

Ascaridoidea: a simple DNA assay for identification of 11 species infecting marine and freshwater fish, mammals, and fish-eating birds

Agnieszka Kijewska,^a Jerzy Rokicki,^{a,b} Jiri Sitko,^c and Grzegorz Węgrzyn^{b,d,*}

^a Department of Invertebrate Zoology, University of Gdańsk, Piłsudskiego 46, 81-378 Gdynia, Poland

^b Institute of Oceanology, Polish Academy of Sciences, Św. Wojciecha 5, 81-347 Gdynia, Poland

^c Moravská Ornitologická Stanica, Bezručova 10, 751 52 Přerov, Czech Republic

^d Department of Molecular Biology, University of Gdańsk, Kładki 24, 80-822 Gdańsk, Poland

Received 16 November 2001; accepted 4 June 2002

Abstract

Eleven species belonging to superfamily Ascaridoidea, which infect marine and freshwater fish, mammals, and fish-eating birds, were analyzed using a PCR-RFLP method. The following species were investigated: *Anisakis pegreffi*, *A. physeteris*, and *A. simplex* (parasites of fish and mammals), *Contracaecum osculatum*, *C. radiatum*, and *C. rudolphi* (parasites of mammals and fish-eating birds), *Hysterothylacium aduncum* (a parasite of fish), *Porrocaecum angusticolle*, *P. crassum*, *P. depressum*, and *P. ensicaudatum* (parasites of fish-eating birds). PCR-amplified rDNA regions encompassing ITS1, 5.8S rDNA, and ITS2 produced on templates of genomic DNA isolated from all investigated species were digested with *TaqI*, *AluI*, *BsuRI*, and *RsaI* endonucleases. Restriction patterns showed that endonuclease *TaqI* is the most useful enzyme for identification of all investigated species. No variations in restriction patterns within each species were detected. Therefore, we propose that the PCR-RFLP assay described in this report may be used for identification of marine and freshwater parasites from superfamily Ascaridoidea.

Index Descriptors and Abbreviations: Nematoda; Secernantea; Ascaridoidea; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; rDNA, ribosomal DNA; ITS, internal transcribed spacer. © 2002 Elsevier Science (USA). All rights reserved.

1. Introduction

Many parasitic helminths are of major socio-economic importance due to the diseases they cause in humans and animals (Iglesias et al., 1996; Ishikura et al., 1995; van Thiel, 1962). Endoparasites from the superfamily Ascaridoidea (Nematoda: Secernantea) include species that are of veterinary, medical, and economic importance. Some of them are parasites of hosts living in marine and freshwater environments, including fish, mammals or fish-eating birds. Occasionally, they may also infect humans (van Thiel, 1962). Adults of most of these parasites live in the alimentary tract of a specific vertebrate host and many taxa have a life cycle involving

development of larval stages in one or more intermediate hosts (Anderson, 1992).

Accurate identification of a parasite at any stage of its development has important implications for studying parasite epidemiology and resolving taxonomic problems. However, proper identification of parasites is often problematic as adults, and especially larvae, of both freshwater and marine species of Ascaridoidea are similar to each other. Serologic methods for identification of some of these species have been proposed, but such tests are often confounded by cross-reaction with antigens from other worms (Akao et al., 1990; Iglesias et al., 1996; Rodero et al., 2001 and references therein). Protein and DNA technologies have provided strategies for identification of species when morphological features are of limited value. Recent taxonomic studies of endoparasitic helminths, making extensive use of

* Corresponding author. Fax: +48-58-301-0072.

E-mail address: wegrzyn@biotech.univ.gda.pl (G. Węgrzyn).

genetic markers, have shown that in the superfamily Ascaridoidea various taxonomic groups of morphospecies often include a number of morphologically similar, but genetically and ecologically different species (Arduino et al., 1995; Gasser and Monti, 1997; Nadler, 1992; Nadler et al., 2000; Nascetti et al., 1986). Such species, even when sympatric, are reproductively isolated in the field. For example, genetic studies performed by Mattitucci et al. (1997, 2001), Nadler et al. (2000), and Zhu et al. (2000) indicated that *Anisakis simplex* and *Contracaecum osculatum* are two complexes of sibling species.

There are several reports indicating successful attempts to identify members of the superfamily Ascaridoidea by genetic means. It was shown that restriction fragment length polymorphism (RFLP) analysis of 25S rDNA could aid in identifying *A. physeteris* (Sugane et al., 1989). Ribosomal DNA (rDNA) can be a useful genetic marker, as it is generally considered that rDNA maintains sequence homogeneity within a species (Gasser and Monti, 1997). Recently, it was proposed that RFLP analysis of the ITS-1–5.8S–ITS-2 fragment of rDNA might be used to distinguish *A. simplex* from *Hysterothylacium aduncum* and *C. osculatum* (Zhu et al., 1998), as well as to identify members of the genus *Anisakis* (D'Amelio et al., 2000). Moreover, samples of *A. simplex* collected from different geographical regions (Baltic Sea, Norwegian Sea, Bering Sea, and Sea of Okhotsk) revealed the same restriction patterns, indicating intraspecies homogeneity (Kijewska et al., 2000).

Contrary to isoenzymatic studies, DNA analysis does not require fresh material and tissues can be fixed in many kinds of media (e.g., ethanol, formaldehyde). DNA-based methods require only a small piece of biological material, thus, allowing parallel identification of the same organism by classical methods. Moreover, it was reported previously that a PCR-RFLP assay for detection of *A. simplex* is highly sensitive because it was possible to detect specific PCR products, even when the ratio of parasite tissues to host-derived material used to isolate DNA was as low as 1:200 (Kijewska et al., 2000). Thus, even a small part of a worm isolated from an animal or human body together with a relatively large piece of the host tissue should still be sufficient for detection and identification of the parasite. The previous successes (described above) in identification of ascaridoid species by PCR-RFLP prompted us to develop a molecular assay for identification of parasites belonging to four ascaridoid genera, which infect marine and freshwater fish, mammals, and fish-eating birds. We found that all investigated species, i.e., *A. pegreffi*, *A. physeteris*, and *A. simplex* (parasites of fish and mammals), *C. osculatum*, *C. radiatum*, and *C. rudolphi* (parasites of mammals and fish-eating birds), *H. aduncum* (a parasite of fish), *Porrocaecum angusticolle*, *P. crassum*, *P. depressum*, and *P. ensicaudatum* (para-

sites of fish-eating birds) may be unambiguously identified using a simple PCR-RFLP assay.

2. Materials and methods

2.1. Parasites

Worms were taken from definitive or intermediate hosts from different geographic regions (Table 1). Morphological characters used to distinguish adult worms were: (i) structures of labiae and ventriculus, (ii) the shape of a tail, (iii) shape of lobes (iv) interlabia/lip length ratio, (v) patterns of caudal papillae, and (vi) length and shape of spicules in males (Baruš et al., 1978; Fagerholm, 1988; Hartwich, 1974). In the case of larvae, the body length and width, the ratio of length and width of stomach, and absence or presence of mucron were also considered (Moravec, 1994). Material was fixed in the following media: 70% ethanol, 70% ethanol with 5% glycerol, 70% ethanol with 1% benzalconium, glycerol, and 4% formaldehyde (in this case, the cuticle was removed and parenchymatic tissues were used only).

2.2. DNA isolation and amplification

Samples fixed in ethanol with small amounts of other fixatives were washed a few times in 50% ethanol and/or in 1% SDS. Genomic DNA from worms (40 mg adult body or, alternatively, one larva per sample) was then isolated by an SDS/proteinase K procedure as described previously (Sambrook et al., 1989). DNA samples were stored in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) at -70°C . For amplification of the rDNA region containing ITS-1, 5.8S rRNA gene, ITS-2, and ~ 70 bp of 28S rRNA gene, the previously described (Zhu et al., 1998) primers NC5 (5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3') and NC2 (5'-TTA GTT TCT TTT CCT CCG CT-3') were used. Each reaction mixture (total volume of 50 μl) contained 1 μl solution of genomic DNA isolated as described above, 1 U DyNAzyme II DNA Polymerase (Finnzymes), dNTPs (250 μM each), and 200 pmol of each primer. The following program was employed: one cycle of denaturation at 94°C for 5 min, annealing at 60°C for 30 s, and extension at 72°C for 90 s, then 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s, and then the final cycle of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 5 min. PCR products were separated electrophoretically on 1% agarose gels and visualized by staining with ethidium bromide. If necessary, to obtain higher amounts of PCR products and to remove saccharides that inhibit amplification, PCR products were passed through a low-melting agarose gel (BioWhittaker) or recovered by using Qiagen

Table 1

Parasites from genera *Anisakis*, *Contracaecum*, *Hysterothylacium*, and *Porrocaecum* isolated from hosts living in different geographic regions

Parasite ^a	Code	Host	Origin
<i>A. pegreffi</i>	Ape	<i>Theragra chalcogramma</i>	S-W Atlantic
<i>A. physeteris</i>	Aph	<i>Physeter catodon</i>	Azores
<i>A. simplex</i>	Asi	<i>Theragra chalcogramma</i>	S-W Atlantic
		<i>Micromesistius potassou</i>	Sea of Okhotsk
		<i>Pleurogrammus monopterigus</i>	Bering Sea
		<i>Scomber scombrus</i>	Norwegian Sea
<i>C. osculatum</i>	Cos	<i>Halioherchus grypus</i>	Baltic Sea
		<i>Phocoena phocoena</i>	Baltic Sea
<i>C. radiatum</i>	Cra	<i>Laptonychotes weddelli</i>	South Shetlands
<i>C. rudolphi</i>	Cru	<i>Phalacrocorax carbo</i>	Poland
		<i>Phalacrocorax carbo</i>	Italy
		<i>Phalacrocorax carbo</i>	Czech Republic
<i>H. aduncum</i>	Had	<i>Zoarces viviparus</i>	Baltic Sea
		<i>Platichthys flesus</i>	Baltic Sea
<i>P. angusticolle</i>	Pan	<i>Buteo buteo</i>	Czech Republic
		<i>Circus aeruginosus</i>	Czech Republic
<i>P. crassum</i>	Pcr	<i>Aythya ferina</i>	Czech Republic
<i>P. depressum</i>	Pde	<i>Buteo buteo</i>	Czech Republic
		<i>Strix aluco</i>	Czech Republic
<i>P. ensicaudatum</i>	Pen	<i>Turdus merula</i>	Czech Republic
		<i>Sturnus vulgaris</i>	Czech Republic

^a Three to 10 worms from each species were investigated.

columns and re-amplified (see BioWhittaker and Qiagen protocols).

2.3. Restriction fragment length polymorphism (RFLP) analysis

The amplified DNA fragments were digested with *TaqI*, *AluI*, *BsuRI*, and *RsaI* restriction enzymes and the products were separated electrophoretically on 4% NuSieve agarose (BioWhittaker) gels and visualized by staining with ethidium bromide (Sambrook et al., 1989). The ϕ X174 DNA digested with *BsuRI* (*HaeIII*) was used as a DNA marker (Marker 9, Fermentas).

3. Results and discussion

Total DNA was isolated from worms belonging to 11 species of parasitic Ascaridoidea, which infect marine and freshwater fish, mammals, and fish-eating birds, namely: *A. pegreffi*, *A. physeteris*, *A. simplex*, *C. osculatum*, *C. radiatum*, *C. rudolphi*, *H. aduncum*, *P. angusticolle*, *P. crassum*, *P. depressum*, and *P. ensicaudatum*. These parasites were taken from different hosts found in various geographic regions (Table 1). A chromosomal DNA region encompassing ITS-1, 5.8S rDNA, and ITS-2 was amplified using PCR with previously described (Zhu et al., 1998) primers. The lengths of the PCR products in all samples ranged from ~930 to ~1150 bp (Fig. 1, Table 2). For each species, the length of the specific PCR product was the same for all samples (data not shown). In control experiments, tissue samples from herrings and cormo-

MW Asi Ape Aph Cos Cru Cra Had Pen Pcr Pan Pde MW

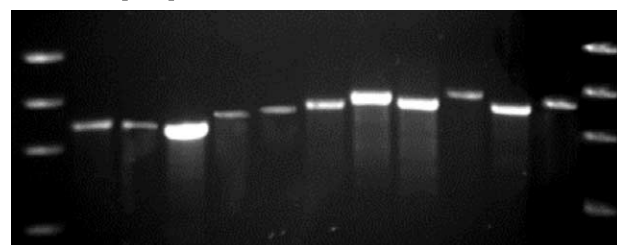


Fig. 1. Products of PCR amplification of the rDNA fragment encompassing the ITS-1–5.8S–ITS-2 region from different species of Ascaridoidea. Agarose gel electrophoresis was performed and the gel was stained with ethidium bromide (0.5 μ g/ml) and photographed over a transilluminator (312 nm wavelength). Abbreviations of samples correspond to codes presented in Table 1. MW—molecular weight standards (from the top: 1353, 1078, 872, and 603 bp).

Table 2

Sizes of PCR-amplified DNA fragments encompassing the ITS-1–5.8S–ITS-2 region from different parasites

Parasite	PCR product size (bp)
<i>Anisakis pegreffi</i>	~960
<i>Anisakis physeteris</i>	~930
<i>Anisakis simplex</i>	~960
<i>Contracaecum osculatum</i>	~1000
<i>Contracaecum radiatum</i>	~1050
<i>Contracaecum rudolphi</i>	~1020
<i>Hysterothylacium aduncum</i>	~1100
<i>Porrocaecum angusticolle</i>	~1030
<i>Porrocaecum crassum</i>	~1150
<i>Porrocaecum depressum</i>	~1050
<i>Porrocaecum ensicaudatum</i>	~1050

MW Ape Aph Asi Had MW MW Cra Cos Cru MW MW Pen Pan Pde Pcr MW

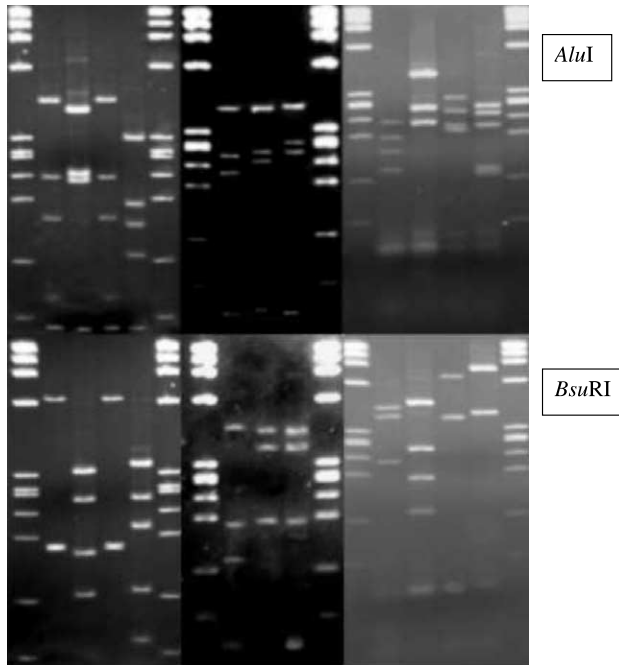


Fig. 2. Restriction fragment length polymorphism of the PCR-amplified DNA fragments encompassing the ITS-1–5.8S–ITS-2 region from different species of Ascaridoidea. The PCR products (see Fig. 1) were digested with *AluI* or *BsuRI* (upper and lower panels, respectively, as indicated near corresponding electrophoregrams) and separated by agarose gel electrophoresis. The gels were stained with ethidium bromide (0.5 µg/ml) and photographed over a transilluminator (312 nm wavelength). Abbreviations of samples correspond to codes presented in Table 1. MW—molecular weight standards (from the top: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp).

rants were used with negative results, i.e., no PCR amplification products were observed (data not shown).

Amplified DNA fragments were digested with *AluI*, *BsuRI*, *RsaI*, and *TaqI* restriction endonucleases and separated electrophoretically in 4% agarose gels (Figs. 2 and 3). The results of the experiments indicate that in most cases characteristic restriction patterns may be ascribed to particular species. The most useful enzyme

MW Ape Asi Aph Had MW MW Cos Cra Cru MW MW Pen Pan Pde Pcr MW

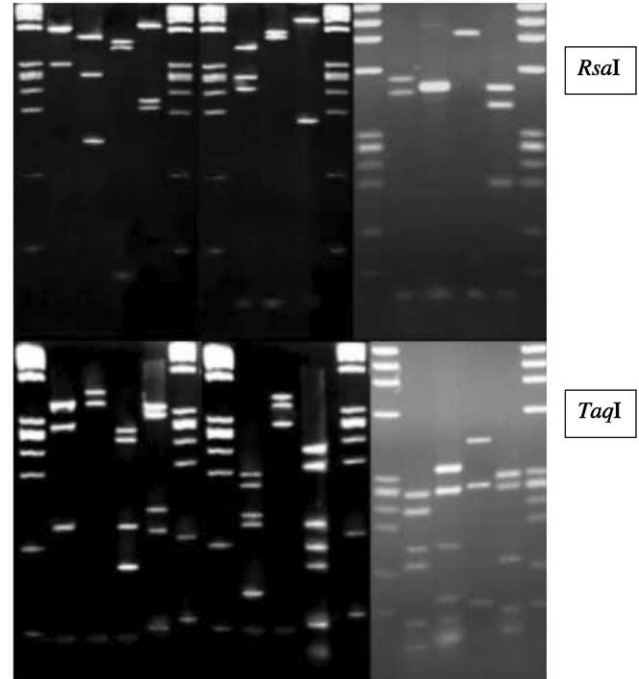


Fig. 3. Restriction fragment length polymorphism of the PCR-amplified DNA fragments encompassing the ITS-1–5.8S–ITS-2 region from different species of Ascaridoidea. The PCR products (see Fig. 1) were digested with *RsaI* or *TaqI* (upper and lower panels, respectively, as indicated near corresponding electrophoregrams) and separated by agarose gel electrophoresis. The gels were stained with ethidium bromide (0.5 µg/ml) and photographed over a transilluminator (312 nm wavelength). Abbreviations of samples correspond to codes presented in Table 1. MW—molecular weight standards (from the top: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp).

for identification of species of marine and freshwater Ascaridoidea appears to be *TaqI*, as after digestion with this endonuclease of the PCR-amplified ITS-1–5.8S–ITS-2 DNA fragment, restriction profiles unique for each species were observed (Table 3). Again, in the case of each species, the restriction patterns were the same for all samples (data not shown).

Table 3

Restriction fragment length polymorphism of the PCR-amplified DNA fragments encompassing the ITS-1 - 5.8S - ITS-2 region from different parasites belonging to genera *Anisakis*, *Contracaecum*, *Hysterothylacium*, and *Porrocaecum*

Parasite	Restriction pattern (approximate lengths of major DNA fragments in bp)			
	<i>AluI</i>	<i>BsuRI</i>	<i>RsaI</i>	<i>TaqI</i>
<i>A. pegreffi</i>	440, 230, 160, 80, 70	630, 180	590, 310	390, 290, 130, 70
<i>A. physeteris</i>	390, 240, 220, 70	320, 260, 170, 120	510, 450, 60	280, 260, 130, 90, 70
<i>A. simplex</i>	440, 230, 160, 80, 70	630, 180	540, 280, 150	440, 400, 70
<i>C. osculatum</i>	420, 270, 240, 60	440, 360, 190	450, 270, 240, 50	190, 180, 140, 130, 80, 70
<i>C. radiatum</i>	420, 250, 220, 60	470, 180, 130	550, 490, 50	370, 340, 290, 70
<i>C. rudolphi</i>	430, 280, 260, 60	440, 360, 190, 60	850, 180, 50	220, 190, 130, 110, 100, 70
<i>H. aduncum</i>	310, 190, 150, 120, 70	340, 260, 210, 120, 80	700, 220, 210	320, 300, 150, 130, 70
<i>P. angusticolle</i>	400, 270, 230, 60	450, 260, 190, 120, 60	510, 500, 60	340, 280, 160, 80, 60
<i>P. crassum</i>	270, 240, 210, 120, 60	770, 370, 60	500, 420, 190	310, 280, 120, 70, 60
<i>P. depressum</i>	300, 250, 210, 200, 60	690, 360, 60	930, 60	420, 280, 70
<i>P. ensicaudatum</i>	230, 190, 160, 130, 60	420, 370, 220, 60	550, 480, 60	270, 230, 150, 120, 70

In conclusion, parasites from the superfamily Ascaridoidea can be easily distinguished using PCR amplification of the ITS-1–5.8S–ITS-2 DNA fragment, its digestion with *TaqI* restriction endonuclease, and separation of the reaction products by agarose gel electrophoresis. For such an analysis, only a small part of the worm body is required. Moreover, using this assay it is possible to identify both larvae and adult parasites. Previous isoenzymatic (Mattitucci et al., 1997; Nascetti et al., 1986) and DNA (Kijewska et al., 2000) studies indicated that there is a remarkable genetic homogeneity within *A. simplex* from different geographical regions. Results presented in this report suggest that this may be the case also in other species of marine and freshwater parasites from superfamily Ascaridoidea. Since no intraspecies variations in the restriction patterns of the PCR-amplified DNA fragment were found in any of the investigated parasites, it seems that the use of the assay described in this report may be generally useful rather than restricted to local parasite populations.

Acknowledgments

We thank Dr. Bahram S. Dezfali for providing samples of parasites. We are grateful to Dr. Hanna Szpilewska for discussions. This work was supported by the Polish State Committee for Scientific Research (Project No. 6 P04B 026 16). G.W. acknowledges financial support from the Foundation for Polish Science (subsidy 14/2000).

References

- Akao, N., Ohyama, T.A., Kondo, K., 1990. Immunoblot analysis of serum IgG, IgA and IgE responses against larval excretory–secretory antigens of *Anisakis simplex* in patients with gastric anisakiasis. *Journal of Helminthology* 64, 310–318.
- Anderson, R.C., 1992. *Nematode Parasites of Vertebrates: their Development and Transmission*. C.A.B. International and Cambridge University Press, Cambridge.
- Arduino, P., Nascetti, G., Cianchi, R., Plötz, J., D'Amelio, S., Paggi, L., Orecchia, P., Bullini, L., 1995. Isozyme variation and taxonomic rank of *Contracaecum radiatum* (v. Linstow, 1907) from the Antarctic Ocean (Nematoda, Ascaridoidea). *Systematic Parasitology* 30, 1–9.
- Baruš, V., Sergeeva, T.P., Sonin, M.D., Ryzhikov, K.M., 1978. Helminths of fish-eating birds of the Palearctic region. In: Ryšavý, B., Ryzhikov, K.M. (Eds.), *Nematoda*. Publishing House of the Czechoslovak Academy of Sciences, Prague, p. 320.
- D'Amelio, S., Mathiopoulos, K.D., Santos, C.P., Pugachev, O.N., Webb, S.C., Picanco, M., Paggi, L., 2000. Genetic markers in ribosomal DNA for the identification of members of the genus *Anisakis* (Nematoda: Ascaridoidea) defined by polymerase chain reaction-based restriction fragment length polymorphism. *International Journal for Parasitology* 30, 223–226.
- Fagerholm, H.P., 1988. Patterns of caudal papillae in *Contracaecum osculatum* (Nematoda) and some related species from different regions of the world. *International Journal for Parasitology* 8, 1039–1051.
- Gasser, R.B., Monti, R.J., 1997. Identification of parasitic nematodes by PCR-SSCP of ITS-2 rDNA. *Molecular and Cellular Probes* 11, 201–209.
- Hartwich, G., 1974. Keys to genera of the Ascaridoidea. In: Anderson, R.C., Chabaud, A.G., Wilmott, S. (Eds.), *CIH Keys to the Nematode Parasites of Vertebrates*. Farnham Royal, Commonwealth Agricultural Bureau, Richmond, p. 153.
- Iglesias, R., Leiro, J., Ubeira, F.M., Santamarina, M.T., Navarette, I., Sanmartin, M.L., 1996. Antigenic cross-reactivity in mice between third-stage larvae of *Anisakis simplex* and other nematodes. *Parasitology Research* 82, 378–381.
- Ishikura, H., Takahashi, S., Sato, N., Kon, S., Ohtani, S., Ishikura, H., Yagi, K., Yamamoto, N., Ito, T., Sato, T., Mizuma, K., Tokogawa, O., Joong-Moon, C., Minase, T., Kikuchi, K., 1995. Alteration of parasite–host relationship in anisakiasis. *Sapporo Medical Journal* 64, 239–252.
- Kijewska, A., Słomińska, M., Węgrzyn, G., Rokicki, J., 2000. A PCR-RFLP assay for identification of *Anisakis simplex* from different geographical regions. *Molecular and Cellular Probes* 14, 349–354.
- Mattitucci, S., Nascetti, G., Cianchi, R., Paggi, L., Arduino, P., Margolis, L., Bratley, J., Webb, S., D'Amelio, S., Orecchia, P., Bullini, L., 1997. Genetic and ecological data on the *Anisakis simplex* complex with evidence for a new species (Nematoda, Ascaridoidea, Anisakidae). *Journal of Parasitology* 83, 401–416.
- Mattitucci, S., Paggi, L., Nascetti, G., Abollo, E., Webb, S.C., Pascual, S., Cinachi, R., Bullini, L., 2001. Genetic divergence and reproductive isolation between *Anisakis brevispiculata* and *Anisakis physeteris* (Nematoda: Anisakidae). *International Journal for Parasitology* 31, 9–14.
- Moravec, F., 1994. *Parasitic Nematodes of Freshwater Fish of Europe*. Kluwer Academic Publishers and Publishing House of the Academy of Sciences of the Czech Republic, Dordrecht, Prague, p. 473.
- Nadler, S.A., 1992. Phylogeny of some ascaridoid nematodes, inferred by comparison of 18S and 28S rDNA sequences. *Molecular Biology and Evolution* 9, 932–944.
- Nadler, S.A., D'Amelio, S., Fagerholm, H.-P., Berland, B., Paggi, L., 2000. Phylogenetic relationships among species of *Contracaecum* Railliet & Henry, 1912 and *Phocascaris* Host, 1932 (Nematoda: Ascaridoidea) based on nuclear rDNA sequence data. *Parasitology* 121, 455–463.
- Nascetti, G., Paggi, L., Orecchia, P., Smith, J.W., Mattitucci, S., Bullini, L., 1986. Electrophoretic studies on the *Anisakis simplex* complex (Ascaridida. Anisakidae) from the Mediterranean Sea and North-East Atlantic. *International Journal for Parasitology* 6, 633–640.
- Rodero, M., Jimenez, A., Chivato, T., Laguna, R., Cuellar, C., 2001. Purification of *Anisakis simplex* antigen by affinity chromatography. *Parasitology Research* 87, 736–740.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sugane, K., Quing, L., Matsuura, T., 1989. Restriction fragment length polymorphisms of Anisakinae larvae. *Journal of Helminthology* 63, 269–274.
- van Thiel, P.H., 1962. Anisakiasis. *Parasitology* 52, 16–17.
- Zhu, X., Gasser, R.B., Podolska, M., Chilton, N.B., 1998. Characterisation of anisakid nematodes with zoonotic potential by nuclear ribosomal DNA sequences. *International Journal for Parasitology* 28, 1911–1921.
- Zhu, X., D'Amelio, S., Paggi, L., 2000. Assessing sequence variation in the internal transcribed spacers of ribosomal DNA within and among members of the *Contracaecum osculatum* complex (Nematoda: Ascaridoidea: Anisakidae). *Parasitology Research* 86, 677–683.