly more closely (Krueger et al.,
405 2007) related to *O. ochengi* than is *O. dukei*. This opens the possibility that there might also be
406 limited gene flow between *O. volvulus* and *O. ochengi*. Should this be the case, this would create
407 the possibility for genetic features, for example resistance against ivermectin, that arise in *O.*
408 *ochengi* in cattle to spread into the human pathogen *O. volvulus*.
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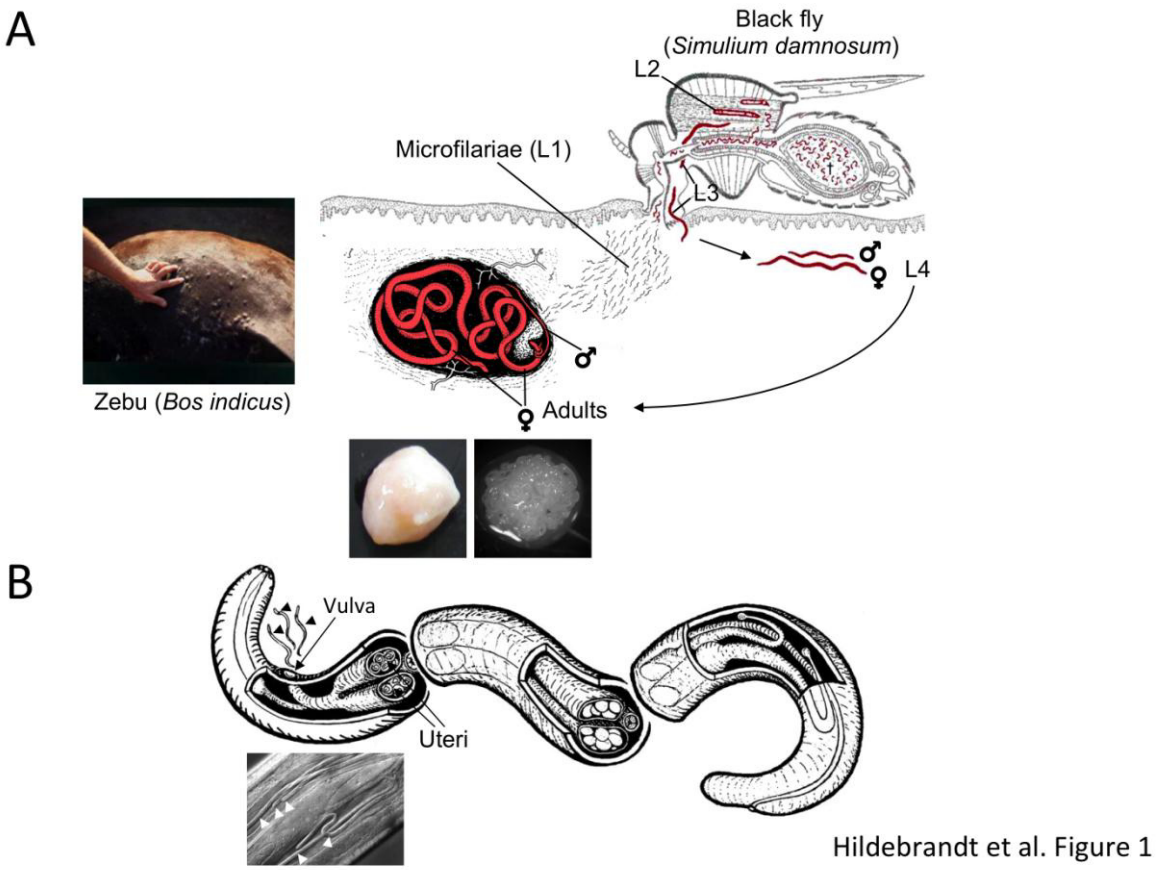


Figure 1: Anatomy and life cycle of *O. ochengi*

(A) The life cycle of *O. ochengi*. For explanation see text. The photographs show (from left) the belly of a zebu with numerous *O. ochengi* nodules, an indidvidual nodule (diameter about one cm) dissected from the skin of a zebu and an adult *O. ochengi* female after the digestion of the nodule with collagenase. (B) Schematic representation of an adult *O. ochengi* female. Anterior is to the left. The total length of the worm is about 20 - 30 cm. The embryos develop in the two-armed gonad. The first stage larvae (microfilariae, arrowheads) hatch within the uterus (Differential Interference Contrast image) and leave their mother through the anteriorely located vulva. The figure was compiled using copyright protected drawings and photographs. Permission was granted by the copy right holder A. Renz.

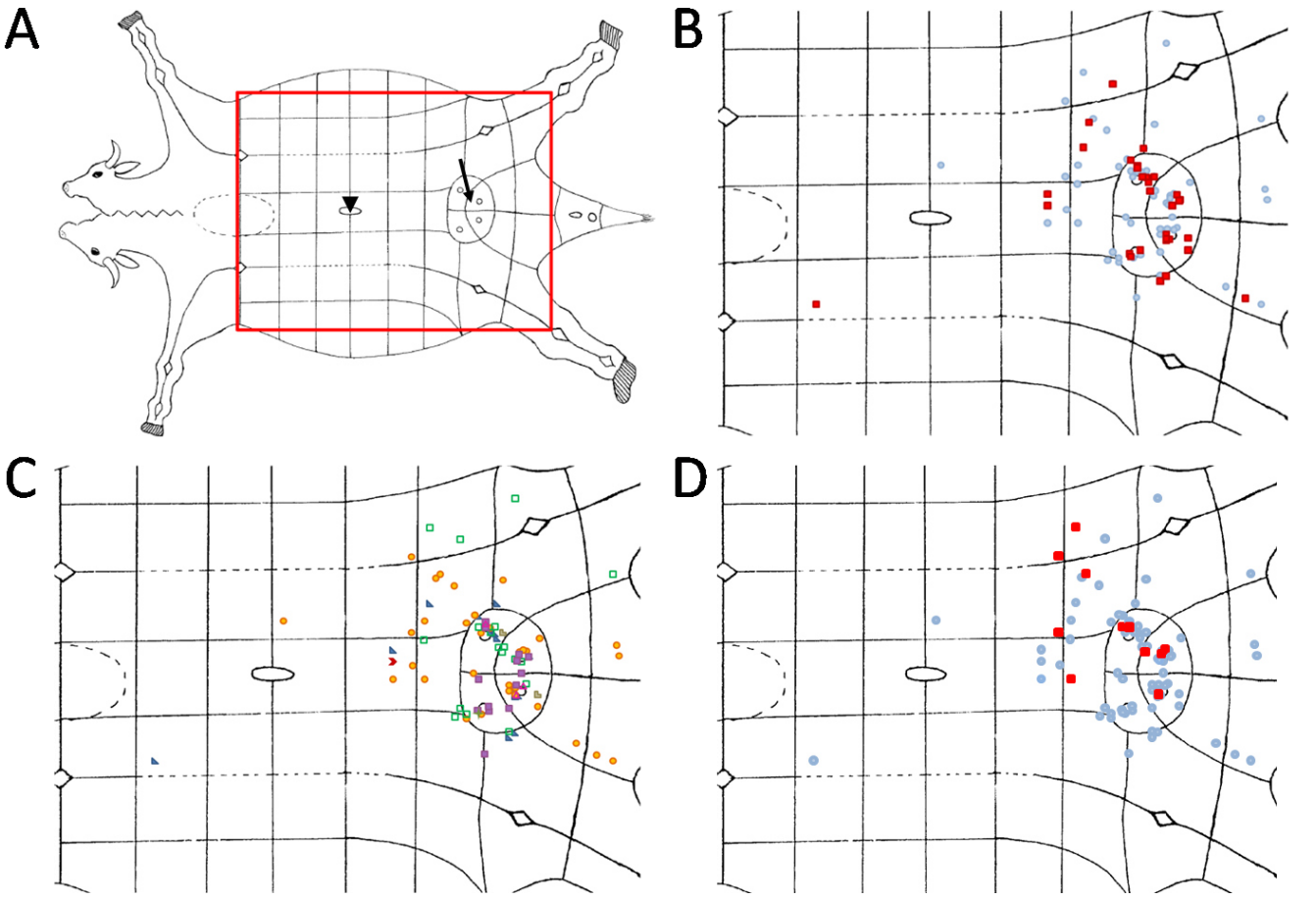
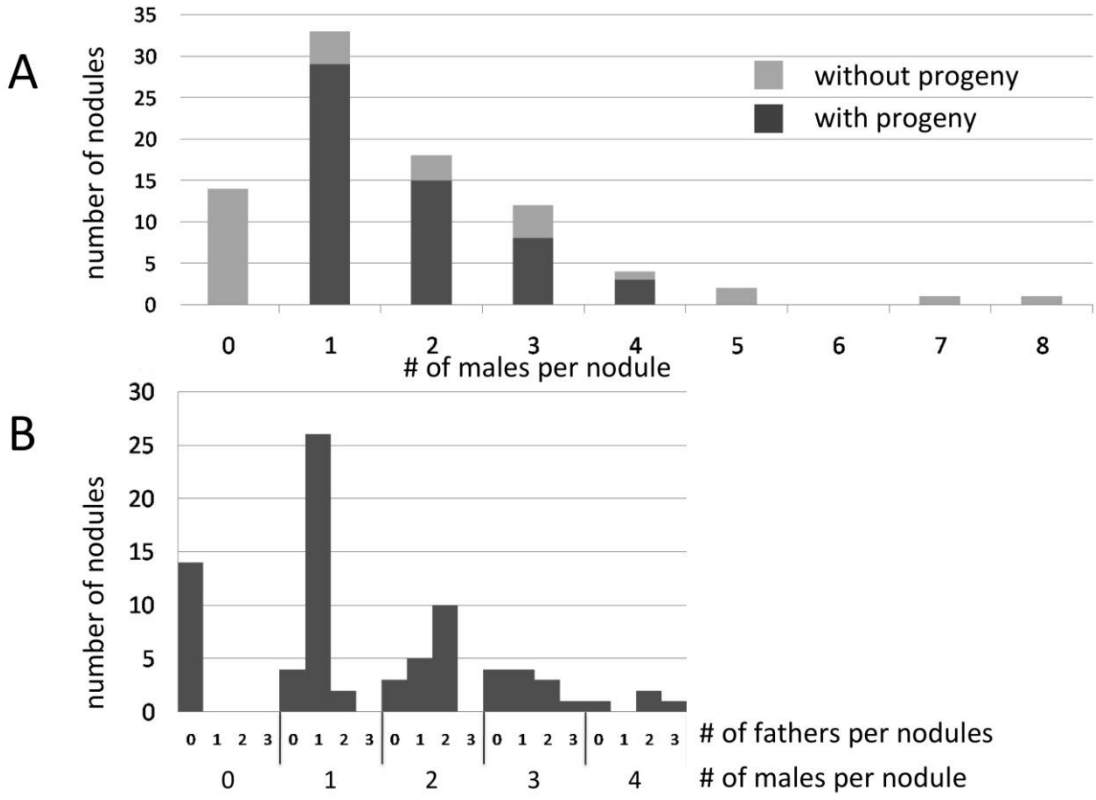


Figure 2: Location of the *O. ochengi* nodules

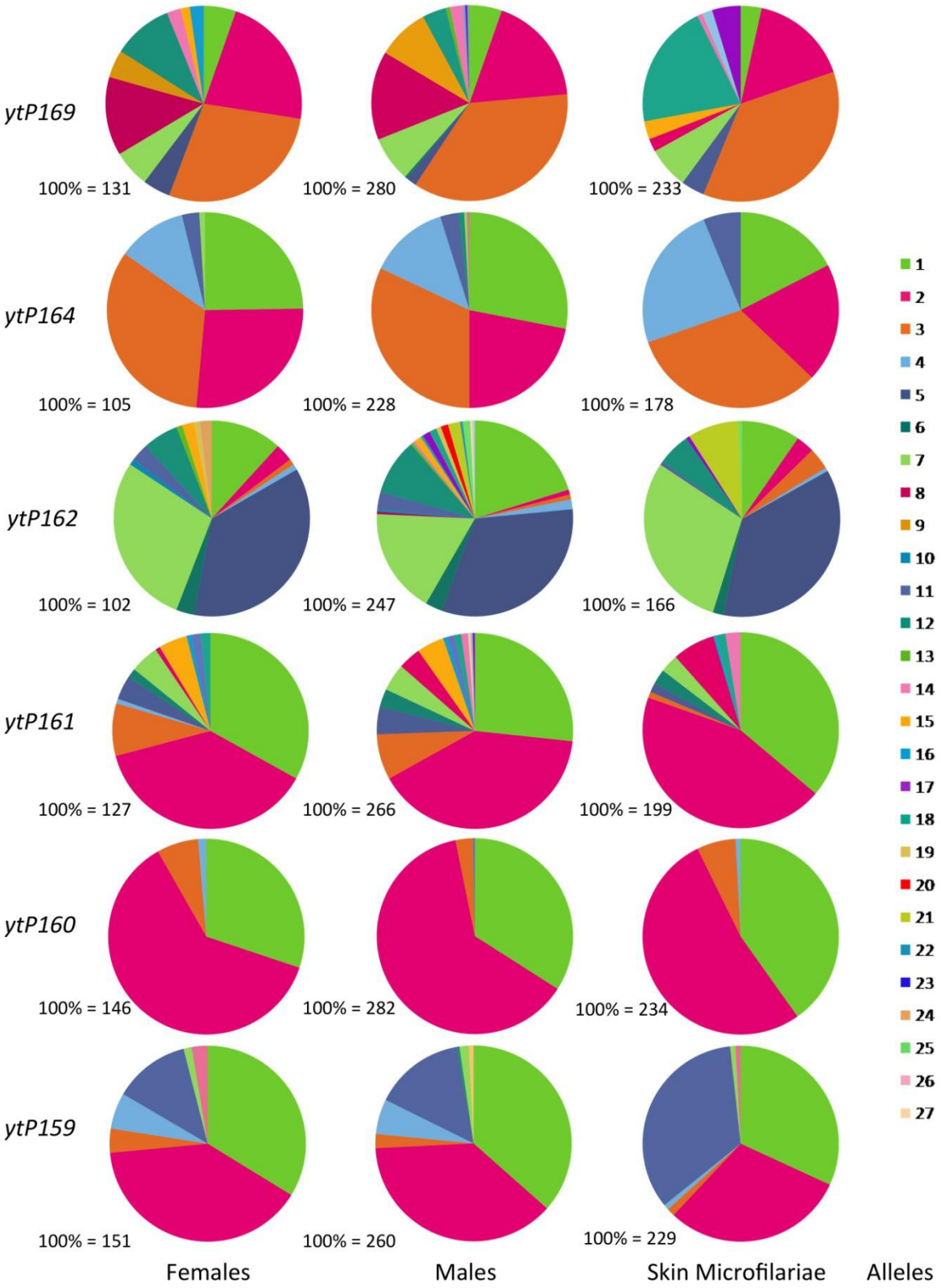
(A) Schematic representation of the skin of a zebu. Anterior is to the left, the skin is opened at the dorsal side. The arrow head and the arrow point to the umbilicus and the udder respectively. The section shown in B-D is framed. (B) Distribution of nodules containing reproductive and non-reproductive females. Blue (in the bw version light) circles: nodules with offspring, red (dark) squares: nodules without offspring. (C) Distribution of the males. The following symbols indicate nodules with the number of males indicated: 0 males 1 male 2 males 3 males 4 males 5 males 7 males 8 males. (D) Distribution of nodules (mothers), to which microfilariae isolated from the skin could be assigned. Red (filled) squares: nodules with assigned microfilariae, blue (open) circles: other nodules. At least six more females must have contributed to the pool of sampled microfilariae.



Hildebrandt et al. Figure 3

Figure 3: Number of males per nodule

(A) Histogramm showing the number of males per nodule (X-axis) and number of nodules with the corresponding number of males (Y-axis). The bars are subdivided; dark grey indicates nodules with progeny while light grey indicates nodules without progeny. (B) Minimum number of males required to explain the progeny found in a nodule. X-axis bottom label: number of males present in the nodule; X-axis upper label: minimal number of fathers; Y-axis: number of nodules with the corresponding number of males in the nodule and minimum number of fathers. Notice that one of the reproductively active females found in a nodule with a single male carried only a very small number of very young embryos; determination of fatherhood of these embryos failed, and this nodule is included in A but not in B.



Hildebrandt et al. Figure 4

Figure 4: High genetic diversity

Apparent allele distributions in females, males, and skin microfilariae (columns) are given for the six markers tested (rows). Colors (shades of grey) indicate the allele numbers. Between four (*ytP160*) and 24 (*ytP162*) different alleles per locus were present in this particular host individual. Notice: not all alleles we know of were found in the host individual described here; therefore the allele numbers go up to 27. The allele distributions shown are approximations but not accurate measures of the allele frequencies (see discussion). Only unambiguously identified alleles are included in the figure (*c. f.* materials and methods). Therefore uneven numbers are possible. We were not able to genotype all individuals at all six markers. Therefore the numbers differ between markers.

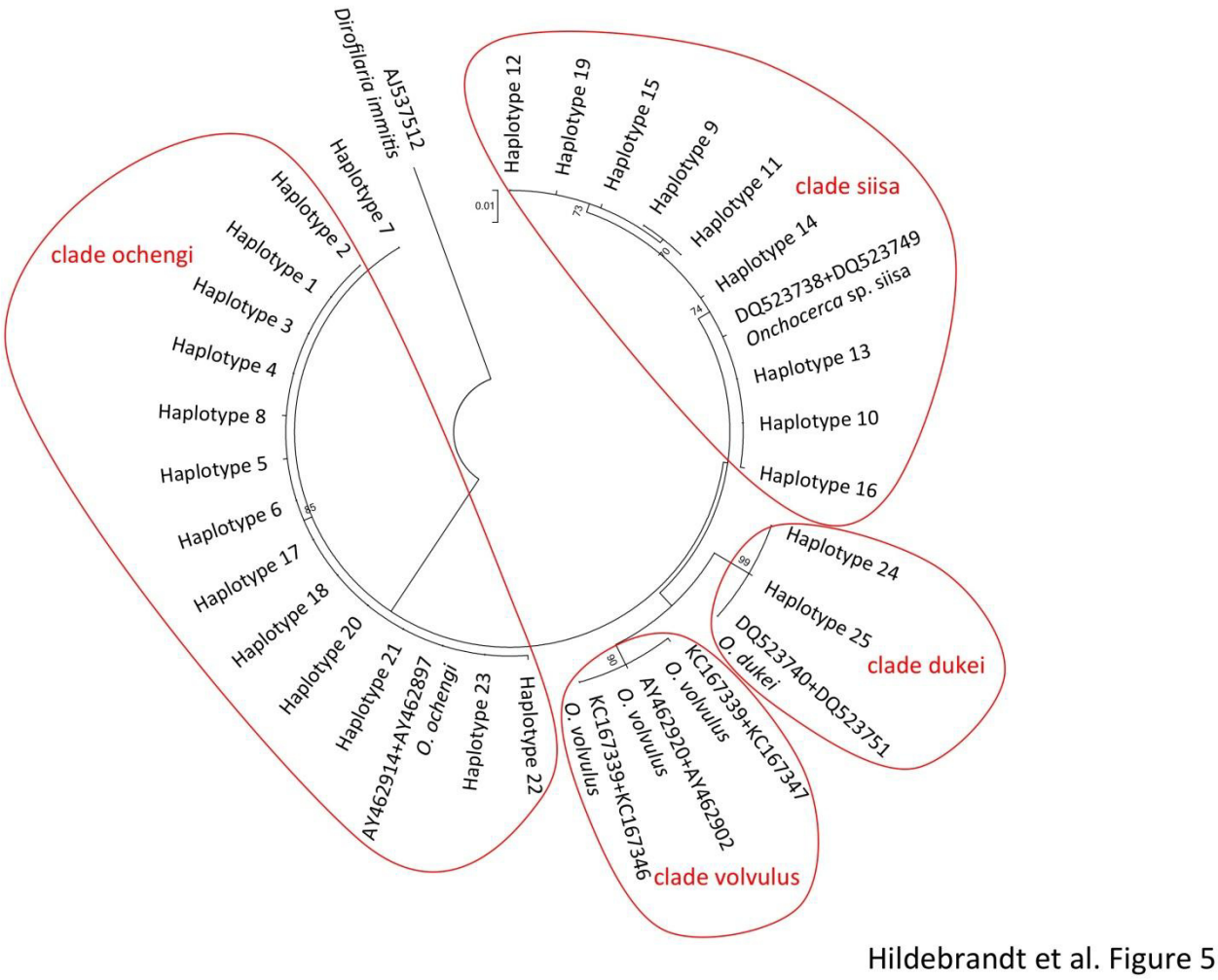
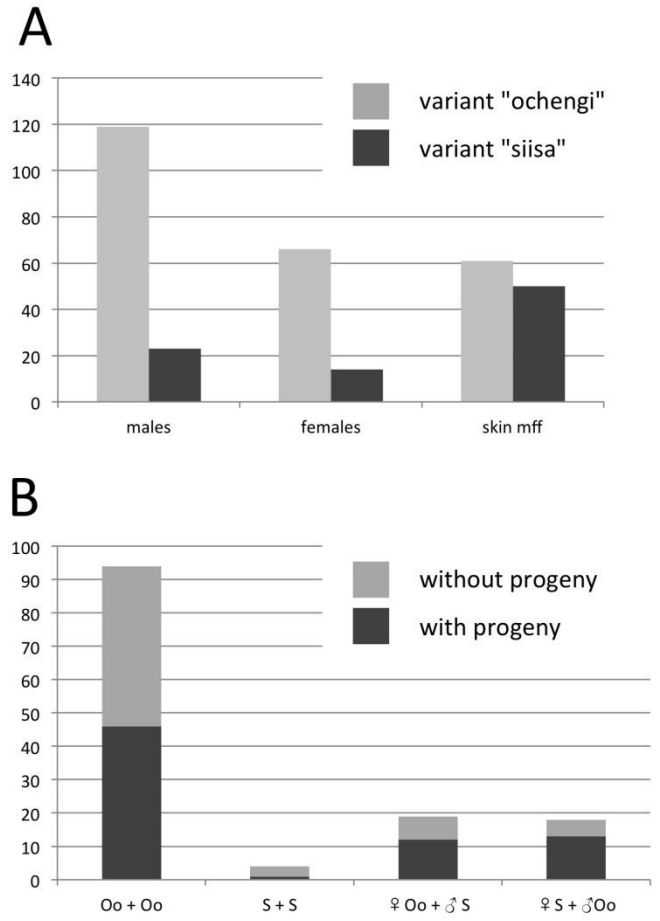


Figure 5: Mitochondrial haplotypes found

Unrooted maximum likelihood tree reconstructed from concatenated portions of the 12S and the 16S mitochondrial rDNA genes. “Haplotype …” indicates sequences observed in this study; sequences taken from Genbank are labeled by accession number and the species name they belong to. Only bootstrap values of 70 and above are shown. Similar trees were also calculated using different models (neighbour joining, maximum parsimony) and including or excluding gaps. The separation into the major groups, also referred to as "clades" (framed), was very robust. The resulting relationships of the major clades and of haplotype 7 changed with the exact parameter settings and the model used. Therefore, the phylogenetic relationships of these major groups cannot be resolved with the available data.



Hildebrandt et al. Figure 6

Figure 6: Interbreeding of the two mitochondrial clades

(A) Number of males, females, and skin microfilariae with a mitochondrial haploptype variant "ochengi" (light grey) and variant "Siisa" (dark grey). (B) Number of male/female pairs with a particular combination of mitochondrial haplotypes. Every male and female found in the same nodule were considered a pair; as such, individuals can contribute to more than one pair when more than one male or female is present. Pairs were considered to have produced progeny together if we found offspring of the male in the uterus of the female (c.f. 2.3.). Oo variant "ochengi"; S: variant "Siisa". Pairs with progeny are in dark grey, pairs without progeny are in light grey. Note that we were not able to establish the mitochondrial haplotypes for 2 males, 7 females, and 10 microfilariae.

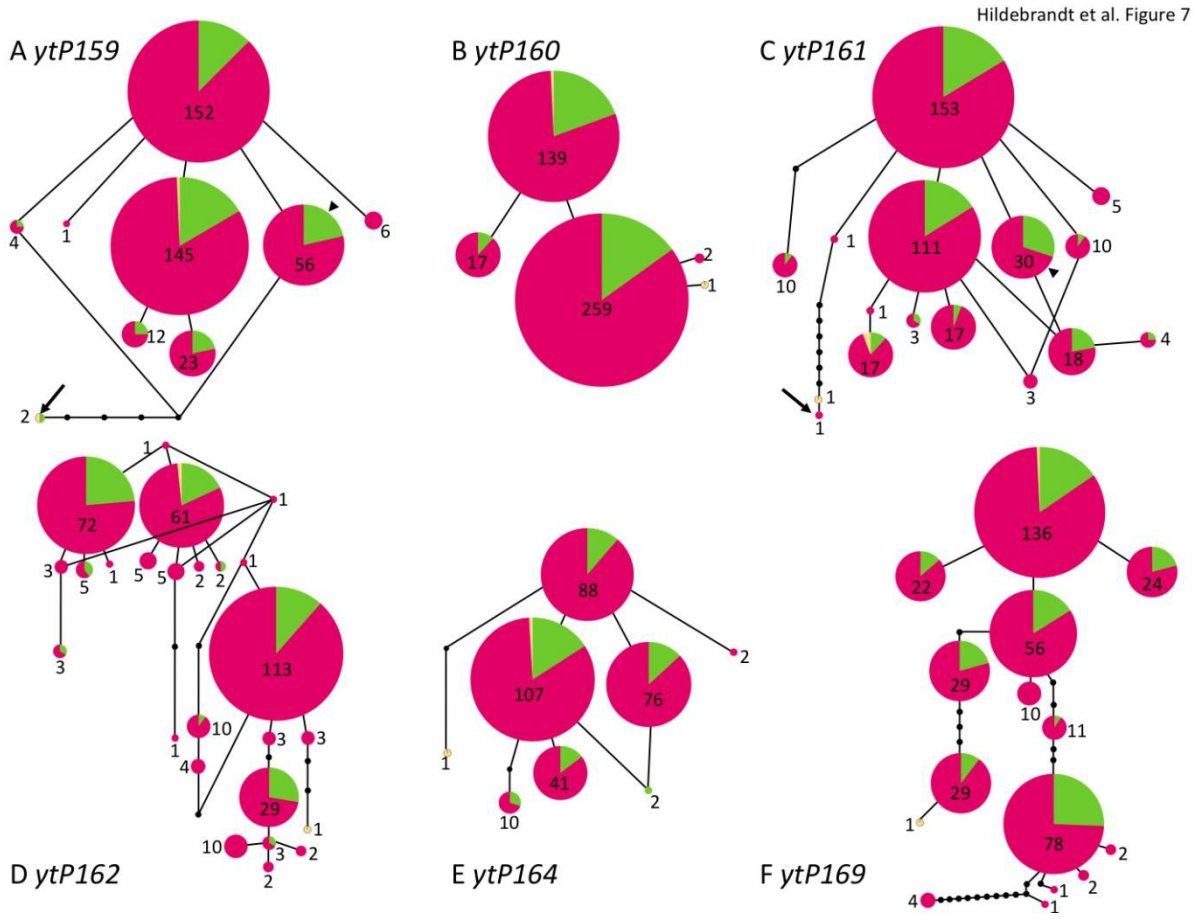


Figure 7: Nuclear allele distribution in the different mitochondrial clades

Relationship of the different alleles at the six nuclear loci analyzed as determined by the program TCS (Clement et al., 2000) (see Materials and Methods). Every edge represents one difference (one different nucleotide or one nucleotide inserted/deleted). The length of an edge is not informative. Colored disks represent alleles (haplotypes) present in our samples. The size of the disk roughly represents the allele frequencies (see also caption to Fig. 4). The absolute number of occurrences is indicated in or next to the disks. The mitochondrial haplotype clades (Fig. 5) were mapped onto the allele networks. For each allele the fraction present in animals of a particular mitochondrial clade is represented by color/shade of grey (type ochengi - red/dark, type siisa - green/intermediate, type dukei - yellow/light). The only animal with the mitochondrial haplotype 7 is not included in this figure. In A and C the possible *O. dukei* derived alleles in animals with non-*dukei* mitochondrial genotypes (arrows) and the common alleles they were heterozygous with (arrowheads) are indicated (see section 3.5). (A) marker *ytP159*. (B) marker *ytP160*. (C) marker *ytP161*. (D) marker *ytP162*. (E) marker *ytP164*. (F) marker *ytP169*.

Supplementary Table 1

Marker	Primers	Sequence[1]	Alleles[1]
ytP159	fw: TGCGTTTTCTGATCGTATTT rev: CCCTTTTGAATCAATGATGA seq: TGCGTTTTCTGATCGTATTT	TGCGTTTTCTGATCGTATTTCGGAA TTCAAAAAATTAGATGTAGCTGTT ATGGCGTGTTCAACTGACTCGCATT TCTCGCATCTTGCATGGGTAAATAC CGACCGAAAAATGGGTGGACTTGG TCAGATGAATATACCAATTCTTGCT GATACCAATCATGCAATCAGCAAG GCATATGGTGTGCTCAAGGAAGAT GAAGGAATTGCTTATCGGTACGTA TTCTTTGATATGAGTAAGATGTGAA GCCATCGAAGGCA**R**CGAGCGATTT GAA**R**ATATGTGGCATCAACTT**Y**AT GACTTTTTTAGAGTTATTGTTCTTC AGTTCTTGCGAATACTT**Y**CTCTTCT TTGTTGTGTT**R**TGATTGAAATGGTT GAAATCAGATTGTCATAGTTTATtG AAAACAAT**R**TTTGAACTTATTTCAG TGGA**Y**TATTCATCATTGATTCAAAA GGG	1 GACTAAC 2 GACTAGC 3 GACTGAC 4 AACTAAC 5 GACTAGT 6 GGCTAGC 7 GACCAGC 8 GATTAGC
ytP160	fw: CGCGCCAAATTGTTCATATC rev: ACATATTGCCATTGGTATGC seq: ACATATTGCCATTGGTATGC	CGCGCCAAATTGTTCATATCATCAT TTAATTTCACTCCGTTCT**Y**TATTCT GAATTTTGaAGATTGGCTTAGCAGA CAAAGTGAGT**R**TAGAA**K**AATACGA TAAGCTTTACCGTCCAGTACTCCTC GAGAAGTTGCCACATTGTCCGAAA ATGCCCGGAGCATTACGATTAGTG CAACATTTTCATAATCACAGCATAC CAATGGCAATATGT	1 TAG 2 TGG 3 CAG 4 TGT
ytP161	fw: TATCTCCTCTTTCGGTGTCA rev: ATTCTGCTGAAGCTTTCCTT seq: TATCTCCTCTTTCGGTGTCA	TATCTCCTCTTTCGGTGTCAACTTC ACTTTTTATGACTTATCTTGCGGCA GATGGCACAACAAAGCAACAATTG CAAGATGTTCTTGGAGGAAGTAAT TACATATTGAAATTTTTTTAATTCG AAATACTGAAAAAG**S**AATAATCAC GCA**M**ATTACCTCAAAGTTG**R**AA**K** **W**TTGGACATCAAGAATGCT**R**TAAC TGTAA**S**GATAGATTTCATAAATG**W** TAAAATAATCGTTTCTAAATTA**R**CA TAAAATCAATTTTTCAG**M**TGCAAG **Y**G**K**AAGCGAATTTCGATTACACTT TGCTA**R**GCTACTGGTAGAGATGGC AAATGTGGAAAACGA**W**AATTATA CGTTAAATTTAGCAAATCGCCTTTA CGTAGAGCAAAACTTTCCGACAAA GGAAAGCTTCAGCAGAAT	1 CAATTAGTAACTAA 2 CAATTAGTACCTAA 3 CAATTAGTACCTGA 4 CAATTAGAACCTAA 5 CAATTAGTAATTAT 6 CAATTGGTACCTAA 7 CAATTAGTAACTGA 8 CAAGTAGTGCCTAA 9 CAATTAGTAACGAA 10 CAGTTAGTAACTAA 11 CCATTAGTACCTAA 12 GAATTAGTAACTGA 13 CAATTAGTGCCTAA 14 CAATTGGTAACTAA 15 CAATTAGTAACTAT
ytP162	fw: AGGCACATGTTTTGGTAGTGG rev: AGTTTGCCGGTCATTGATTC seq1: CCTATAGAACTTCTCTTGAG seq2: CTCAAGAGAAGTTCTATAGG	AGGCACATGTTTTGGTAGTGGAAA GTACGATATATGATTTG**R**TACTAA **R**ACTTGCCCCGACGAGCTGTAAAA TGAAGGTATGTTTCAACTATCCGAT TGCTGACCGTAATATAAAATTTGC ATCATTCTTTCGTTTTTATTTCCGAC AAATTTCTTGCCTCTATTCAAGAGG A**Y**TCTGATGTCGCATTTTTGGAAGG AAACATGAGAAAAAGTC**Y**A**M**GTA CAACAAATTTTTCTATTGACTTTTT GATTG**S**GAAAATATAATACGC**W**AA A**M**TA**Y**TGGCTGTATTCCAAAGCTT TAC**K**AAAATTTTGTA**R**ATATAATC GCAAAAA**R**TATGC**Y**GCAAAGAAA ATCTACAGAATCTCGAT**Y**TTTCGCT TATTTTACAGGGTCTCAAGAGAAG TTCTATAGGGAAATGTAAAAGAAA	1 GGTCACTATGAACYATATTTTAT 2 GGTCACTCTGAACYATATTTTAT 3 AGTTACTATTAGCYATGTTTCCC 4 AGTCACTATGAACYATATTTTAT 5 AGTCACTATTAGCYATATTTTAC 6 AGTCACTATTAACYGTGTTTTAC 7 GGTCACTATGAACYATATTTTCC 8 AGTCACTATGAACYATGTTTCAT 9 AGTCAGTACGAACYATATTTTAT 10 GGTCACTACGAACYATATTTTAT 11 AGTTCCTATTAGCYATGTTTCCC 12 AGTCACTATTAGCYATGTTTCCC 13 AATTACTATTAGCYATGTTTCCC 14 GGTCACTATGAACYATATTTTAC 15 GGTCACTATGAACYATAGTTTCC 16 AGTCACTATTAACYATATTTTAC

		CATGAAGCAAAAACCGAAGGTTAG AGAATTATTCCRCAAAAAGCAAKT ATTAATGATTTCTGAGRKCGCTAT YTGATACATTTAGACCGTTTTTTGT AYCAAAGAYAATAACAGTATCCTT GCTGAGTTTATCTTGACACAGTGTA TTTGCTATTAAAATTMTGATAYTTT TCAGGATGTTGCTCTTAGAATATCT GAATCAATGACCGGCAAACT	17 AGTCAGTATGAACYATATTTTCC 18 GGTCACTATTAGCYATATTTTAC 19 AGTCACTATTAGCYATATTTTCC 20 GGTTACTATTAGCYATGTTTCCC 21 AGTCACTATTAGCYGTGTTTTAC 22 GGTCACTATGAACYGTATTTTCC 23 AGTCAGTATGAACYATATTTTCC 24 GGTCACAATGAACYATATTTTAT 25 AGTCACTATGAACYATATTTTCC 26 AGTCACTATTAGCYATATTTTAT 27 AGTCACTATGAACYATATTTTAC
ytP164	fw: GCATCTTCGCTATCCTTTGC rev: CGAATGGAAACAGCAGCAG seq: AGACTTATCCGTGGTT	GCATCTTCGCTATCCTTTGCTGCAC AAAGTCCAACTGCGACTGCTTCCT AAATCATAAAAWTCAATCAATTTA AGTAATTCGCTTTAACAAAARTAA TTTAAAATAATTTTTTAATAAAGAA TATAGAAGATTTAAAAGAAAAACC CGAAATRAAGGAAGATTTTTGATT GGTATTTTGGATGAATTGTCATAAA AAGTTTTTCATGAATTAATTAACTA TTAATTCAAYACATACAAATTATCC AAYAATTATTGCAAATAAACATTA ATTAATTACACGATACATATTTTGK TAGTCATACGAACACATCAAATGT TGCTAAACTTATTCGATTTATAATT ACAAAAACAAAAAAAAGAAAAAT TTATCACCTGTCCGGTATATAAATG GCAAAAACCACGGATAAGTCTTTC TTTGTAAAGATTTCCTGCTGCTGTT TCCATTCG	1 TAGTTG 2 TAATTG 3 TAGTTT 4 AAGTTT 5 TGGCTT 6 TAGTCG 7 TAATTT
ytP169	fw: CGACATTTGCTATGGGAAGC rev: CACCATCGCAGCTGTGTACT seq: CGACATTTGCTATGGGAA	CGACATTTGCTATGGGAAGCATTA AAAATGTAACTGTCAAAGGTCAGG TCGCATGCAGCGATCGATCGCAAA AAGATGTTGAAATACARTTRTGGG ARCGTGATACACGTAAGTTCGACT TWTCTCATTYTGATCGAATACRAA GTTCTATYTTTCYTTTTCCTATRAA YTGATATTTGTATCTGATAATAAW TKARGTGAATTTTAASCTAATYKG TGATATAAAGTTTTAAATTTAATTT CTAGTGGATCCGGATGATTTGCTG AATACGACGAAGACCGAYGCTCGT GGAAATTTCAAGATATATGGAGAA GAGAATGAAGTAAACAACATTGAA CCGTATCTAATAATAGTACACAGC TGCGATGGTG	1 AGGACGTCGTATACTGT 2 AGAACACCACTTAGCTT 3 AGAACGTCGTATACTGT 4 AGGTTATTGTAGGCTGT 5 AGAACACCACTTACTGT 6 AGAACACCACTGGGCTT 7 AGAACATCGTAGGCTGT 8 AGAACATCGTATACTGT 9 AGATTATTATAGGCTGT 10 AGGACATCACTTACTGT 11 AGGACACCACTTAGCTT 12 GGAACGTCGTATACTGT 13 AGAACACCACATAGCTT 14 AGAACATCGTATACTGC 15 AAAACACCACTTAGCTT 16 AGAACATCGTTTAdel 17 AGAACACCGTTTAGCTT
12S	fw: GTTCCAGAATAATCGGCTA rev: ATTGACGGATGRTTTGTACC seq: GTTCCAGAATAATCGGCTA	CCTTATTTATTAAATTCATTAARAC ATTAARAAAAAATTACTTTCTTTTC CAATTTCAAAAAAAAATAAAAAAC TAATCCAAAAAAAATTCATAATAG TAACACATGAARCATAAATTCATA AGCCAAATATATATCTGTTTTTAAT GCCAAAACAAYAAAGCATTAC*AA AATAAARTAAAAACTAAACAATCA TACATGTGCCAACAAAATTCACCA AAAAAGAGGGCTCTCCARCAAATC ACATRTTCCAAAGAAAAATCTAAA GTCAGTCAATATTTTTTCGGTTTAA AYAAAACTTTACTCCCGAATTATTA AAYTTTGATTACCTGGGTACTAATC CAGTTCAAAAAACAAATTTTTGAT ACCAYAAGAAACT***AAAAAAAA TYAAA*AYATTTTAC*AAAAAACA	*O. ochengi* 1 AAAC*AGGCTC**AC*T*CCA* 2 AAAC*AGGCTC***C*T*CCA* 3 GAAC*AGGCTC**AC*T*CCA* 4 AAAC*AGGCTC*AAC*T*CCA* 5 AAAC*AGGCTCAAAC*T*CCA* 6 AAAC*AGGTTC**AC*T*CCA* 7 GAGC*AGGCTC**AC*T*CCA* 8 AGAC*AGACTC**AC*T*CCA* 9 AAAC*AGGCCC**AC*T*CCA* 10 AAAC*AGGCTC**AC*T*CC** 11 AAACAAGGCTC**AC*T*CCA* *O. ochengi* 'Siisa' form 1 AGAC*AGACTC***T*C*CAA* 2 AGAC*AGACTC***C*C*CAA* 3 AGAC*AGACTC**ACTCACAA* 4 AGAC*AGACTC**AC*C*CAA*

		AYATAAAAACAAAMTAAAA**CT	5 AGAC * AAACTC* * * T* C* CAA* 6 AGAC * GGACTC* * * T* C* CAAA *O. volvulus* 1 AGAT * AGATTT* * A T* T* TCA* 2 AGAC * AGATTT* * A T* T* TCA*
16S fw: TGGCAGCCTTAGCGTGATG rev: CAAGATAAACCGCTCTGTCT CAC seq: TGGCAGCCTTAGCGTGATG		**Y**TTTTTTATTTACTTTTT**R**TTTGAAT TA*TTTTTTT**R**ATTAAAAATTATTA GTTAAGGTATTACAAAGATAAGTC TTCGGAAATTTTGTTTTGAATTTTG AAATTTTT**R***TTTTTAATTTTTTCTT GGGGATGGATTTTAAGAAAGTTTT ATACTATT**R**TTATT**R**TTAA**R**AATTA CTCCGGAGTTAACAGGGTTGTAGA CATATAAATAG**R**T**K**TTTATATTAGT G*TGCTGCGCTACATCGATGTTGTA TATTTTTTTTGATAATGGAGAGG*T TTTTTTT**R**TTTTGAGACTGTTCTTCT **Y**GTATAAAAAATT**R**ACTTGATATT AGTTTAGTTCGTCG	*O. ochengi* 1 TA* GG* GAAAT** ATG 2 TA* GG* GAAAT** ATG 3 TA* GGT GAAAT** ATG 4 TA* GG* GAAAT*T ATG 5 TA* GG* GAAATT* ATG 6 TAT GG* GAAAT* *ATG 7 TA* GG* GAAAT* *ATA *O. ochengi* ‘Siisa’ form 1 CA* GA* AAAGT** A TG 2 CA* GG* AAAGT** A TG 3 CA* GG* AAAGT** A CG 4 CA* GG* AAAGG** A TG 5 CA* GA* AGAGT** A TG *O. volvulus* 1 CG*A A* GAAGT** GTG 2 TG*A A* GAAGT** GTG 3 CG*A A* GAGGT** GTG