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SHORT COMMUNICATION

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

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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.

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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).

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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 seq: TGCGTTTTCTGATCGTATTT	446	8
ytP161	fw: TATCTCCTCTTTCGGTGTCA rev: ATTCTGCTGAAGCTTTCCTT seq: TATCTCCTCTTTCGGTGTCA	405	14
ytP162	fw: AGGCACATGTTTTGGTAGTGG rev: AGTTTGCCGGTCATTGATTC seq1: CCTATAGAACTTCTCTTGAG seq2: CTCAAGAGAAGTTCTATAGG	629	25
ytP164	fw: GCATCTTCGCTATCCTTTGC rev: CGAATGGAAACAGCAGCAG seq: AGACTTATCCGTGGTT	448	7
ytP169	fw: CGACATTTGCTATGGGAAGC rev: CACCATCGCAGCTGTGTACT seq: CGACATTTGCTATGGGAA	372	15

[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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