Renifer aniarum (Digenea: Reniferidae), an introduced North American parasite in grass snakes *Natrix natrix* in Calabria, southern Italy

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ABSTRACT: Over the past decades, as a result of various human activities involving intentional or unintentional movement of animals, many helminth species have been introduced to new regions with several ecological and epidemiological implications for the native species. A high prevalence of infection with an introduced digenean *Renifer aniarum*, previously known only from North America, was found in the grass snake *Natrix natrix* in the Calabria region, southern Italy. Morphological and molecular comparison with North American *R. aniarum* has confirmed the identity of the Italian specimens. A total of 41 grass snakes were studied for *R. aniarum* infection. Of 24 snakes sampled between 2009 and 2010, 22 were positive for this parasite. In contrast, all 17 snakes sampled from museum collections between 1983 and 1994 were negative. Our results support the hypothesis that *R. aniarum* was perhaps introduced into this area during the 1990s by the translocation of the American bullfrog *Lithobates* (*Rana*) *catesbeianus*, a normal second intermediate host of the digenean in its native range in North America. Although the life cycle of *R. aniarum* is complex and includes 3 host stages, this parasite has found suitable first and second intermediate hosts as well as definitive hosts in Italy. *Renifer aniarum* was second only to the very common grass snake tapeworm *Ophiotaenia europaea* in both prevalence and abundance among 9 species of helminths recovered in our study.

KEY WORDS: *Renifer aniarum* · *Natrix natrix* · Grass snake · Introduced species · Molecular identification · Calabria region · Southern Italy

INTRODUCTION

The grass snake *Natrix natrix* Linnaeus, 1758 has a broad distribution extending from Europe and north-western Africa to Middle Asia, and is the most common aquatic snake in southern Italy. It is strongly associated in its distribution with wetlands and other freshwater habitats where it feeds mainly on amphibians and fishes (Arnold & Burton 1978).

To date, a total of 59 helminth species have been reported from the grass snake across its entire geographical range. However, most studies have been conducted in eastern and central Europe (Grabda-Kazubska 1961, Sharpilo 1976, Lewin 1992, Buchvarov et al. 2000, Kirin 2002, Shimalov 2010). Sharpilo (1976) reported 50 helminth species found in *Natrix natrix* from the former Soviet Union, including numerous larval stages. Yildirimhan et al. (2007) reported helminths from Turkey and listed a total of 56 helminth species reported from the entire range of this snake species including 27 nematodes, 20 trematodes, 5 cestodes, and 4 acanthocephalans. In Italy, only 10 helminth species have been reported from *N. natrix*, namely *Leptophallus nigrovenosus*, *Macrodera longicollis*, *Parale-*

poderma cloacicola, *Telorchis assula*, *Spirometra erinaceieuropaei*, *Capillaria mingazzini*, *Dracunculus oesophageus*, *Oswaldocruzia filiformis*, *Centrorhynchus aluconis*, and *C. buteonis* (Rizzo 1902, Lühe 1909, Joyeux & Baer 1927, Desportes 1938). To the best of our knowledge, there are no recent studies of *N. natrix* parasites in Italy.

Over the past decades, human activities have led to intentional or unintentional introductions of numerous animal species into new geographic areas. As a result, many helminth species have been introduced to new regions or have expanded their distribution following the anthropogenic breakdown of biogeographic barriers, with different ecological and epidemiological implications for the native species (Blanc 2001, Torchin et al. 2002, Taraschewski 2006, Dubey & Shine 2008, Gherardi et al. 2008). Management of invasive species requires detailed information about their distribution and potential effects. Such information regarding invasive helminth species in reptiles, especially snakes, is practically non-existent. Gherardi et al. (2008) provided an excellent up to date review of introduced freshwater animals in Italy. The list includes 112 species of invertebrates and vertebrates and includes only a single species of parasitic platyhelminths, namely *Gyrodactylus salaris* Malmberg, 1957, a parasite of salmonid fishes now broadly distributed as a result of introductions with fish.

In the present study, we compare helminth parasitism in freshly collected and museum specimens of grass snakes. We found 9 helminth species, including a newly discovered introduced digenean *Renifer aniarum*, which parasitizes the mouth cavity of snakes in North America. It has never been reported in Europe or anywhere outside the New World. The high prevalence of infection suggests that this introduced species has now successfully established its life cycle in the new area. This is the first report of a digenean of reptiles from another continent expanding its range into Europe. We discuss the probable source of invasion and prevalence of this parasite and other helminth species in grass snakes in southern Italy.

MATERIALS AND METHODS

Necropsied grass snakes. Between May and September of 2009 and 2010, a total of 15 grass snakes, all road-killed or killed by predators, were collected in the Calabria region of southern Italy. Of 15 snakes, 13 came from the Crati River, ~5 km north of Cosenza, 1 from the Neto River, along the ss107 road (Crotone province), and 1 from the Corace River (Catanzaro) (Fig. 1). Individuals were frozen until necropsy was performed. Snout-to-vent length (svl; cm) was re-
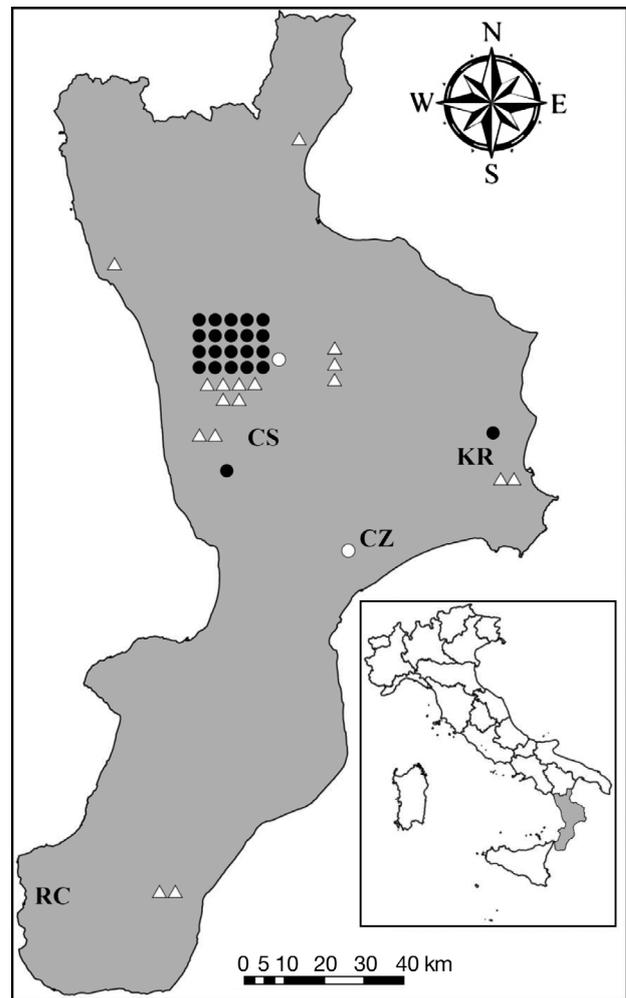

Fig. 1. *Natrix natrix.* Grass snake sampling sites in the Calabria region of southern Italy. *Renifer aniarum*-infected (●) and uninfected (○) grass snakes sampled between 2009 and 2010. Uninfected grass snakes from the Herpetological Collection (△) of the Department of Zoology, University of Arcavacata, sampled between 1983 and 1994. CS: Cosenza; KR: Crotone; CZ: Catanzaro; RC: Reggio Calabria

corded by a digital caliper to the nearest 0.01 mm. Snakes were dissected and sexed by gonadal observation. The body wall was opened by a longitudinal incision and the digestive tract, including oral cavity, esophagus, stomach, and intestine, was then inspected for helminth parasites using stereomicroscopy. Worms were washed in saline and fixed in 70% ethanol. For morphological studies and identification, cestodes and trematodes were stained with Mayer's acid carmine, alum carmine or Mayer's hematoxylin, dehydrated in a graded ethanol series, cleared in methyl salicylate or clove oil, and mounted permanently in Canada balsam or Damar gum. Nematodes were studied in temporary mounts cleared in lactophenol. Drawings were made

on a differential interference contrast-equipped compound Olympus BX-51 microscope using a drawing attachment. Two voucher specimens of *Renifer aniarum* from Italy and 2 specimens from Tennessee, USA, were deposited in the US National Parasite Collection (Beltsville, Maryland) under accession numbers USNPC 104 274 and 104 275. Voucher specimens of other helminths recovered were deposited under accession numbers 104 260 to 104 267. Infection parameters were calculated following Bush et al. (1997).

Oral rinsed snakes. Between May and September 2010, 9 grass snakes including 8 from the Crati River and 1 from the Savuto River (Fig. 1) in Altilia (Cosenza Province) were collected alive, measured, and sexed (DeNardo 1996). Because the grass snake is a protected species in Italy, and it is forbidden to euthanize it for parasitological purposes, the oral cavity of live snakes was first visually examined, then an oral rinse was performed 3 times with physiological saline using a 60 ml syringe. Washed material was observed under a stereomicroscope. At the end of this procedure, snakes were marked and released into the wild.

An additional 17 preserved grass snakes from the Herpetological Collection (HC) of the Department of Zoology, University of Arcavacata in Rende (Cosenza), collected between 1983 and 1994 from different localities of the Calabria region (Fig. 1) were examined for *Renifer aniarum* infection. The majority of these preserved grass snakes had been housed individually in glass jars filled with 90% ethanol, except for 3 snake specimens preserved together in 1 jar. For the study, the temporo-mandibular joint of preserved grass snakes was disjointed, and the oral cavity was first inspected for helminths using stereomicroscopy. Then, a scraping of the oral mucosal surfaces and an oral rinse was performed 3 times with physiological saline using a 60 ml syringe, and washed material was observed under a stereomicroscope. Finally, the preservation liquid from each jar was inspected for helminths that could have potentially dropped to the bottom. Because all *R. aniarum* specimens in necropsied grass snakes were found in the oral cavity except for only 4 specimens found in the esophagus of a single grass snake that had 85 specimens of *R. aniarum* in its oral cavity, the oral rinse method used here was considered a valid way to reveal *R. aniarum* infection in both preserved and live grass snakes.

Comparative material. Specimens of *Renifer aniarum* collected from a diamondback water snake *Nerodia rhombifer* caught on 16 May 2001 at Reelfoot Lake, Lake County, Tennessee, USA, were used for morphological and molecular comparison with specimens collected in Italy. In addition, specimens from a plain-bellied water snake *N. erythrogaster* caught on 15 August 2010 at Fish Lake, Jackson County, Missississippi, USA, were also used for molecular comparison. Living worms were rinsed in saline, killed with hot water (for molecular analyses) or formalin (for morphological examination), fixed in 70% ethanol, and stored in a freezer until further processing.

Molecular identification of *Renifer aniarum*. Genomic DNA for molecular analysis was isolated from *Renifer* specimens collected from Italy, Mississippi and Tennessee, according to Tkach & Pawlowski (1999). A single adult worm was used for each DNA extraction after preliminary morphological examination. DNA fragments approximately 2400 base pairs long spanning the 3′ end of the 18S nuclear rDNA gene, internal transcribed spacer region (ITS1+5.8S+ITS2) and 5′ end of the 28S gene (including variable domains D1 to D3) were amplified by PCR on an Eppendorf Master Gradient thermal cycler using forward primer ITSf (5′-CGC CCG TCG CTA CTA CCG ATT G-3′) and reverse primer 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′). PCR primers and several internal primers were used in sequencing reactions. Internal forward primers were digl2 (5′-AAG CAT ATC ACT AAG CGG-3′), 300F (5′-CAA GTA CCG TGA GGG AAA GTT G-3′), and 900F (5′-CCG TCT TGA AAC ACG GAC CAA G-3′); internal reverse primers were 300R (5′-CAA CTT TCC CTC ACG GTA CTT G-3′), digl2r (5′-CCG CTT AGT GAT ATG CTT-3′), ECD2 (5′-CTT GGT CCG TGT TTC AAG ACG GG-3′) and d58r (5′-CAC GAG CCG AGT GAT CCA CCG C-3′). PCR reactions were performed according to protocols described by Tkach et al. (2003).

PCR products were purified directly using Qiagen Qiaquick™ columns, cycle-sequenced using ABI BigDye™ chemistry, alcohol-precipitated, and run on an ABI Prism 3100™ automated capillary sequencer. Contiguous sequences were assembled and edited using Sequencer™ (GeneCodes, v4.1.4) and submitted to GenBank under accession numbers HQ665459– HQ665460. Sequences of all 3 forms were aligned using Clustal W as implemented in the BioEdit software v7.0.1 (Hall 1999) and compared using BioEdit.

RESULTS

Identity of *Renifer* collected from grass snakes in Italy

Morphological identification

Morphological identification of the specimens collected from grass snakes in Italy indicated that they were *Renifer aniarum*, previously known from snakes only in North America. All morphological features of the specimens from 2 continents were essentially identical with rather minor variability resulting from differences in age and fixation (Fig. 2). Italian specimens

have demonstrated the presence of 'mirror forms' regarding the position (dextral and sinistral) of the genital atrium (Fig. 2A,B).
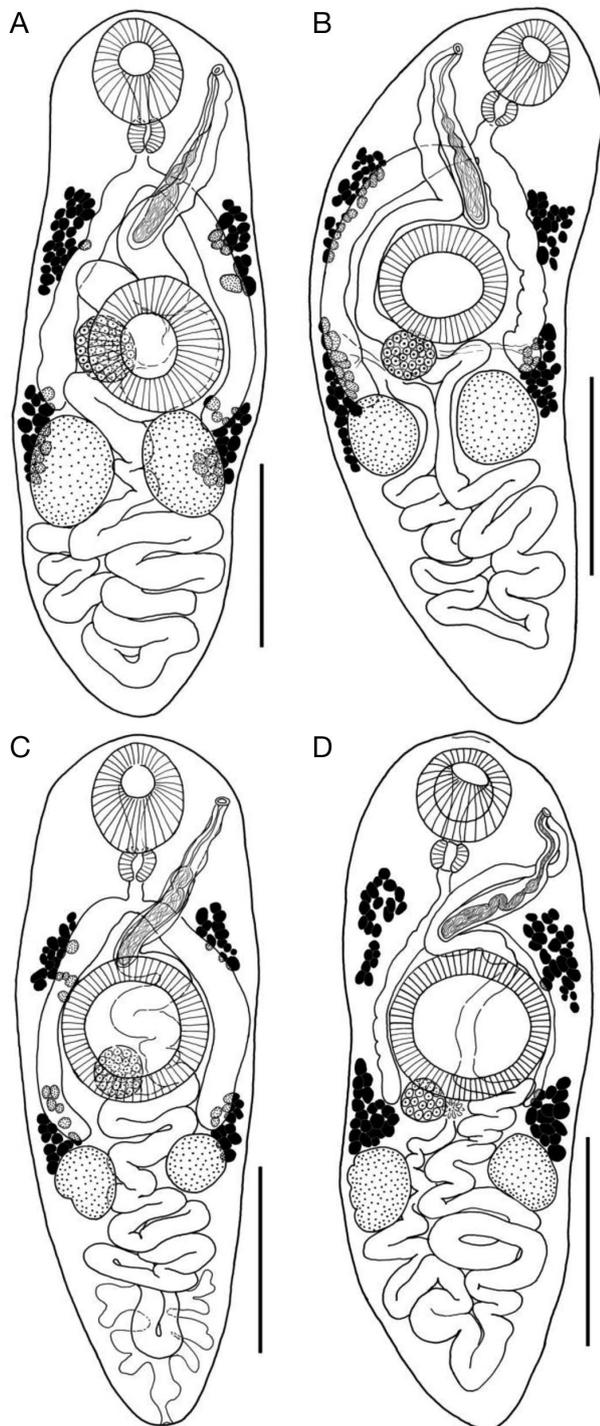

Fig. 2. *Renifer aniarum*. Morphology (ventral view) of specimens from (A,B) grass snake *Natrix natrix* from Italy showing mirror symmetry in the genital pore position, (C) plain-bellied water snake *Nerodia erythrogaster* from Mississippi, USA, and (D) diamondback water snake *Nerodia rhombifer* from Tennessee, USA. Scale bars = 500 µm

Molecular analysis

In order to compare interspecific sequence variability, we aligned sequences of specimens from *Natrix natrix* collected in Italy and from *Nerodia rhombifer* collected in Tennessee and *N. erythrogaster* in Mississippi. No differences were detected between the sequences of specimens from the 2 continents and different hosts. Taking into account that the sequenced DNA fragment comprised several regions characterized by different variability including the highly variable ITS1 region, our molecular data confirm the morphological identification of Italian specimens as *Renifer aniarum*.

Infection rates of grass snakes with helminths

All necropsied grass snakes were adult individuals including 9 females and 5 males (svl range: 52 to 96 cm) except for a single juvenile female (svl: 33 cm). A total of 9 helminth species were found including 5 digeneans, 3 nematodes, and 1 cestode (Table 1). *Renifer aniarum*, *Ratzia parva* and *Orneoascaris chrysanthemoides* in *Natrix natrix* represent new host records for these parasites and *R. aniarum* and *O. chrysanthemoides* are recorded from Eurasia for the first time.

The most prevalent species were *Ophiotaenia europaea* (93.3%) and *Renifer aniarum* (86.6%). *Renifer aniarum* was absent only from 2 necropsied females including one from the Crati River (svl: 91 cm) and the other from Catanzaro (svl: 83 cm) (Fig. 1). In the oral cavity of the latter snake, 2 specimens of *Leptophallus nigrovenosus* were found.

Live grass snakes sampled in 2010 from the Crati and Savuto Rivers (6 females and 3 males; svl ranging from 65 to 88 cm) were all positive for *Renifer aniarum* and all negative for *Leptophallus nigrovenosus* (Table 2). In contrast, the 17 snakes (9 female and 7 male adults; svl ranging from 21 to 120 cm, plus 1 juvenile) from the HC were all negative for *R. aniarum* (Table 2), while 5 of them (29.4%), all adult individuals, were positive for *L. nigrovenosus*. The 3 museum grass snakes preserved in a single jar were all negative for oral helminths. Thus, oral rinse proved to be an efficient method of screening both live and preserved snakes for oral digenean infections.

DISCUSSION

Members of the genus *Renifer* (syn. *Ochetosoma*) parasitize the upper digestive tract of snakes. *Renifer aniarum* (Family Reniferidae, see Tkach 2008) was originally described by Leidy (1890) as *Distomum*

Table 1. *Natrix natrix*. Prevalence, abundance (mean ± SD) and infection intensity of the helminth parasites found in the digestive tract of 15 necropsied grass snakes from the Calabria region of southern Italy. Esophagus (E); Upper (U) and lower (L) intestines (I); Oral cavity (OC)

Helminth	Location in host	Prevalence (%)	Abundance	Infection intensity Mean ± SD	Range
Digenea					
Renifer aniarum	OC, E	13 (86.6)	33.6 ± 31.8	38.7 ± 31.1	2–89
Telorchis assula	UI	2 (13.3)	0.2 ± 0.7	2 ± 1.4	1–3
Leptophallus nigrovenosus	OC	1 (6.6)	0.2 ± 0.5	2	2
Ratzia parva	UI	1 (6.6)	0.06 ± 0.2	1	1
Nematoda					
Paracapillaria sonsinoi	I	9 (60)	3.5 ± 4.7	5.8 ± 4.8	2–16
Orneoascaris chrysanthemoides	LI	2 (13.3)	0.1 ± 0.3	1	1
Oswaldocruzia filiformis	UI	1 (6.6)	0.06 ± 0.2	1	1
Cestoda					
Ophiotaenia europaea	I	14 (93.3)	51.8 ± 49.1	55.5 ± 48.8	9–183

Table 2. *Natrix natrix*. Grass snakes found infected by the digenean *Renifer aniarum* from the Calabria region of southern Italy

Geographical locality	n	Necropsied specimens	Oral-rinsed specimens Live	Museum
Crati River	21	12 of 13	8 of 8	–
Neto River	1	1 of 1	–	–
Savuto River	1	–	1 of 1	–
Corace River	1	0 of 1	–	–
Museum specimens	17	–	–	0 of 17

aniarum from the Northern water snake *Nerodia sipedon* in Pennsylvania. Since its original description, it has been reported in viperid (*Agkistrodon* spp.) and colubrid snakes (*Coluber* spp., *Farancia* spp., *Heterodon* spp., *Lampropeltis* spp., *Nerodia* spp., and *Seminatrix* spp.) from the United States (see McAllister & Bursey 2008) and in plain-bellied water snakes in Mexico (Jimenez & Caballero 1975). Most of these hosts are semi-aquatic or aquatic snakes feeding occasionally on frogs (McAllister & Bursey 2008).

As mentioned above, some of the natural hosts of *Renifer aniarum* in North America belong to *Nerodia*, a genus closely related to *Natrix*. Most of the species currently included in *Nerodia* were once members of *Natrix* until the revision of Rossman & Eberle (1977) who restricted *Natrix* to the Old World and erected *Nerodia* to include all the North American species previously included within the genus *Natrix*.

Life cycles of all members of the Reniferidae Pratt, 1902 studied so far include amphibians as second intermediate hosts (Byrd 1935, Yamaguti 1975, Prudhoe & Bray 1982, Schell 1985, V. Tkach unpubl. data). Eggs of *Renifer aniarum* containing miracidia are ingested by pulmonate snails belonging to the genus

Physa where miracidia then develop into sporocysts. Sporocysts develop into cercariae, then leave the snail and penetrate and encyst in tadpoles of the genera *Hyla*, *Lithobates* and *Pseudacris*. When tadpoles are ingested by snakes, adult flukes develop in the mouth and esophagus of definitive hosts and produce eggs in 35 d (Byrd 1935, Walker 1939).

Introduced species can alter the organization and the functioning of resident communities through various processes such as predation, parasite transfer, or competitive exclusion (Torchin et al. 2002, Cadi & Joly 2004, Taraschewski 2006, Peeler et al. 2010). Ability of an introduced parasite to adapt to native hosts varies by helminth species depending on several factors. According to Torchin et al. (2002) and Taraschewski (2006), a limiting factor for invasion by helminths in a new area is the presence of a suitable habitat itself, i.e. a susceptible host. If suitable hosts for all parasite life-cycle stages are not present, the parasite will not become established. In the case of *Renifer aniarum*, a digenean with a complex, 3-host life cycle, all 3 suitable hosts are available in the new area. In the freshwater environment of the Calabria region, snails of *Physa* spp., frogs of *Hyla* spp. and *Rana* spp., and snakes of *Natrix* spp. occur together.

In Italy, where the American bullfrog *Lithobates* (*Rana*) *catesbeianus* was introduced, the largest population occurs within the Po River Valley in the northern part of the country, where intentional introductions, mainly from Louisiana (USA) continued at least until 1937 (Albertini & Lanza 1988, Santos-Barrera et al. 2009). In Cosenza province in the 1990s, commercial farms of American bullfrogs were established in Roggiano Gravina on the Esaro River (which is an important tributary of the Crati River) and in Altilia on the Savuto River (Fig. 1). We were unable to obtain more information about the farm on the Esaro River. We

know, however, that in the spring of 1992, a total of 600 000 tadpoles of this anuran species were imported from California (USA) by a farm located on the Savuto River. A few weeks later, almost all metamorphosing tadpoles escaped from the basins of the farm to the Savuto River. A few months after this episode, the farm was closed (A. Pagliusi, Savuto River farm owner, pers. comm.). We hypothesize that *Renifer aniarum* was likely introduced into this area by the translocation of American bullfrogs, one of the natural second interme- diate hosts of reniferids in North America. Our exami- nation of the museum specimens of the *Natrix natrix* collection deposited in the HC before the introduction of bullfrogs suggests the absence of *R. aniarum*, which supports the above hypothesis regarding the source and time of the new parasite introduction. Unfortu- nately, we are not aware of any helminthological study of helminth fauna from the introduced bullfrog in Italy or elsewhere in Europe. Thus, there is a remote possi- bility that *Renifer* was introduced in Italy with earlier introductions of bullfrogs, but was undetected until our study. Therefore, the introduction of this new parasite in Italy with bullfrogs is the most likely route because no other North American frog or snake species is known in Italy's wildlife (Capula et al. 2005, Gherardi et al. 2008) and, as mentioned above, amphibians are obligatory intermediate hosts of *Renifer* and the rest of the Reniferidae.

The establishment of the *Renifer aniarum* life cycle in southern Italy may have been favored by the pres- ence of *Physa acuta* Draparnaud, 1805 (*Haitia acuta* according to some malacologists) in the region (Man- ganelli et al. 2000, Cianfanelli et al. 2007, Gherardi et al. 2008). *Physa acuta* is a globally distributed invasive snail species that was apparently introduced by humans into many countries on different continents, primarily with the aquarium trade (Paraense & Pointie 2003, Gherardi et al. 2008, Albrecht et al. 2009). Although this species was initially described from France and was thought to be a Mediterranean species introduced into North America and elsewhere (Burch 1989, Smith 1989), there is growing biological and fos- sil evidence that this species originated from North America (Dillon et al. 2002, Taylor 2003, García- Berthou et al. 2007). Thus, *R. aniarum* could have found in Italy not only closely related physid snail spe- cies that could potentially be suitable as intermediate hosts, but actually a North American species of *Physa* to which this parasite could be evolutionary adapted. This could have created an optimal condition for the establishment of a *R. aniarum* life cycle as long as they could mature in native snakes. At the same time, intro- duction of *Renifer* to the studied region with *P. acuta* seems to us unlikely because these snails were intro- duced in Italy more than 150 yr ago (Gherardi et al.

2008). Since *Natrix natrix* is a very ubiquitous snake species across Europe and its helminths have been studied repeatedly in many countries, it is highly unlikely that a *Renifer* infection would have gone undetected for such a long time.

Although the exact route of the *Renifer aniarum* introduction to Calabria may always remain somewhat questionable, the parasite has certainly found suitable intermediate and definitive hosts in the region. Although additional studies are necessary to find out whether *R. aniarum* also uses native snail and frog spe- cies as intermediate hosts, the presence of more than a single species of congeneric potentially susceptible hosts in each host category (e.g. *Physa* spp., *Hyla* spp., *Rana* spp., and *Natrix* spp.) probably favored the spread of the parasite in the Calabria region where the parasite seems to be particularly well established in the grass snake. Moreover, in both prevalence and abundance among 9 species of helminths recovered in our study, this introduced parasite was second only to the very common grass snake tapeworm *Ophiotaenia europaea* (Table 1).

Of the 17 examined museum grass snake specimens preserved before the time of alleged introduction of *Renifer aniarum*, 5 were infected with *Leptophallus nigrovenosus*, a common digenean parasite of the oral cavity of the grass snake in Europe. However, only 1 of the 15 grass snakes necropsied after the introduction of *R. aniarum* harbored *L. nigrovenosus*. Interestingly, this snake was one of only 2 individuals free of *R. aniarum*. Although more data is necessary to evaluate this possibility with any level of confidence, the observed pattern allows us to hypothesize that the invasive *R. aniarum* could be displacing a native para- site species *L. nigrovenosus*. This interesting question certainly deserves further investigation and once again highlights the importance of museum collections as a key resource of knowledge, especially at times of rapid environmental and faunal changes.

The majority of known cases of human-mediated introductions of parasitic worms with complex life cycles into new distribution areas are in domestic or game animals, and fish (Blanc 2001, Torchin et al. 2002, Taraschewski 2006). On some occasions, para- sites of amphibians were reported to have established in new distribution areas, e.g. the toad lung nematode *Rhabdias pseudosphaerocephala* that was introduced into Australia with the cane toad *Bufo marinus* (Dubey & Shine 2008). However, the latter does not require intermediate hosts. We are not aware of other exam- ples of introduction of reptilian digeneans into new regions with amphibians or other kinds of intermediate hosts. This makes the above situation with the intro- duction of *Renifer aniarum* in southern Italy quite remarkable.

Orneoascaris chrysanthemoides has been reported just from several species of amphibians and reptiles in Africa (Sprent 1985, Goldberg & Bursey 2010, McAllister et al. 2010). *Ratzia parva* (Syn. *Brachymetra parva*) has been previously reported in Africa and Europe from France, Spain and Croatia in snakes (*Natrix maura* and *Coluber hippocrepis*) and frogs (*Discoglossus pictus, Rana esculenta, R. perezi*, and *R. ridibunda*) (see Lluch et al. 1985). This latter is another species with a complex life cycle that requires 3 hosts; however, we believe that in this case, the lack of previous records of this species in Italy is rather a result of inadequate data than recent expansion of the parasite distribution area.

Due to numerous barriers that prevent establishment of life cycles, introduced animals usually leave their specific parasites behind (Torchin et al. 2002, Taraschewski 2006). This makes the above situation with the introduction of *Renifer aniarum* in southern Italy quite remarkable. Unfortunately, the parasitological aspect of the biological invasions is rather rarely studied. In most cases parasites are simply overlooked as an important element of such invasions. As already mentioned above, the list of introduced freshwater animals in Italy (Gherardi et al. 2008) includes only a single species of monogenean, which is clearly an underestimation. Our study demonstrates the importance of parasitological studies of wildlife, especially in areas of known accidental or purposeful introductions of nonnative species and highlights the value of museum collections in monitoring faunal and ecological changes.

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