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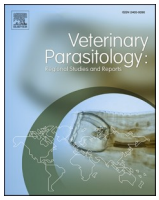

Short Communication

Molecular identification of *Contracaecum rudolphii* A and B (Nematoda: Anisakidae) from cormorants collected in a freshwater ecosystem of the pre-alpine area in Northern Italy

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ABSTRACT

Contracaecum rudolphii (s.l.) is a complex of sibling species with different genetic structure and ecological preference. This study reports the presence of specimens of *Contracaecum rudolphii* (s.l.) from sedentary and wintering cormorants (*Phalacrocorax carbo sinensis*) from the pre-mountain area of the Alps in Northern Italy, an important crossroads for most of the bird migration routes. A total of 48 specimens of cormorants collected from two adjacent freshwater habitats were analysed and *C. rudolphii* nematodes were retrieved in 100% of the examined specimens. A subsamples of 115 *C. rudolphii* individuals were genetically characterized and found to belong to the sibling species *C. rudolphii* B ($n = 90$) and *C. rudolphii* A ($n = 25$). *C. rudolphii* B were retrieved from both locations and included adults as well as larvae, while only adults of *C. rudolphii* A were detected, and in just one location. As expected for a freshwater environment, *C. rudolphii* B constitutes the largest sibling fraction, indicating that this likely is the endemic species, while cormorants originating from the breeding brackish lagoons and marine coastal environments of central and northern Europe could have brought *C. rudolphii* A from their breeding sites or migration stopovers.

Contracaecum rudolphii (s.l.) Hartwich, 1964 is a complex of five sibling species of anisakid nematodes, with an indirect life cycle in aquatic ecosystems, involving various hosts at different levels in food webs (D'Amelio, 1990; D'Amelio et al., 2012, 2007; Garbin et al., 2011; Li et al., 2005; Mattiucci et al., 2002; Shamsi et al., 2009). These multiple sibling species were recently discovered across the world, thanks to the application of molecular tools with high discriminatory power, and, in spite of their morphological similarity, they differ in genetic structure, geographical distribution, ecological niche, host preference and life cycle (D'Amelio, 1990; Mattiucci et al., 2020, 2002).

Both sibling species reported in Europe, *C. rudolphii* A and *C. rudolphii* B, have been frequently retrieved in association with their main definitive host, the continental subspecies of the great cormorant (*Phalacrocorax carbo sinensis*), from various areas of north-eastern Italy (Li et al., 2005; Mattiucci et al., 2002), central Italy (Li et al., 2005; Mattiucci et al., 2020) and across the coasts of Sardinia Island (Amor et al., 2020). Although evidence of the sympatric co-occurrence of these two sibling species had been previously reported across the distribution range of their migrating bird hosts (Amor et al., 2020; Li et al., 2005; Mattiucci et al., 2020; Szostakowska and Fagerholm, 2012), recent studies focused on their complex life cycles biology provided new insights, revealing different ecological preferences. Namely, *C. rudolphii* B was found to be capable of completing the entire life cycle in the freshwater environment, while *C. rudolphii* A is predominantly found inhabiting the marine and brackish water environment (Amor et al., 2020; Mattiucci et al., 2020; Szostakowska and Fagerholm, 2007).

In the last decades, the area of pre-alpine lakes in Lombardy (northwestern Italy) has become an important crossroads for bird migration routes, especially for wintering cormorants coming from the breeding areas of Northern Europe, resulting in the occupation of new breeding

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areas and in the establishment of sedentary colonies (Bregnballe et al., 2014; Gagliardi et al., 2009).

The aim of this study is investigate the presence of *C. rudolphii* in the great cormorant in this specific area, and determine the pattern of distribution of the sibling species using morphological and molecular tools.

From February to March 2019, a total of 48 specimens of cormorants were collected during the controlled abatement plan conducted in Lombardy region (Regional Decree No. 529 of 17/09/2018 and EEC Directive 2009/147, article 9; European commission, 2013, p. 9) from two distinct localities in the pre-mountain areas of the Alps: i) the Isella Peninsula of Lake Annone (45°48′57.8"N 9°20′30.7"E) ($n = 29$) and the adjacent area of Valmadrera – Parè on the banks of Lake Como (45°51′28.7"N 9°22′12.2"E) ($n = 19$). The entire digestive tract of each specimen was examined post-mortem for the presence of nematodes. Collected nematodes were washed in saline solution and then preserved in 70% ethanol until morphological and molecular analyses.

After preliminary morphological identification using taxonomic keys (Baruš et al., 2000, 1978; Kanarek and Bohdanowicz, 2009; Li et al., 2013; Moravec, 1994), the middle part of the nematode body was excised and used for molecular characterization. Genomic DNA was extracted individually from a representative subsample of 115 nematodes (at least 2 from each individual cormorant) using the NucleoSpin® Tissue Kit (Macherey Nagel, Duren, Germany), according to the manufacturer's instructions.

In order to obtain an accurate identification of sibling species, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was performed on the nuclear ITS ribosomal region and the mitochondrial *rrnS* gene, as previously described (D'Amelio et al., 2007; Farjallah et al., 2008).

The entire ITS region (ITS1, 5.8S rDNA gene and ITS2) was amplified using primers NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) (Zhu et al., 2000) in a volume of 20 µl, as previously described (Zhu et al., 2007). The amplification of the *rrnS* region was performed using the primers MH3 (5′-TTGTTCCAGAATAATCGGCTAGACTT-3′) and MH4.5 (5′-TCTACTTTACTACAACTTACTCC-3′), in a volume of 20 µl, following the protocol reported by D'Amelio et al., 2007.

The RFLP procedure was carried out by digesting 10 µl of ITS and *rrnS* PCR-amplified products for 90 min at 37 °C, respectively with 10 U/µl of *Mlu*CI and *Rsa*I restriction enzymes in their appropriate buffer (New England Biolabs) (Farjallah et al., 2008). RFLP patterns were identified in 2% agarose gel; and a subsample of PCR digested restriction fragments were also separated by means of Qiaxcel capillary electrophoresis (Qiagen GmbH, Hilden, Germany) using a high-resolution gel cartridge, to ascertain a perfect resolution of the multiple banding pattern (Fig. 1).

Bidirectional sequencing of 17 representative samples for each of the two different restriction patterns was performed (Eurofins Genomics, Ebersberg, Germany), in order to verify and analyze the interspecific diversity, after purification with the QIAquick® Gel Extraction Kit (Qiagen GmbH) following the manufacturer's instructions. Obtained sequences were compared with those available in the NCBI GenBank database using Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Representative consensus sequences obtained in this study were deposited in GenBank database under the accession numbers MW596000-MW596002 and MW596602-MW596604.

For each marker (ITS or *rrnS*), a genetically and geographically representative set (see below for details) of newly obtained sequences was employed for phylogenetic analyses, together with selected database sequences. Sequences of each dataset were aligned using MUSCLE software version 3.8 (Edgar, 2004). The best sequence evolution model was established using jModelTest software version 2 (Darriba et al., 2012) (GTR + I + G for both), and maximum likelihood (ML) phylogenetic trees were inferred using PhyML (Guindon and Gascuel, 2003) with 100 bootstrap pseudo-replicates.

After assignment of nematodes to each sibling species, the effect of the site of collection on the frequency of *C. rudolphii* A and *C. rudolphii* B was analysed by the chi square test using the statistical package Stats (3.6.1 Version) in R, the Chi-square function "chisq.test". A *P*-value of less than 0.05 was considered statistically significant.

The necropsy of the stomachs of cormorants spotted the presence of nematodes morphologically identified as *Contracaecum rudolphii* in 100% of examined specimens, with a high number of worms detected in each stomach.

Restriction analysis of ITS and *rrnS* amplicons performed on a subsamples of *C. rudolphii* nematodes ($n = 115$) yielded restriction profiles corresponding to *C. rudolphii* B ($n = 90$) and *C. rudolphii* A ($n = 25$) (Table 1 and Fig. 1). *C. rudolphii* A infection was significantly more common ($p < 0.005$) (25/42; 59.5%) than that of *C. rudolphii* B (17/42; 40.5%) in cormorants caught around the banks of Lake Como. In contrast, in birds from Lake Annone, only infestation with *C. rudolphii* B was detected (73/73; 100%), and clearly resulted significantly more common ($p < 0.005$). Additionally, while *C. rudolphii* B specimens included adults as well as larvae from both locations (Table 1),

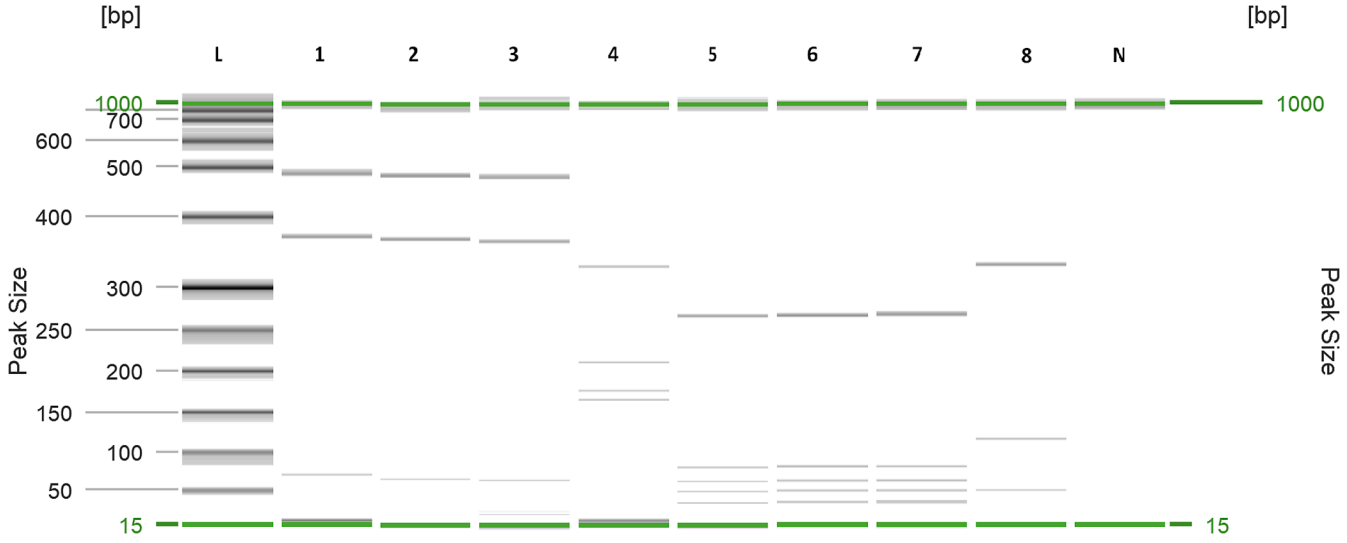

Fig. 1. Example of capillary electrophoresis analysis of PCR-RFLP ITS fragments generated by the enzyme *Mlu*CI (channels 1–4) and *rrnS* fragments generated by the enzyme *Rsa*I (channels 5–8). L: Ladder 50–800 bp; 1–3, 5–7: *Contracaecum rudolphii* B; 4 and 8: *Contracaecum rudolphii* A; 9: negative control. Alignment marker: 15–1 k bp.

Table 1
number (n) and prevalence (P%) of Adult and L4 specimens of *Contracaecum rudolphii* A and *C. rudolphii* B identified by PCR/RFLP analysis in the definitive host (*Phalacrocorax carbo sinensis*) according to the site of collection.

Sampling site	Stage	Number of specimens examined	*C. rudolphiii* A		*C. rudolphiii* B	
			n	n (P%)	n	n (P%)
Annone Lake Shore	L4	10	0	0 (0.0%)	10	73 (100%)
	Adult	63	0		63	
Como Lake shore	L4	5	0	25 (59.5%)	5	17 (40.5%)
	Adult	37	25		12	
Total		115	25 (21.3%)		90 (78.3%)	

C. rudolphii A from Lake Como were only adults.

As expected for a freshwater environment, *C. rudolphii* B constitutes the largest sibling fraction. However, while cormorants from Lake Annone were exclusively infested by *C. rudolphii* B (29/29, 100%), those from Lake Como harboured both sibling species with a higher prevalence of *C. rudolphii* A that was detected both as single infection (11/19; 57.9%) and in coexistence with *C. rudolphii* B (5/19; 26.3%).

The sequencing of a representative subsample ($n = 17$) showed two clusters with identities in the range of 99.5–99-7% for *C. rudolphii* A ($n = 3$), 99.7–100% for *C. rudolphii* B from Lake Annone ($n = 5$) and 99.5–100% for *C. rudolphii* B from Lake Como (n = 9), confirming in all cases the RFLP assignments. Phylogenetic analyses further confirmed the assignments to each sibling species and resulted consistent with previous studies (Supplementary Fig. 1a,b) (Amor et al., 2020; Garbin et al., 2011; Mattiucci et al., 2020).

The obtained results are in accordance with previous studies, both in terms of geographical and environmental distribution (Amor et al., 2020; Li et al., 2005; Mattiucci et al., 2002; Mattiucci et al., 2020). On one side, the environmental conditions, as well as the structure of fish, planktonic and benthonic communities of the two analysed lakes (ARPA, https://www.arpalombardia.it/Pages/Acque-Superficiali/Rapporti-Annuali.aspx), likely provide suitable conditions for the development of *C. rudolphii* B, typical in freshwater. Considering that cormorants are opportunistic feeders that can shift their diet in relation to fish availability (Leopold et al., 1998), they are likely to be locally adapted in this regard, providing the definitive host to complete the nematode life cycle. Moreover, the high prevalence of *C. rudolphii* B seems to indicate a high abundance in the environment, and specifically in intermediate hosts, possibly favoured by the stable availability of the resident cormorants as definitive hosts.

On the other side, finding also the typically brackish water *C. rudolphii* A in cormorants collected in the freshwater environment of Lake Como is interesting. Considering that cormorants can reflect the parasite community structure of their native region, a plausible explanation can be found in the presence of migrant individuals experiencing distinct environments and consequent different foraging behaviours during their seasonal migration cycles (Gagliardi et al., 2009). Accordingly, individuals originating from the breeding brackish lagoons and marine coastal environments of central and northern Europe could be bringing *C. rudolphii* A from their breeding sites or migration stopovers, where it is described to be predominant species (Li et al., 2005; Szostakowska and Fagerholm, 2012). All retrieved larval stages belonged to *C. rudolphii* B, giving further supports to this hypothesis. Indeed, larvae likely represent recently acquired infections, occurred in the study area, while adult nematodes represent pre-existing infections potentially acquired in previously visited areas.

In this regard, the detection of both sibling species of *C. rudolphii* (s. l.) even as double infections in single host individuals is noteworthy, and in accordance with previous reports (Amor et al., 2020; Mattiucci et al., 2020; Szostakowska and Fagerholm, 2012).

In any case, for a better understanding of the population dynamics within the *C. rudolphii* complex in this peculiar area, further in-depth studies would be necessary to address the genetic characterization of larval stages of *C. rudolphii* in the aquatic intermediate hosts and compare it with long-term investigation of the nematode presence in the definitive host.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vprsr.2021.100674.

Ethical statement

No ethical approval was required, as this study did not involve clinical trials or experimental procedures.

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Declaration of Competing Interest

The authors have nothing to disclose.

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