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# Molecular evidence for the occurrence of *Contracaecum rudolphii* A (Nematoda: Anisakidae) in shag *Phalacrocorax aristotelis* (Linnaeus) (Aves: Phalacrocoracidae) from Sardinia (western Mediterranean Sea)

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ABSTRACT

Specimens of *Contracaecum rudolphii* Hartwich, 1964 (Nematoda: Anisakidae) from *Phalacrocorax aristotelis* (Linnaeus) from the Archipelago of La Maddalena (Sardinia, western Mediterranean Sea) were characterised genetically and compared with *C. rudolphii* A *sensu* D'Amelio et al. 1990 and *C. rudolphii* B *sensu* D'Amelio et al. 1990 from *Phalacrocorax carbo sinensis* (Blumenbach) from north-eastern Italy, and with *C. rudolphii* C *sensu* D'Amelio et al. 2007 from *Phalacrocorax auritus* (Lesson) from west-central Florida, USA. The sequencing of the small subunit of the mitochondrial ribosomal RNA gene (*rrn*S) and by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis of the same gene and of the internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) allowed the identification of all specimens of *C. rudolphii* from *P. aristotelis* as *C. rudolphii* A. The results confirmed that the definition of genetic markers, following the analysis of nuclear ribosomal and mitochondrial DNA, provides quick and practical diagnostic tools for the detection of the 3 sibling species of *C. rudolphii*. The occurrence of *C. rudolphii* in *P. aristotelis* is reported for the first time from the Mediterranean area, improving the picture of the dispersal patterns of the populations of these piscivorous birds, and confirming the existence of different and isolated populations between the North and South European waters.



Anisakid nematodes of the genus *Contracaecum* Raillet et Henry, 1912 in their adult stage infect a wide range of fish-eating birds and sea mammals [1] and some of them are also transmissible to humans, where they can cause anisakidosis [2]. Avian *Contracaecum* spp. are reported in all regions of the world in a very large number of host species [1,3–5] and among these species, *Phalacrocorax* spp. (Aves: Phalacrocoracidae) are known to be frequent hosts [6]. These birds are vulnerable to large-scale mortality incidents caused by various factors, e.g. prolonged adverse weather, dinoflagellate blooms, oil spills, netting, shooting, anisakid infections [6–10]. In this last case, the knowledge of epidemiological data provides useful information for management and conservation of seabird populations. The shag *Phalacrocorax aristotelis* (Linnaeus) compared to other *Phalacrocorax* spp. has a much more restricted geographical distribution, being found only in the western Palearctic. It breeds from North Atlantic coastal areas of Iceland to Morocco, including the Norwegian coastline and the Kola Peninsula. It has also a restricted and discontinuous breeding area along the shores of the Mediterranean and Black Seas [11]. The European shag is an inshore and offshore bottom feeder, preying mainly on demersal or benthic fish species [12]. This species plays an important ecological role as regulator of populations of some fish with marked site fidelity [13]. Data on the *Contracaecum* spp. harboured by this species are available only for Iceland, the Artic Boreal and the Spanish Atlantic populations [6,14,15] and no data are available for the Mediterranean. From this area, previous multilocus enzyme electrophoretic studies were carried out on *Contracaecum rudolphii* Hartwich, 1964 harboured by the Eurasian subspecies of the great cormorant *Phalacrocorax carbo sinensis* (Blumenbach) [6,16,17]. These studies showed a marked genetic heterogeneity in several loci for specimens collected from different geographical localities. The genetic differentiation among the *Contracaecum* species was also detected in the ITS (Internal Transcribed Spacers) region (ITS1, 5.8 subunit rRNA gene and ITS2) of the nuclear ribosomal DNA, providing additional support for the hypothesis that *C. rudolphii* represents a complex of at least 2 sibling species (*C. rudolphii* A *sensu* D'Amelio et al. 1990 and *C. rudolphii* B *sensu* D'Amelio et al. 1990) and that *Contracaecum septentrionale* Kreis, 1955, a morphologically distinct congener from shag, is a valid species [15].

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The aim of the present work was to investigate for the first time the taxonomic status of *Contracaecum* sp. infecting *P. aristotelis* from Archipelago of La Maddalena (Sardinia, western Mediterranean Sea), using PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms) and sequence analysis of the ITS and of the *rrn*S (the small subunit of the mitochondrial ribosomal RNA gene).

## 1. Materials and methods

### 1.1. Hosts and parasites

A total of 88 nematodes, at larval and adult stage (M=18; F=20; L3=35; L4=15), from *P. aristotelis* (N=6) from Archipelago of La Maddalena (41°14'N, 9°26'E) (Sardinia, western Mediterranean Sea) were used in the present study. Worms, collected from the stomach at necropsy, were placed in 70% ethanol. The anterior and posterior ends of nematode specimens were removed and cleared in lactophenol for morphological studies. The remaining part of each worm was used for molecular analysis. For morphological identification, diagnostic characters for anisakids and for *Contracaecum* from birds [5] were considered (e.g., morphology of lips and interlabial tips, length of spicule and morphology of the spicule tip, and patterns of the male caudal papillae, etc.). For comparative purposes, some additional specimens belonging to *C. rudolphii* A and *C. rudolphii* B from the great cormorant *P. carbo sinensis* from north-eastern Italy, and to *C. rudolphii* C *sensu* D'Amelio et al. 2007 from the double-crested cormorant *Phalacrocorax auritus* (Lesson) from west-central Florida, USA, were identified by microscopy and by using genetic markers defined previously in the internal transcribed spacers (ITS) of nuclear ribosomal DNA [15] and in the small subunit of the mitochondrial ribosomal RNA gene (*rrn*S) [18].

### 1.2. Isolation of genomic DNA

Total DNA was extracted using the Wizard® Genomic DNA purification kit (Promega) according to the manufacturer's protocol. Body portions from individual nematodes were each placed in 600 µl of a mixture containing 0.5 M ethylene diamine tetraacetic acid (EDTA) plus Nuclei Lysis solution and then crushed employing a sterile pestle. An aliquot of 17.5 µl of proteinase K (20 mg/ml, Promega) was added to each tube, which was incubated at 55 °C for 3 h. An aliquot of 3 µl of RNase solution (4 mg/ml) was added, and the tubes were incubated at 37 °C for 30 min. Subsequently, 200 µl of protein precipitation solution were added, the tubes vortexed and chilled on ice for 5 min, and the DNA precipitated with ethanol. Each DNA pellet was air-dried for 20 min and dissolved in 100 µl of DNA rehydration solution.

### 1.3. PCR amplification

The region spanning the ITS1, the 5.8S and the ITS2 of the nuclear ribosomal DNA was amplified by PCR using 5 µl of the extracted DNA as a template (20–40 ng) and 50 µl reaction mixture containing 0.5 µl AmpliTaq Gold (AmpliTaq Gold, Applied Biosystems, USA), 5 µl of 10×PCR buffer II (Applied Biosystems), 5 µl of $MgCl_2$ (3 mM, Applied Biosystems), 4 µl of dNTPs (dCTP, dGTP, dATP, dTTP) (Promega) and 0.5 µl each primer (50 pmol/µl). The entire ITS (ITS1, 5.8S rDNA gene and ITS2) was amplified using the primers NC5 (forward: 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (reverse: 5′-TTAGTTTCTTCCTCCGCT-3′) [15] under the following conditions: 10 min at 95 °C (initial denaturation), then 30 cycles of 30 sec at 95°C (denaturation), 30 sec at 55 °C (annealing) and 75 sec at 72 °C (extension) followed by a final elongation of 7 min at 72 °C.

The amplification of the *rrn*S was performed using 5 µl of template DNA (20–40 ng), and 50 µl reaction mixture containing 0.5 µl AmpliTaq Gold (AmpliTaq Gold, Applied Biosystems, USA), 5 µl of 10×PCR buffer II (Applied Biosystems), 5 µl of $MgCl_2$ (3 mM, Applied Biosystems), 4 µl of dNTPs (dCTP, dGTP, dATP, dTTP) (Promega) and 0.5 µl each primer (50 pmol/µl); forward MH3 (5′-TTGTTCCAGAATAATCGGCTAGACTT-3′), reverse MH4.5 (5′-TCTACTTTACTACAACTTACTCC-3′) [19]. The conditions of PCR were as follows: 10 min at 95 °C (initial denaturation), 35 cycles of 30 sec at 95 °C (denaturation), 30 sec at 55 °C (annealing) and 30 sec at 72 °C (extension), and a final elongation step of 7 min at 72 °C. A negative control (no DNA) was included in all PCR amplifications.

Reactions were carried out using a Gene Amp PCR System 2400 (Applied Biosystems). Five microliters of the amplification products were visualised on 1% ethidium-bromide-stained agarose gels to check the quality of amplification.

### 1.4. PCR-RFLP analysis

The restriction enzymes, *Tsp509*I and *Rsa*I, were used in RFLP analysis of the ITS and of the *rrn*S, respectively. Restrictions were performed using 10 µl of PCR products, 3 µl of distilled water, 0.5 µl of restriction enzyme, 1.5 µl of a specific buffer and 0.2 µl bovine serum albumin up to a final volume of 15.2 µl. The digestion was performed for 90 min at 37 °C, and the digestion products were evaluated on 2% ethidiumbromide-stained agarose gels.

### 1.5. DNA sequencing and analysis

The ITS and *rrn*S, amplicons were purified using the commercial kit SureClean (Bioline), following the manufacturer's instructions. Sequencing was performed by MWG-BIOTECH AG (Ebersberg, Germany). Nucleotide sequences were aligned, with previously characterised sequences of *Contracaecum*, using the program ClustalX [20] and adjusted manually after careful checking for misalignments.

## 2. Results

All the adult nematodes recovered from *P. aristotelis* from Sardinia were morphologically identified as *C. rudolphii* (s.l.). The ITS and *rrn*S amplicons (~1000 bp and 540 bp, respectively), from individual worms of studied specimens and of specimens for comparative purposes, were amplified individually and subjected to agarose gel electrophoresis. For each genetic marker no size variation was detectable on agarose gel among any of the amplicons examined. PCR-RFLP analysis of the ITS amplicons using the endonulease *Tsp509*I produced fragments of 330, 220, 170 and 80 bp for *C. rudolphii* A and for studied specimens, compared with fragments of 480, 220, 170 and 80 bp for *C. rudolphii* C and fragments of 480, 360 and 80 bp for *C. rudolphii* B (Fig. 1). PCR-RFLP analysis of representative *rrn*S amplicons using *Rsa*I produced three different patterns, corresponding

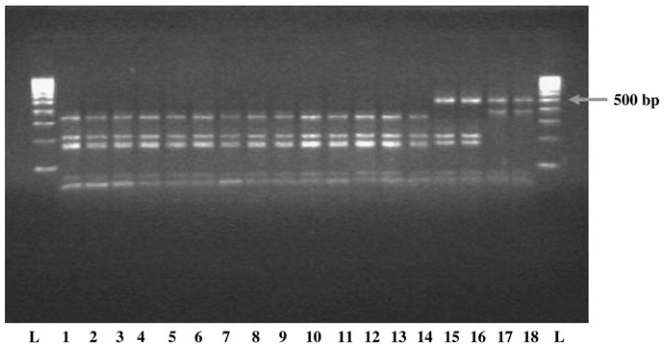


**Fig. 1.** RFLP patterns obtained by digestion of the ITS region of the rDNA (ITS-1, 5.8S gene and the ITS-2) with the restriction enzyme *Tsp509*I shown by the species *Contracaecum rudolphii* s.l. Lanes: 1-12 *C. rudolphii* A from *P. aristotelis* from Archipelago of La Maddalena; 13- 14 *C. rudolphii* A from north-eastern Italy; 15-16 *C. rudolphii* C from west-central Florida (USA); 17-18 *C. rudolphii* B from north-eastern Italy; L 100 bp DNA ladder.