

SHORT REPORT

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Merging species? Evidence for hybridization between the eel parasites *Anguillicola crassus* and *A. novaezelandiae* (Nematoda, Anguillicolidea)

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Abstract

Background: The eel parasitic nematodes *Anguillicola crassus* (originating from Asia) and *Anguillicola novaezelandiae* (originating from New Zealand) were both introduced to Europe, but occurred in sympatry only in Lake Bracciano in Italy, where they both infected the European eel (*Anguilla anguilla*). *A. novaezelandiae* was introduced to the lake in 1975 and disappeared soon after *A. crassus* was also found there in 1993. We tested the hypothesis if hybridization of the two species might be an explanation for the findings at Lake Bracciano.

Findings: After laboratory infection of one European eel with 10 third stage larvae of each parasite, two living female and 4 male adults of each species were found to co-occur in the swim bladder after 222 days post exposure. In 9 out of 17 eggs, isolated in total from uteri of the two *A. novaezelandiae* females, alleles were detected by microsatellite analysis that are characteristic for *A. crassus*, suggesting the hybrid origin of these eggs. In contrast, none of the eggs isolated from *A. crassus* females possessed alleles different from those found in *A. crassus* adults, but it was revealed that one female can be inseminated by several males.

Conclusion: Our results show that *A. crassus* and *A. novaezelandiae* can co-infect a single eel and can mature together in the same swim bladder. We also provide evidence for the possibility of hybridization of *A. crassus* males with *A. novaezelandiae* females. Therefore, hybridization might be an explanation for the disappearance of *A. novaezelandiae* from Lake Bracciano.

Keywords: Nematode, *Anguillicola*, Hybridization, Invasive species, Eel parasite, Lago Bracciano

Findings

Background

Nematodes of the genus *Anguillicola* are eel-specific swim bladder parasites using copepods as intermediate hosts (some *Anguillicola* spp. were moved to the genus *Anguillicoloides* according to [1], but recent molecular findings support the original taxonomy [2]). In eel species as final hosts, larval stages are found in the swim bladder wall while adult stages migrate into the swim bladder lumen and feed on the host's blood [3]. The best studied and most abundant species is *Anguillicola crassus* [4], which was introduced to Europe in the early 1980s through the importation of living eels from Asia [5-8]. The original host of *A. crassus*, the Japanese eel

(*Anguilla japonica*), is well adapted to the parasite and can limit parasite load, for example by massive encapsulation of larval stages [9,10]. In contrast, high infection intensities and prevalences were found in European eels (*Anguilla anguilla*), in which the nematode causes severe damage of the swim bladder wall, a general stress response and mortality of the host [5,11-13]. Therefore, the parasite is expected to threaten European eel populations, especially as the loss of swim bladder function might impair the spawning migration of eels [12,14].

A. crassus seems to be the most efficient invader of the genus, but it was not the first to be introduced to Europe. As early as 1975, Short-finned eels (*Anguilla australis*) from New Zealand infected with *Anguillicola novaezelandiae* were released into Lake Bracciano in Italy [15,16], where the parasite was able to infect European eels and built a stable population in the lake [17,18]. *A. novaezelandiae* showed high prevalence and

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intensity of infection in European eels, but there are no records of any damage to the swim bladder. To the best of our knowledge, there is no record of *A. novaezelandiae* being established in waters outside of Lake Bracciano, as this lake is not connected to other waterbodies. In 1993, *A. crassus* was described for the first time in Lake Bracciano, but no mixed infections of eels with both *Anguillicola* species were recorded [17].

Subsequently, *A. crassus* became the dominant species in the lake and so far *A. novaezelandiae* was not found any more [17,19]. One possible explanation is that *A. crassus* outcompeted and replaced *A. novaezelandiae*, but the two species might have also formed viable hybrids that resemble *A. crassus* morphologically. To assess the latter hypothesis we performed a laboratory infection experiment with subsequent microsatellite analysis, to test i) if *A. crassus* and *A. novaezelandiae* can co-infect the same eel and ii) if they are able to mate and produce hybrid offspring.

Methods

Infection experiment

European eels (*Anguilla anguilla*) were obtained from a commercial fish farm (Albe Fischfarm, Haren/Rütenbrock, Germany), where no cases of *Anguillicola crassus* infections were ever reported. To confirm the absence of parasites before the experiment, ten eels from the stock were killed, dissected and examined for the presence of parasites.

Anguillicola novaezelandiae collected from *Anguilla australis* originating from New Zealand and *A. crassus* collected from *A. anguilla* from lake Müggelsee in Berlin, Germany, were used for the laboratory life cycles. Infective third stage larvae (L3) of *A. crassus* and *A. novaezelandiae* were produced by the method of Haenen et al. [20].

For the experiment, one European eel (40 cm, 106 g) was infected with 10 L3 of *A. crassus* and 10 L3 of *A. novaezelandiae* by administering L3 with a stomach tube (1.5 mm diameter; B. Braun Melsungen AG) as described in [21]. The eel was kept in a 80 L fish tank at 20°C and was fed twice a week for a period of 222 days in order to guarantee the presence of second stage larvae for molecular analyses (for details on the life cycle see [22]). After 222 days post infection (dpi), the eel was killed by decapitation and examined immediately for infection of the swim bladder. Adult parasites were counted and sex was determined. For storage, worms were fixed separately in 70% ethanol. *Anguillicola* species were identified morphologically according to [1]. The identification was done without knowledge about the PCR results to avoid bias of the investigator. The infection experiment was conducted in compliance with national and institutional guidelines for the care and use of animals.

Molecular species identification

Small pieces of the cuticle or the pharynx were cut off from each adult nematode recovered from the eel using sterile technique. Care was taken not to carry-over intestinal content of the worms. DNA was extracted from each sample using a JETQUICK DNA Clean-Up Spin Kit (Genomed) according to manufacturer's instructions. Species-specific primers targeting *cox I* were designed for both nematode species according to a multiple alignment including *cox I* sequences from *A. crassus* (GenBank accession no.: EU376921), *Anguillicola globiceps* (JF805673), *Anguillicola papernai* (JF805697), *Anguillicola australiensis* (JF805640), *A. novaezelandiae* isolate from Tasmania (JF805629), *A. novaezelandiae* New Zealand isolate (JX868555) from the present study and European eel (HQ600683). Sequences of *A. crassus* specific primers were: crasscox for 5'-CCT TTT GTT AGG TGA TGG GCA A-3', crasscox rev 5'-TAG CGA GAT CAA CAC TTA TAC CAG-3', amplifying a product of 303 bp and for *A. novaezelandiae* (New Zealand isolate): novcox for 5'-ATT GGG TGA CGG CCA GTT ATA-3', novcox rev 5'-ACT TAT ATG CTC CAG AGT AAT AGA ACT A-3', amplifying a product of 404 bp. PCR conditions were optimized and specificity of primers was tested. One 20 µl PCR reaction mix contained 4 µl of 5x Crimson Taq buffer (New England Biolabs), 0.2 mM dNTP mix (New England Biolabs), 0.5 µM of each primer 0.5 U Crimson Taq (New England Biolabs) and 1 µl template DNA. The mix was topped up to 20 µl with PCR grade water. PCR was conducted on a TGradient thermocycler (Biometra) with the same program for both primer pairs: 95°C for 5 min, 35 cycles of 95°C, 58°C and 72°C each for 45s and a final elongation at 72°C for 5 min. PCR products were analysed by standard agarose gel electrophoresis.

Microsatellite analysis

Microsatellite markers for *A. crassus* were previously developed by [23]. We tested those seven markers also for *A. novaezelandiae* and selected two markers (AcrCT04 and AcrCA102) that produced a clearly distinguishable pattern of PCR products for the two species. Each forward primer was labelled at the 5'-end with a fluorescent dye (FAM for AcrCT04 and HEX for AcrCA102).

To analyse the allelic pattern of the adult worms, their DNA was amplified with AcrCT04 and AcrCA102 according to [23]. For characterization of the offspring, ten eggs were dissected from each uterus of female nematodes. Eggs were washed by repeated transfers to drops of clean distilled water on a sterile petri dish. Subsequently, single eggs were placed in reaction tubes, DNA was extracted as described above and amplified with AcrCT04 and AcrCA102. PCR products were

purified with a JETQUICK PCR Product Purification Spin Kit (Genomed). Products of AcrCT04 and AcrCA102 were pooled and sent for analysis on an Applied Biosystems DNA Analyzer ABI3730 (GATC). Fluorescent peaks were analysed with the Peak Scanner™ Software v1.0 (Applied Biosystems).

To check for contamination of egg DNA from one nematode species with DNA from the other, all eggs were tested with the *cox I*-PCR. As *cox I* is a mitochondrial marker, it should show the identity of the female irrespective of a possible hybrid origin.

Results and discussion

Infection experiment

Both primer pairs designed according to the *cox I* sequence of *A. crassus* and *A. novaezelandiae* were species specific and did not cross react with eel DNA.

In total, 13 adult nematodes were isolated from the swim bladder of the eel, five of which were female and eight male. One of the females was found dead and partly decomposed, therefore this specimen was not used for further analyses. No larval stages (L3 or L4) were detected in the swim bladder wall. The morphological species identification was in accordance with the *cox I* PCR results. Only the DNA from one female produced a strong band for *A. novaezelandiae* and an additional faint band with the *crassus*-primers, indicating contamination of the sample. This problem remained even though new DNA was extracted and tested again twice from this specimen (Table 1). As the *A. crassus*-band was faint, we assumed that this specimen was *A. novaezelandiae*, in compliance with the result of the morphological identification. Two female and four male individuals were identified for each species, which equates to a recovery rate of 60% (ten third stage larvae from each species were used for infection) for each species with an overall recovery rate of 65%, considering also the dead female. This shows that co-infection of the

final host with both species is possible, and might have also occurred in Lake Bracciano.

Microsatellite analysis

According to the analysis of adult nematodes, allele sizes for marker AcrCT04 ranged from 123 bp – 198 bp for *A. crassus*, while a single 109 bp allele was characteristic for *A. novaezelandiae* specimens. AcrCA102 amplified two alleles, one 305 bp and one 325 bp. These were detected only in *A. crassus* samples (Table 1). Analysis of microsatellite patterns of the eggs revealed information on parental combinations. Only “*A. crassus*-alleles” were found in the eggs analysed from the females AC1 and AC2, showing that species boundaries were not crossed. Characteristic alleles of *A. crassus* males, allowed the identification of the potential father in some cases (Table 2). Apparently, one female was inseminated by several males, giving rise to eggs fertilized by different sperm (e.g. AC3 and AC6 for *A. crassus* female 1; see also Table 2).

In the offspring of the two *A. novaezelandiae* females AN1 and AN2, alleles characteristic for *A. crassus* were detected. In five out of nine eggs from AN1 and in four out of eight eggs from AN2, alleles different from the 109 bp allele were amplified by AcrCT04. In all these individuals, either the 305 bp or the 325 bp “*A. crassus*-allele” amplified by AcrCA102 was found as well, strongly indicating hybrid origin of these eggs, attributable to at least three different *A. crassus* males. Contamination was excluded in these cases by *cox I*-PCR that detected only *A. novaezelandiae* (maternal) mitochondrial DNA in the hybrids (Table 2).

In some cases, no PCR product was obtained from single eggs (Table 2). Most likely, DNA-extraction was unsuccessful, because eggs were lost during collection and washing as they easily stick in pipette tips and other plastics. For egg no. 3 and egg no. 7 of *A. crassus* female no. 2, more than two alleles were detected by marker AcrCT04, which revealed contamination of the sample

Table 1 Results of morphological and molecular examination of adult nematodes

No.	<i>A. crassus</i>						<i>A. novaezelandiae</i>						dead ♀
	AC1	AC2	AC3	AC4	AC5	AC6	AN1	AN2	AN3	AN4	AN5	AN6	-
Sex	♀	♀	♂	♂	♂	♂	♀	♀	♂	♂	♂	♂	♀
Morphology	AC	AC	AC	AC	AC	AC	AN	AN	AN	AN	AN	AN	ND
<i>cox I</i> PCR	AC	AC	AC	AC	AC	AC	AN	AN	AN AC*	AN	AN	AN	ND
Alleles AcrCT04	123 198	135 163	123 146	135 159	135 -	135 198	109 -	109 -	109 -	109 -	109 -	109 -	ND
Alleles AcrCA102	325 -	305 325	305 325	305 -	305 325	305 325	- -	- -	- -	- -	- -	- -	ND

AC: *A. crassus*, AN: *A. novaezelandiae*; numbers indicate allele size in bp; ND: not determined; *faint band.

Table 2 Results of microsatellite analysis for eggs

AC ♀ no. 1	egg 1	egg 2	egg 3	egg 4	egg 5	egg 6	egg 7	egg 8	egg 9	egg 10
cox I PCR	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC
Alleles AcrCT04	123 135	146 198	123 198	ND	123 198	123 198	123 198	135 198	198 –	123 135
Alleles AcrCA102	325 –	305 325	305 325	ND	325 –	305 325	305 325	325 –	305 325	305 325
potential fathers	AC4, AC5, AC6	AC3	AC3, AC6		AC3, AC6	AC3, AC6	AC3, AC6	AC4, AC5, AC6	AC6	AC4, AC5, AC6
AC ♀ no. 2	egg 1	egg 2	egg 3	egg 4	egg 5	egg 6	egg 7	egg 8	egg 9	egg 10
cox I PCR	AC	AC	AC	AC	AC/AN	AC	AC	AC	AC/AN	AC
Alleles AcrCT04	163 198	135 –	C	146 163	123 163	135 146	C	135 159	159 163	ND
Alleles AcrCA102	325 –	305 325		325 –	305 325	325 –		305 325	305 –	ND
potential fathers	AC6	AC4, AC5, AC6		AC3	AC3	AC3		AC4	AC4	
AN ♀ no. 1	egg 1	egg 2	egg 3	egg 4	egg 5	egg 6	egg 7	egg 8	egg 9	egg 10
cox I PCR	AN	AN	AN	AN	AN	AN	AN	AN	AN/AC	AN
Alleles AcrCT04	109 123	109 –	109 146	109 123	109 –	109 –	109 135	109 –	ND	109 146
Alleles AcrCA102	325 –	–	325 –	325 –	–	–	305 –	–	ND	305 –
potential fathers	AC3		AC3	AC3			AC4, AC5, AC6			AC3
AN ♀ no. 2	egg 1	egg 2	egg 3	egg 4	egg 5	egg 6	egg 7	egg 8	egg 9	egg 10
cox I PCR	AN	AN	AN	AN	AN	AN	AN/AC	AN	AN	AN/AC
Alleles AcrCT04	109 –	109 –	109 198	109 159	109 –	109 198	ND	109 198	109 –	ND
Alleles AcrCA102	–	–	305 –	305 –	–	325 –	ND	305 –	–	ND
potential fathers			AC6	AC4		AC6		AC6		

AC: *A. crassus*, AN: *A. novaezelandiae*; numbers indicate allele size in bp; C: more than 2 alleles were present, indicating contamination with DNA of 2nd individual; alleles indicating hybrids or PCR results showing contaminations are highlighted in bold.

with DNA of another *A. crassus* individual. The *cox I* PCR also indicated a weak *A. novaezelandiae* contamination (faint bands) in DNA from eggs no. 5 and no. 9 isolated from AC female no. 2, although no AN alleles were detected in these samples (Table 2). Also, contamination of some DNA samples from *A. novaezelandiae* eggs with *A. crassus* DNA was detected by *cox I*-PCR (AN1: egg no. 9, AN2: egg no. 7 and no. 10; see Table 2). These eggs were not taken into account for evaluation of the microsatellite data. Reasons for the contaminations found in one adult nematode (AN3) and several eggs might have been carry over of tissue or eggs from other individuals during sampling or DNA extraction, remainders of sperm in the uteri, or errors during PCR setup. Nevertheless, most individuals were contamination free and allowed reliable interpretation of the results.

These results provide evidence that *A. crassus* and *A. novaezelandiae* can form hybrids, at least in laboratory infections. As *A. crassus* originated from Asia and *A. novaezelandiae* from New Zealand, these species would not have met under natural conditions without human influence. Therefore, evolving a morphological

or behavioural reproductive barrier between the two species was not necessary. It is not clear, whether there is some pre- or postzygotic barrier for *A. novaezelandiae* males to fertilize *A. crassus* females, or if the lack of hybrids from *A. crassus* females was a coincidental result of our study. Further experimental double infections of eels with both *Anguillicola* spp. will help to answer this question.

Hybridization within different groups of helminth parasites is a well-known phenomenon. In laboratory experiments, hybridization was proven for different *Schistosoma* spp. [24-27] and two other trematodes, *Fasciola hepatica* and *Fasciola gigantica* [28]. In most of these laboratory studies, hybrids showed reduced survival and impaired fertility already in the F1 or F2 generation. However, hybridization among parasites also occurs under natural conditions and was observed in monogeneans [29], schistosomes [30] and nematodes (*Anisakis*) [31]. The eggs isolated from the females from the present study were fixed in ethanol and therefore it could not be tested, if the hybrid larvae produced are viable, infective, and if they develop into fertile adults,

but these issues will be investigated in further studies. Therefore, it can only be speculated if hybridization between *A. crassus* and *A. novaezelandiae* is an explanation for what happened in Lake Bracciano.

A case of replacement of one species by another through introgressive hybridization among helminth parasites was described for *Schistosoma haematobium* and *Schistosoma intercalatum* in a part of Cameroon where *S. intercalatum* was completely replaced by the introduced *S. haematobium* and the hybrids of both species within about 30 years [32,33]. Laboratory experiments proved that hybrids of *S. haematobium* and *S. intercalatum* were more successful in mating competition than both *S. intercalatum* and, to a lesser extent, *S. haematobium* [34]. A similar scenario might be possible for Lake Bracciano, though the replacement of *A. novaezelandiae* by *A. crassus* occurred much faster. But incompatibility of *A. novaezelandiae* males with *A. crassus* females might quickly lead to a dominance of *A. crassus* genes in the population. The hybrid offspring would also be a selective disadvantage for *A. novaezelandiae* females, even if these hybrids are not viable. Of course alternative explanations for the disappearance of *A. novaezelandiae* from Lake Bracciano are likely such as a faster development of larval stages in the copepod intermediate hosts or a broader spectrum of suitable intermediate hosts for *A. crassus*. These and other life-cycle parameters have to be studied in detail before introgressive hybridization can be figured out as the main reason for the disappearance of *A. novaezelandiae*.

Conclusions

Results of the present study show that *A. crassus* and *A. novaezelandiae* might occur in the same host in areas of sympatry like Lake Bracciano. According to our results, hybridization is possible between *A. novaezelandiae* females and *A. crassus* males, but not vice versa. This finding would fit well to the scenario of Lake Bracciano, where *A. novaezelandiae* seemed to have disappeared. Further laboratory hybridization-experiments are planned to test if this hybrid offspring is viable and fertile, and to analyse their morphology if adult nematodes develop.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DG was involved in dissecting the nematodes and egg sampling, primer design, molecular work, microsatellite analysis and writing the manuscript. KD and BS established and maintained the laboratory life-cycle of nematodes and contributed to writing by corrections and critical comments. KD conducted the infection experiments, eel and nematode dissection and sampling, as well as morphological identification of nematodes and sequencing of *cox I*. BS had a substantial role in conception of the study and guidance of the practical work. All authors read and approved the manuscript.

Acknowledgements

This study was supported by DFG via Grant SU217/12-1.

Received: 27 September 2012 Accepted: 29 October 2012

Published: 30 October 2012

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doi:10.1186/1756-3305-5-244

Cite this article as: Grabner et al.: Merging species? Evidence for hybridization between the eel parasites *Anguillicola crassus* and *A. novaezelandiae* (Nematoda, Anguillicolidea). *Parasites & Vectors* 2012 **5**:244.

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