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Hybridization between *Anguillicola crassus* and *A. novaezelandiae*, and viability of the F1 generation

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Research Paper

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Abstract

For decades, it has remained unclear how the Asian swim bladder nematode *Anguillicola crassus* was able to supplant the previously stable population of its relative from New Zealand *Anguillicola novaezelandiae* in the Lake Bracciano, Italy. Previously, researchers have hypothesized that *A. crassus* possesses an ecological advantage due to a more efficient life cycle in combination with a pattern of unidirectional hybridization between *A. novaezelandiae* females and *A. crassus* males. The present study focuses on the viability of hybrid offspring and their allelic pattern, particularly in developed adult stages of the hybrid F1 generation. While the percentages of hybrid individuals from *A. novaezelandiae* mothers and *A. crassus* fathers increased from egg to adult stages, it was more distinct in egg stages of *A. crassus* females and *A. novaezelandiae* males, but did not occur in adult F1 individuals at all. Therefore, we corroborate the hypothesis of unidirectional hybridization by differentiating between egg and adult stages, and suggest this as another explanatory factor for the extinction of *A. novaezelandiae* in Lake Bracciano in Italy and the predominance of *A. crassus*.

Introduction

Anthropogenic activities such as worldwide trade or travel led to an increase of invasive species around the world, which is a major driver for shifting species compositions (Colautti *et al.*, 2006; Pejchar & Mooney, 2009) and loss of ecosystem functions (Cardinale *et al.*, 2012). Even though ecosystems are well organized and aligned structures, they are still dynamic and reorganize themselves continuously. If a non-indigenous species enters a habitat, it might face competition with native or other invasive species for space and food. In general, it is difficult to measure the impact of an invasive species on ecosystem functions in a new habitat due to the complexity of interacting factors in an ecosystem (Kumschick *et al.*, 2015). Even though the magnitude of the impact is hard to measure, there are several studies attributing changes to the presence of introduced species (Doody *et al.*, 2017; Atalah & Sanchez-Jerez, 2020; Livingstone *et al.*, 2020). Albins (2013), for example, compared the influence of an invasive predator fish (Lionfish, *Pterois volitans*) on the local diversity of a prey community at Bahamian coral reef, to the influence of a native predator fish (coney grouper, *Cephalopholis fulva*). He illustrated that the presence of the invasive species had an impact on prey communities, regardless of whether the native piscivore was present or not. Nevertheless, it remains difficult to measure the long-term impact of the invader on the conquered ecosystem, as changes in prey community, including herbivores, which are keeping seaweeds under control, or cleaner fish that control ectoparasite density on other fish species, may lead to a complete reconstruction of the coral reef with unpredictable consequences (Albins, 2013).

Comparing the number of introduced species with established invasive ones, it seems by far more common that new species are not able to establish themselves in a new environment. Local species are often more resilient, the amount of introduced individuals and the adaptability of new species to habitats have a decisive impact and lead in few cases to stable, invasive populations (Carlton & Geller, 1993; Kolar & Lodge, 2001). In rare cases, introduced species are able to reproduce quickly, with tremendous consequences for local ecosystems. For example, the introduction of brown snakes (*Boiga irregularis*) to Guam – where they forage on local birds and rodents, but do not face any predator – led to the extinction of many native bird species (Wiles *et al.*, 2003).

Among the multitude of ecological factors that are covered in invasion research, a neglected but important topic are neozoan parasites (Poulin, 2017). On the host–parasite level, the new host can be seen as the newly conquered ecosystem, with the same scenarios being possible as described above for free-living species. Accordingly, if a new parasite species conquers a new region, it is in need of suitable hosts. If such a host is already occupied by other parasites, it either needs to find other suitable hosts, outcompete the existing parasite or co-occur within

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the same host, otherwise it is not able to survive and establish a population. However, if parasites do co-exist in one host and are closely related, it is also possible that they produce hybrid offspring (King *et al*., 2015). Several studies have demonstrated that hybridization is not only possible, but that that hybrid parasites might have a better host exploitation, faster maturation time and a better resistance against the host's immune system (Oey *et al*., 2019).

Natural hybridization is a mechanism that is commonly examined in evolutionary science and considered as one of the major drivers and sources for genetic variance (Arnold, 2004; Barton, 2008; Harrison *et al*., 2017). In some instances, hybrid offspring can develop in both host species of their parental generation, which provides them with a better host range. This was shown, for example, for hybrid offspring of *Schistosoma bovis*, a parasite of cattle, and *Schistosoma haematobium*, a common human parasite, collected from children in Senegal (Webster *et al*., 2013). Schelkle *et al*. (2012) suggest that hybridized monogeneans may exhibit a higher capability to escape the host immune system. In contrast, it is also possible that hybridization between parasites can limit the adaptations that one species develops to a host and, therefore, decreases their infectivity (Dybdahl *et al*., 2008). However, hybrids may face subsequent reproductive challenges, as some may be unable to produce fertile offspring (Al-Ahmad *et al*., 2006; Thomsen *et al*., 2011).

The well-studied swim bladder nematode *Anguillicola crassus* is one example of a parasite that has managed to outcompete an already established invasive parasite, *Anguillicola novaezelandiae*, with the same habitat requirements: the swim bladder of European eels (*Anguilla anguilla*). In the 1970s, the nematode *A. novaezelandiae*, originating from the Shortfin eel *Anguilla australis*, was introduced to Lake Bracciano, Italy, where the parasite was able to establish a stable population in the native European eel (*A. anguilla*) population (Paggi *et al*., 1982). However, because the lake is not connected to other waterbodies, the parasite population remained in this particular lake and did not spread further. After the introduction of the closely related invasive species *A. crassus*, originating from the Japanese eel (*Anguilla japonica*) in the early 1980s, both species were reported to co-occur in Lake Bracciano, even though mixed infections in eels have never been reported (Moravec *et al*., 1994). Nevertheless, a few years later, *A. novaezelandiae* seems to have gone extinct, and *A. crassus* is the only species reported from eels from Lake Bracciano in Italy (Münderle, 2005). Later, Grabner *et al*. (2012) demonstrated that mixed infestations of both nematode species in one eel produce hybrid offspring under laboratory conditions.

The aim of this study is to build on the previous results from Grabner *et al*. (2012), which are based on a single infested eel. We combine infestation hybridization experiments with molecular validation of hybridization to investigate hybridization events more explicitly in the F1 (1st filial) generation of *A. crassus* and *A. novaezelandiae* in European eels. However, in order to validate if the genetic advantage of *A. crassus* might be an explanation for the disappearance of *A. novaezelandiae* from Lake Bracciano, a multi-generation study with several eels has to be performed.

Materials and methods

Animal source

European eels (*A. anguilla*) were purchased from an eel farm (Albe Fischfarm, Haren/Rütenbrock, Germany), where *A. crassus* infections have not been previously recorded (Dangel *et al*., 2013; Hohenadler *et al*., 2018). The general absence of the parasite was verified by the dissection of ten randomly chosen eels, which were checked by light microscopy for infestation of the swim bladder.

Larvae (L2) of *A. crassus* were collected from European eels from the River Rhine caught by fishermen. L2 of *A. novaezelandiae* were obtained from a lab culture, which originated from *A. australis* from New Zealand (see also Grabner *et al*., 2012; Dangel & Sures, 2013). Life cycles were established according to Haenen *et al*. (1994).

To prevent the accidental release of *A. novaezelandiae* or potential hybrid larvae into the waste water system, all used tank water was collected and boiled before being discharged into the sewage system.

Infestation experiments

F0 generation

For the hybridization experiment, four eels were inoculated with ten larvae (L3) of *A. crassus* and *A. novaezelandiae* each by a stomach tube (1.5 mm diameter; B. Braun Melsungen AG, Germany) according to Sures & Knopf (2004). After inoculation, eels were kept for 150 days in aerated 80 l tanks with a PVC tube as environmental enrichment. Twice a week, they were fed ad libitum with eel pellets (DAN-EX 2848, BioMar A/S, Brande, Denmark) and one-third of the water was changed the day after feeding. After 150 days post inoculation (dpi), eels were dissected, adult nematodes were counted and sexes were distinguished by light microscopy.

F1 generation

Each gravid female from the previous hybridization experiment was carefully washed to remove eggs attached to the outer cuticle. Developed eggs containing F1 L2 were removed from the uterus. One batch of eggs was stored in 70% ethanol for further molecular analysis and another batch was transferred to tap water to initiate hatching of L2 that were fed to copepods (*Macrocyclops albidus*). Developed F1 L3 stages were removed from copepods after 14 days, and 20 eels were inoculated with these as described above. Each eel was inoculated with 11–27 L3 individuals originating from a single female nematode. The further procedure was performed as described above for the F0 (parental) generation, including checking gravid females for embryonated eggs.

Molecular analysis

Small pieces of the pharynx or cuticle were cut out of adult individuals, and washed multiple times in Milli-Q water, to remove contaminations of the host tissue. DNA was extracted with a salt precipitation protocol as described in Grabner *et al*. (2015). To verify species identity of the parental generation, molecular barcoding was performed using species-specific primer targeting *cox I* according to Grabner *et al*. (2012). Primer sets for each species were run separately for every individual sample. The polymerase chain reaction (PCR) mix contained 10 µl OneTaq® 2X Master Mix (New England Biolabs, Frankfurt am Main, Germany), 0.5 µM of each primer and 1 µl of sample DNA, and was topped up to 20 µl with PCR-grade water. The PCR was run on a peqStar Labcycler at 95°C for 5 min, 35 cycles of 95°C, 58°C and 72°C each for 45 s, and a final elongation at 72°C for 5 min. PCR products were checked by standard agarose gel electrophoresis (1.5% agarose, 85 V, 100–1000 bp ladder). Bands

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for *A. crassus* are expected at 303 bp, and at 404 bp for *A. novaezelandiae*.

Analysis of microsatellite markers was used to identify a possible hybrid origin of the F1 generation. The markers AcrCT04 and AcrCA102 (Wielgoss *et al.*, 2007) were used as described in Grabner *et al.* (2012). PCR was conducted as described above, with the following conditions: 94°C for 5 min, 35 cycles of 94°C, 55.9°C and 72°C each for 45 s and a final elongation at 72°C for 10 min.

PCR products were further analysed with a Fragment Analyzer™ (Agilent Technologies, Waldbronn, Germany) using a 33 cm capillary and the dsDNA 905 Reagent Kit (Agilent Technologies). DNA concentrations were quantified by Fragment Analyzer™ Automated CE System PROSize® 3.0. Marker fragment sizes were evaluated based on PCR products amplified with the ArcCT04 and ArcCA102 of all F0 adult worms. The resulting fragment sizes were assigned to the respective *Anguillicola* species. Because the microsatellite markers yield fragments of 100–260 bp (ArcCT04) and 297–332 bp (ArcCA102) (Wielgoss *et al.*, 2007), signals <100 bp and >400 bp, as well as signals with a relative intensity of <5%, were not considered further. In addition, fragments between 136 and 139 bp were excluded, as they appear for both species.

To account for the uncertainty of 3–5 bp of the microsatellite measurement in the resulting fragments, closely spaced bands were merged as follows, resulting in fragment sizes that were exclusively found in one or the other species and named accordingly (AC: *A. crassus*; AN: *A. novaezelandiae*): AC1 = 115–117 bp; AC2 = 136–139 bp; AC3 = 146–149 bp; AN1 = 119–124 bp; AN2 = 129–133 bp; AN3 = 141–142 bp.

From the F1 generation, a total of 30 eggs and 48 adult individuals originating from *A. crassus* mothers and 50 eggs and 88 adult individuals originating from *A. novaezelandiae* mothers were individually examined.

Results

F0 generation

All four inoculated eels were infested with various numbers of *Anguillicola* spp. individuals, with recovery rates ranging between 15% and 60%. Initial screening of the nematodes revealed that eel no. I was infested with females only. Since no offspring is possible without male individuals, these nematodes were not considered for further investigation. The infracommunities of the other eels were composed as follows: eel no. II: 6 ♀, 6 ♂; eel no. III: 6 ♀, 4 ♂; eel no. IV: 4 ♀, 4 ♂. All females were gravid, apart from two individuals – one in eel no. II and one in eel no. IV.

Species were determined by species-specific *cox I* primers and microsatellite analysis of the parental generation revealed three distinguishable alleles for both *A. crassus* (AC1-3) and *A. novaezelandiae* (AN1-3) (table 1). The results of species identification by *cox I* primers matched the results of species-specific microsatellite alleles consistently. Only one individual (IIIW6) showed unambiguous alleles of *A. novaezelandiae*, but could not be clearly distinguished by *cox I* primers as bands for both *A.*

Table 1. Molecular analysis of microsatellite markers (AcrCT04) of adult *A. crassus* and *A. novaezelandiae* of all examined eels (I–IV). Fragments were merged as follows: AC1 = 115–117 bp; AC2 = 136–139 bp; AC3 = 146–149 bp; AN1 = 120–124 bp; AN2 = 129–133 bp; AN3 = 141–142 bp.

	A. crassus[a]					*A. novaezelandiae*[a]						
Eel no. I												
Nematode	IW1	IW2	IW3									
Sex	♀	♀	♀									
AcrCT04												
Eel no. II												
Nematode	IIW1	IIW4	IIM2	IIM4	IIM5	IIW2	IIW3	IIW5	IIW6	IIM1	IIM3	IIM6
Sex	♀	♀	♂	♂	♂	♀	♀	♀	♀	♂	♂	♂
AcrCT04			AC1				AN1				AN1	AN1
	AC2	AC2	AC2		AC2	AN2	AN2	AN2	AN2	AN2	AN2	AN2
								AN3	AN3	AN3		
Eel no. III												
Nematode	IIIW1	IIIW2	IIIM2	IIIM3		IIIW3	IIIW4	IIIW5	IIIW6	IIIM1	IIIM4	
Sex	♀	♀	♂	♂		♀	♀	♀	♀	♂	♂	
AcrCT04												
		AC2	AC2			AN2	AN2		AN2	AN2	AN2	
Eel no. IV												
Nematode	IVM1	IVM2	IVM3	IVM4	IVW1	IVW2	IVW3	IVW4				
Sex	♂	♂	♂	♂	♀	♀	♀	♀				
AcrCT04					AC1	AN1	AN1	AN1				
	AC2	AC2				AN2	AN2	AN2				

[a]Species determination targeting *cox I*.

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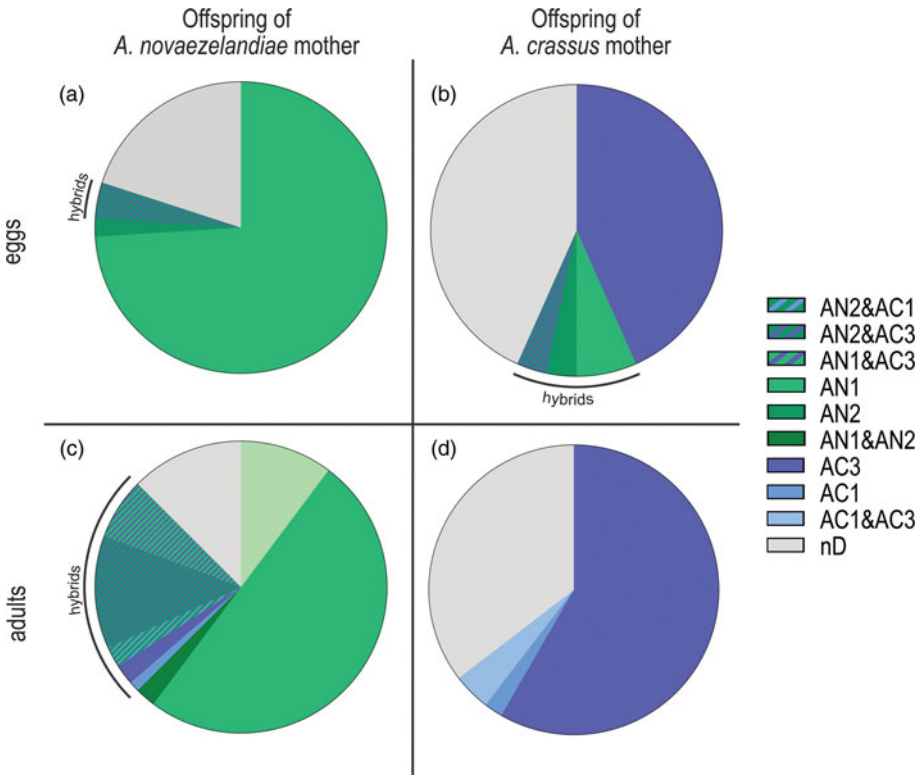

Fig. 1. Ratio of alleles of F1 generation. Shades of blue/purple represent *A. crassus* alleles; shades of green represent *A. novaezelandiae* alleles. (a) Eggs (with L2) from *A. novaezelandiae* mother ($n = 50$); (b) eggs (with L2) from *A. crassus* mother ($n = 30$); (c) adults from *A. novaezelandiae* mother ($n = 88$); (d) adults from *A. crassus* mother ($n = 48$). AC1 = 115–117 bp; AC3 = 144–150 bp; AN1 = 119–126 bp; AN2 = 128–133 bp. nD, no distinct pattern of fragments and, therefore, no species allocation possible.

novaezelandiae and for *A. crassus* were visible. Two individuals (IVW2 and IVW3) showed no band in the *cox I* PCR, but as their microsatellite alleles showed a clear *A. novaezelandiae* pattern, they were considered as such.

For six individuals (IIM4, IIIW1, IIIM3, IIIW5, IVM3, IVM4), no distinct microsatellite pattern was visible. The detected fragments were lying outside the range of the microsatellite alleles located for the two species, which are, therefore, considered as unspecific fragments. This remained the case even after repeating the measurement.

F1 generation

Recovery rates of nematodes originating from *A. crassus* mothers ranged between 4% and 67%, with 3.0 ± 2.2 ♀ and 3.1 ± 3.2 ♂. Recovery rates of nematodes originating from *A. novaezelandiae* mothers ranged between 11% and 65%, with 3.6 ± 2.8 ♀ and 1.7 ± 1.3 ♂. Most of the females had either no eggs or poorly developed/unembryonated eggs, except for three females originating from an *A. crassus* mother, which showed normally developed and embryonated eggs.

The ratio of hybrids and non-hybrids differs between eggs (containing L2) and developed adults of F1 generation (fig. 1). Eggs that originated from *A. crassus* females revealed 13% offspring with alleles of both species and 43% offspring with *A. crassus* alleles only, whereas eggs that originated from *A. novaezelandiae* females revealed 4% offspring with alleles of both species and 76% offspring with only *A. novaezelandiae* alleles. Hybridization was not detected in any of the adult offspring originating from *A. crassus* females. Adult offspring of *A. novaezelandiae* females revealed a percentage of 25% with alleles of both species, whereas 63% showed only alleles of *A. novaezelandiae*. The number of individuals without a distinct pattern for offspring originating from an *A. crassus* mother differs between 43% (eggs) and 35% (adults), and for offspring originating from an *A. novaezelandiae* mother, between 20% (eggs) and 13% (adults).

Discussion

In the present study, we provide additional details on the relevance of hybridization between the two eel swim bladder nematodes *A. crassus* and *A. novaezelandiae*. Previously, Grabner *et al.* (2012) suggested that *A. crassus* may have genetic advantages over *A. novaezelandiae*, as their findings indicated that hybridization appears to be possible only between *A. novaezelandiae* females and *A. crassus* males. Since they described this pattern based on nematodes obtained from one single eel only, it had remained uncertain whether this finding is reproducible. To verify the hypotheses of genetic advantages, the present study provides evidence for the viability of hybrid offspring and indicates that only hybrid offspring of *A. novaezelandiae* females can develop to F1 adults. We distinguish between hybrid larvae, which were released by the mother nematode but not developed further, and those that developed to the adult stage after passage through the copepod and experimental infection of an eel. Our results confirm the findings by Grabner *et al.* (2012) with respect to possible hybridization between both species of *Anguillicola*, and give further information about hybrid development.

In the present study, the length of the fragments amplified with the AcrCT04 primers varied between 120 bp and 142 bp, while previous data showed a uniform pattern for *A. novaezelandiae* of a single allele of 109 bp obtained by the AcrCT04 primers (Grabner *et al.*, 2012). This is due to the fact that a new field isolate of *A. novaezelandiae* was used for the laboratory cycle in the present study, showing a different allelic pattern. Interestingly, alleles of *A. novaezelandiae* were found only in the egg stages

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obtained from *A. crassus* mothers. Those eggs obtained from *A. crassus* mothers that were passed through a copepod and were used to infest an eel showed exclusively *A. crassus* alleles in the developing F1 adults. This indicates that hybrid eggs and larvae originating from the *A. crassus* female/*A. novaezelandiae* male crossing only develop to the larval stages. Even though the length difference between AC1 (115–117 bp) and AN1 (120–124 bp) may be at the limit of measuring accuracy of the method, it was consistently the case in all measurements performed that samples of both species never exceeded this limit. Therefore, we consider the size assignment of the alleles as valid.

Nevertheless, we cannot exclude the possibility that those hybrid nematodes develop to adult stages, as the examined sample size is still too small to give complete proof. Among individuals that derived from an *A. novaezelandiae* mother, the number of hybrids increased from 4% in the egg stage to 25% in adult nematodes, which is a strong indication that the development proceeds with greater success in this hybrid crossing. Recovery rates of hybrid offspring originating from both species are not known, as we cannot detect if an infectious larva is a hybrid beforehand without dissecting it; however, the further development into adult stages can be seen as a good predictor. In addition, the number of offspring without any distinct allelic pattern is higher in individuals originating from *A. crassus* mothers (35–43%), compared to those from *A. novaezelandiae* mothers (13–20%). This could be due to a different binding efficiency of the AcrCT04 primers for the two species, resulting in a lower density of bands for *A. crassus*.

The finding of the present study that *A. novaezelandiae* female/*A. crassus* male crossings exist and can even develop to the adult stage adds to the information provided by Grabner *et al.* (2012), who analysed only egg stages and, thus, could only speculate about further development. Applying our results to the situation in Lake Bracciano in the late 1980s, the interpretation depends on the fate of the F1 adults, which we still cannot predict with certainty. Basically, there are two possibilities – either hybrid offspring is viable and fertile, or hybridization leads to a dead end of reproduction. If hybrids are fertile, it might even be a disadvantage for *A. crassus* to produce hybrid offspring with *A. novaezelandiae*, as the former has by far the better adaptation to the eel's immune response (Knopf *et al.*, 2000; Keppel *et al.*, 2014), and there is the possibility that hybrid offspring will lack some of those adaptations. The life cycle of *A. crassus* is also more efficient compared to *A. novaezelandiae*, as the larvae are released over a longer period of time. Accordingly, these larvae are capable of infesting the intermediate host for a longer period of time as well (Dangel *et al.*, 2013). Therefore, it may be worse for hybrid offspring of *A. crassus* to lose this efficiency – although this is only speculation according to current knowledge, since no valid data on hybrid offspring performance are available. The effect of the hybrids on the populations of the two *Anguillicola* species would also depend on the potential differential reproductive success of each of the two species with the hybrids. However, we can only speculate about the further development and fertility of the F1 generation. In other species, male hybrids in particular are often facing sterility, as has been found for *Drosophila*, mice and other animals (Haldane, 1922; Price & Bouvier, 2002; Sun *et al.*, 2004; Thomsen *et al.*, 2011; Kagawa & Takimoto, 2018; Widmayer *et al.*, 2020); however, there are also a variety of known studies showing that hybrids can indeed be fertile (Wallis & Beardmore, 1980; Close & Bell, 1997; Volf *et al.*, 2007). If hybrid offspring are not fertile, this is to some extent a disadvantage for both species, as some of their reproduction effort leads to a dead end. Yet, it seems reasonable that *A. crassus* was able to combine its ecological advantage of a more efficient life cycle (Dangel *et al.*, 2013), underlined by theoretical modelling of the population growth rate of the two species (Dangel *et al.*, 2015), with some genetic advantage to contribute to the extinction of *A. novaezelandiae* in Lake Bracciano. The latter had to face an additional fitness impairment as it lost some reproductive output to non-viable or non-fertile hybrids, which it was not able to cope with. Nevertheless, existing in constant competition with another species is an energy-consuming process, such that in the long run it was more beneficial to eliminate a competitor and accept possible minor disadvantages – for example, a slightly worse adaptation to the immune response of the host.

Conclusively, this research contributes to a better understanding of what happened in the Lake Bracciano in the late 1980s and early 1990s – hybridization between the two species might have decreased the reproductive fitness of both, but due a more efficient life cycle and population growth rate, *A. crassus* could eventually make up for this disadvantage, while *A. novaezelandiae* has gone extinct.

Future experimental studies should focus on the viability and fertility of the F2 (2nd filial) generation to further clarify the fate of hybrid individuals in a population. Furthermore, the gene flow between the two *Anguillicola* species should be measured using a high number of genomic markers, using double-digest restriction-site-associated DNA, which has been shown to be efficient in detecting hybridization in previous studies (e.g. Xu & Hausdorf, 2021; Paulus *et al.*, 2022).

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