



## Genetic markers in the study of *Anisakis typica* (Diesing, 1860): larval identification and genetic relationships with other species of *Anisakis* Dujardin, 1845 (Nematoda: Anisakidae)

S. Mattiucci<sup>1</sup>, L. Paggi<sup>1</sup>, G. Nascetti<sup>2</sup>, C. Portes Santos<sup>3</sup>, G. Costa<sup>4</sup>, A.P. Di Benedetto<sup>5</sup>, R. Ramos<sup>5</sup>, M. Argyrou<sup>6</sup>, R. Cianchi<sup>7</sup> & L. Bullini<sup>7</sup>

<sup>1</sup> Department of Public Health Science – Section of Parasitology, University of Rome ‘La Sapienza’, P. le Aldo Moro, 5, I-00185 Rome, Italy

<sup>2</sup> Department of Environmental Sciences, Tuscia University, Largo dell’Università, Blocco D, I-01100 Viterbo, Italy

<sup>3</sup> University of Santa Ursula, Institute of Biological and Environmental Sciences, R. Fernando Ferrari, 75-P.IV Rio de Janeiro, RJ, 22.231-040, Brazil

<sup>4</sup> Department of Biology, University of Madeira, L.go do Colegio, 9000, Funchal, Portugal

<sup>5</sup> Universidade Estadual do Norte Fluminense, Av Alberto Lamego 2000, Campos, Rio de Janeiro, 28015-620, Brazil

<sup>6</sup> Marine Biology and Ecology Section, Department of Fisheries and Marine Research, 13 Aeolou Street, 1416 Nicosia, Cyprus

<sup>7</sup> Department of Genetics and Molecular Biology, University of Rome ‘La Sapienza’, Via dei Sardi, 70, I-00185 Rome, Italy

Accepted for publication 31st May, 2001

### Abstract

Genetic variation at 21 gene-enzyme systems was studied in a sample of an adult population of *Anisakis typica* (Diesing, 1860) recovered in the dolphin *Sotalia fluviatilis* from the Atlantic coast of Brazil. The characteristic alleles, detected in this population, made it possible to identify as *A. typica*, *Anisakis* larvae with a Type I morphology (*sensu* Berland, 1961) from various fishes: *Thunnus thynnus* and *Auxis thazard* from Brazil waters, *Trachurus picturatus* and *Scomber japonicus* from Madeiran waters, *Scomberomorus commerson*, *Euthynnus affinis*, *Sarda orientalis* and *Coryphaena hippurus* from the Somali coast of the Indian Ocean, and *Merluccius merluccius* from the Eastern Mediterranean. Characteristic allozymes are given for the identification, at any life-stage and in both sexes, of *A. typica* and the other *Anisakis* species so far studied genetically. The distribution of *A. typica* in warmer temperate and tropical waters is confirmed; the definitive hosts so far identified for this species belong to delphinids, phocoenids and pontoporidae. The present findings represent the first established records of intermediate/paratenic hosts of *A. typica* and extend its range to Somali waters of the Indian Ocean and to the Eastern Mediterranean Sea. A remarkable genetic homogeneity was observed in larval and adult samples of *A. typica* despite their different geographical origin; interpopulation genetic distances were low, ranging from  $D_{Nei} = 0.004$  (Eastern Mediterranean *versus* Somali) to  $D_{Nei} = 0.010$  (Brazilian *versus* Somali). Accordingly, indirect estimates of gene flow gave a rather high average value of  $Nm = 6.00$ . Genetic divergence of *A. typica* was, on average,  $D_{Nei} = 1.12$  from the members of the *A. simplex* complex (*A. simplex s.s.*, *A. pegreffii*, *A. simplex C*) and  $D_{Nei} = 1.41$  from *A. ziphidarum*, which all share Type I larvae; higher values were found from both *A. physeteris* ( $D_{Nei} = 2.77$ )

and *A. brevispiculata* ( $D_{Nei} = 2.52$ ), which have Type II larvae (*sensu* Berland, 1961). Genetic relationships among these species are shown using multidimensional scaling ordination (MDS). The genus *Anisakis* appears to be phylogenetically heterogeneous and includes two distinct groups of species, which are morphologically and genetically differentiated.

## Introduction

*Anisakis typica* (Diesing, 1860) was described from the common dolphin *Delphinus delphis* in the Central Atlantic Ocean. Davey (1971) considered this taxon as one of three valid species of the genus *Anisakis* Dujardin, 1845, together with *A. simplex* (Rud., 1809, det Krabbe, 1878) and *A. physeteris* Baylis, 1923. According to Davey, *A. tursiopsis* (Crusz, 1946), recovered from *Delphinus delphis* off Sri Lanka, is a synonym of *A. typica*.

*A. typica* has been so far reported only at the adult stage, as a parasite of various dolphin species from warmer temperate and tropical waters, belonging to the families Delphinidae, Phocoenidae and Pontoporiidae (Stiles & Hassall, 1899; Shipley, 1905; Baylis, 1929; Kagei, 1967, 1976; Davey, 1971; Forrester & Robertson, 1975; Smith & Wootten, 1978; Praderi, 1984, 1985; Conti & Frohlich, 1984; Raga & Balbuena, 1987; Santos et al., 1996; Andreade et al., 1997; Zerbini & Santos, 1997; Mignucci-Giannone et al., 1998). Larval stages of *A. typica* have not previously been identified. Bagrov (1982) attempted to link *Anisakis* larvae referred to as Type IA, which were collected from the fish *Gempylus serpens* in Philippine waters, with *A. typica*; however, no evidence was presented for such an identification.

In the present study, an adult population of *A. typica* recovered from the dolphin *Sotalia fluviatilis* off the Atlantic coast of Brazil was analysed for 21 gene-enzyme systems. A number of allozymes, found to be characteristic of *A. typica*, allowed us to identify larval samples of this species, collected from various fish hosts in different geographical areas. The aim of this paper is: (i) to provide diagnostic allozyme keys for the identification, at any life-history stage, of *A. typica*, but also of other *Anisakis* species which have been studied genetically; (ii) to compare the genetic variation of *A. typica* in adult and larval samples from different geographical areas and from different hosts; (iii) to investigate the genetic relationships between *A. typica* and the other *Anisakis* species considered; and (iv) to give a provisional picture of the life-cycle and host preference of *A. typica*.

## Materials and methods

The collection data for *A. typica* adults and *Anisakis* spp. L4 and L3 larvae Type I (*sensu* Berland, 1961) are given in Table 1. Horizontal starch gel electrophoresis was performed on homogenates obtained from single specimens following the same procedures as reported in previous papers (Nascetti et al., 1986; Mattiucci et al., 1997, 2001). Eighteen enzymes, putatively encoded by 21 loci (*Iddh*, *Mdh-1*, *Icdh*, *6Pgdh*, *Gapdh*, *NADHdh*, *Sod-1*, *Sod-2*, *Np*, *Aat-2*, *Adk-2*, *cEst-1*, *fEst-2*, *Lap-1*, *Lap-2*, *PepB*, *PepC-1*, *PepC-2*, *Mpi*, *Gpi*, *Pgm-2*), were routinely tested on all the specimens. Buffer systems and staining procedures used followed Poulik (1957), Selander et al. (1974), Brewer & Sing (1970), Harris (1966) and Richardson et al. (1989), and are detailed in a recent paper (Mattiucci et al., 1997).

Departures from the Hardy-Weinberg equilibrium were estimated by chi-square test ( $\chi^2$ ), with Fisher's correction for small samples. Genetic divergence among populations and species was estimated by Nei's standard genetic distance,  $D_{Nei}$  (Nei, 1972), Rogers genetic distance,  $Dt$ , (1972, modified by Wright, 1978) and Cavalli Sforza & Edwards' (1967) chord distance,  $Dc$ . Genetic relationships among populations and species was given by multidimensional scaling ordination (MDS) carried out using the method of Guttman (1964) from  $Dc$  values. The genetic diversity among populations was estimated with  $Fst$  (Wright, 1978). Indirect estimates of gene flow ( $Nm$ , where  $m$  is the fraction of migrant individuals in a population of size  $N$ ) were obtained from the values of  $Fst$ , with the formula proposed by Wright (1943, 1951). The following parameters of genetic variability were estimated: proportion of polymorphic loci, by the 0.99 ( $P_{99}$ ) and 0.95 ( $P_{95}$ ) criteria; mean number of alleles per locus ( $A$ ); and expected mean heterozygosity per locus ( $He$ ). Data analysis was carried out using BIOSYS-1 (Swofford & Selander, 1981) and SYSTAT (Wilkinson & Leland, 1989) software packages.

Table 1. Collecting data of *Anisakis typica* adults and *Anisakis* spp. larvae L4 and L3 Type I (*sensu* Berland, 1961) analysed genetically.

Parasite	Life-history stage	<i>N<sub>p</sub></i>	Host	<i>N<sub>h</sub></i>	Collecting site	Date of collection
<i>A. typica</i>	Adult	2	<i>Sotalia fluviatilis</i> (Delphinidae)	1	Atlantic coast of Brazil, 21°37'S, 41°02'W	1998
<i>A. typica</i>	Adult	48	<i>Sotalia fluviatilis</i> (Delphinidae)	1	Atlantic coast of Brazil, 22°55'S, 40°18'W	1999
<i>Anisakis</i> sp.	L4	20	<i>Stenella coeruleoalba</i> (Delphinidae)	1	Eastern Mediterranean Sea, near Cyprus	2000
<i>Anisakis</i> Type I	L3	30	<i>Axixis thazard</i> (Scombridae)	3	Atlantic coast of Brazil, 22°55'S, 40°18'W	1999
<i>Anisakis</i> Type I	L3	9	<i>Thunnus thynnus</i> (Scombridae)	1	Atlantic coast of Brazil, 22°55'S, 40°18'W	1999
<i>Anisakis</i> Type I	L3	23	<i>Scomber japonicus</i> (Scombridae)	10	Atlantic Ocean, off Madeira	2000
<i>Anisakis</i> Type I	L3	23	<i>Trachurus picturatus</i> (Carangidae)	10	Atlantic Ocean, off Madeira	2000
<i>Anisakis</i> Type I	L3	10	<i>Euthynnus affinis</i> (Scombridae)	2	Indian Ocean, Somali coast	1984
<i>Anisakis</i> Type I	L3	10	<i>Scomberomorus commerson</i> (Scombridae)	2	Indian Ocean, Somali coast	1984
<i>Anisakis</i> Type I	L3	4	<i>Sarda orientalis</i> (Scombridae)	1	Indian Ocean, Somali coast	1984
<i>Anisakis</i> Type I	L3	5	<i>Coryphaena hippurus</i> (Coryphaenidae)	1	Indian Ocean, Somali coast	1984
<i>Anisakis</i> Type I	L3	156	<i>Merluccius merluccius</i> (Merlucciidae)	40	Eastern Mediterranean Sea, off Crete	1998-1999
<i>Anisakis</i> Type I	L3	10	<i>Merluccius merluccius</i> (Merlucciidae)	5	Eastern Mediterranean Sea, off Cyprus	1998-1999

*N<sub>p</sub>*, number of parasites tested; *N<sub>h</sub>*, number of infected hosts studied.

Table 2. Diagnostic allozymes allowing the correct identification at the 99% level (\*) or at the 95% level (●) of *Anisakis typica* from the other *Anisakis* species studied genetically.

Locus	<i>A. typica</i>	<i>A. simplex s.s.</i>	<i>A. pegreffii</i>	<i>A. simplex C</i>	<i>A. ziphidarum</i>	<i>A. physeteris</i>	<i>A. brevispiculata</i>
* <i>Mdh-1</i>	79	100	100	90	78	105	105
* <i>Sod-1</i>	104	92	100	85	98	97	90
● <i>Np</i>	90,100	100	100,90	100	110	60	70
● <i>Aat-2</i>	95,110	93,100,88	100,93	110,100	120	97,90,107	85,80
* <i>Adk-2</i>	95	105	100	100	90	97	103
* <i>fEst-2</i>	130,140	100,108	100	100	125	115	90
* <i>Lap-2</i>	85	100,96	100, 98	100,104	105	80	80
* <i>PepC-1</i>	98	90	100,110,94	92	108	105,95	95
* <i>Gpi</i>	88	100	100	100	97	98, 90	98, 90
* <i>Pgm-2</i>	110	100	100	100,106	113	106	106

## Results

### Genetic characterisation of *A. typica* from other *Anisakis* spp. and larval identification

The genetic comparison of an adult sample of *A. typica* with populations of other *Anisakis* species so far studied genetically (Nascetti et al., 1986; Mattiucci et al., 1997, 2001) showed alleles at 8 loci (*Mdh-1*, *Sod-1*, *Adk-2*, *fEst-2*, *PepC-1*, *Lap-2*, *Gpi* and *Pgm-2*) not previously detected in the other *Anisakis* species. These enzyme loci allow an easy and reliable identification of *A. typica*, at any life-history stage and of both sexes. Characteristic allozymes for this species, with those observed in the other *Anisakis* species considered, are given in Table 2. These alleles extend the diagnostic keys previously provided for *Anisakis* species to include *A. typica* (see Mattiucci et al., 2001).

By using the above diagnostic markers, the 20 *Anisakis* L4 larvae recovered from the striped dolphin (*S. coeruleoalba*) (see Table 1) were found to correspond to *A. typica*; the 280 Type I L3 larvae collected from various fish and listed in Table 1 were identified as: *A. typica* (120), *A. pegreffii* (146), *A. simplex (sensu stricto)* (11) and *A. ziphidarum* (3). The different *Anisakis* species found in the fish hosts examined are listed in Table 3. *A. typica* was found in several mixed infections: with *A. pegreffii* in 9 specimens of *M. merluccius* from the Sea of Crete; with *A. pegreffii* and *A. simplex (s.s.)* in 4 *Trachurus picturatus*, and with *A. pegreffii*, *A. simplex s.s.* and *A. ziphidarum* in 3 *Scomber japonicus* from the Atlantic Ocean off Madeira (Table 3). No F<sub>1</sub> hybrids, backcrosses or recombinant genotypes were found in sympatric areas, supporting the specific status of *A. typica* and the other

*Anisakis* species (Mattiucci et al., 1986, 1997, 2001; Nascetti et al., 1986; Paggi et al., 1998).

### Host preferences and geographical distribution of *A. typica*

The host fishes of *A. typica* listed in Table 3 represent the first record of intermediate/paratenic hosts for this species.

Considering the definitive hosts of *A. typica* detected in the present study, the boto cinza dolphin *Sotalia fluviatilis* was previously recorded for *A. typica* by Santos et al., (1996), while the striped dolphin *Stenella coeruleoalba* was indicated by several authors (Kagei, 1971; Abril et al., 1986; Raga & Balbuena, 1987). Other definitive hosts are: the bottlenose dolphin *Tursiops truncatus* and the rough-toothed dolphin *Steno bredanensis* (see Forrester & Robertson, 1975), the short-finned pilot whale *Globicephala macrorhynchus* and the spotted dolphin *Stenella frontalis* (see Raga & Balbuena, 1987; Mignucci-Giannone et al., 1998), the melon-headed whale *Peponocephala electra* (see Cannon, 1977), the pygmy killer whale *Feresa attenuata* (see Conti & Frohlich, 1984; Zerbini & Santos, 1997), the harbour porpoise *Phocoena phocoena* (see Stile & Hassall, 1899) and the franciscana river dolphin *Pontoporia blainvillei* (see Kagei et al., 1976; Praderi, 1984, 1985; Aznar et al., 1995; Andreade et al., 1997). These, plus other dolphin species recorded as definitive hosts of *A. typica*, are given in Table 4.

The present findings, based on larval and adult stages of *A. typica*, extend the geographical range of *A. typica* to Somali waters of the Indian Ocean and to the Eastern Mediterranean Sea.

Table 3. Larvae of *Anisakis typica* and other *Anisakis* species genetically identified, with their fish host (see Table 1).

Fish species	Collecting site	<i>n</i>	<i>A. typica</i>	<i>A. simplex s.s.</i>	<i>A. pegreffii</i>	<i>A. ziphidarum</i>
<i>Auxis thazard</i>	Atlantic Ocean, coast of Brazil	30	30	–	–	–
<i>Thunnus thynnus</i>	Atlantic Ocean, coast of Brazil	9	9	–	99	–
<i>Scomber japonicus</i>	Atlantic Ocean, off Madeira	23	8	6	6	3
<i>Trachurus picturatus</i>	Atlantic Ocean, off Madeira	23	10	5	8	–
<i>Euthynnus affinis</i>	Indian Ocean, Somali coast	10	10	–	–	–
<i>Scomberomorus commerson</i>	Indian Ocean, Somali coast	10	10	–	–	–
<i>Sarda orientalis</i>	Indian Ocean, Somali coast	4	4	–	–	–
<i>Coryphaena hippurus</i>	Indian Ocean, Somali coast	5	5	–	–	–
<i>Merluccius merluccius</i>	Eastern Mediterranean Sea, off Crete	156	24	–	132	–
<i>Merluccius merluccius</i>	Eastern Mediterranean Sea, off Cyprus	10	10	–	–	–

*N*, number of Type I larvae identified.

Table 4. Definitive hosts of *Anisakis typica* so far reported.

Host	References
<b>Delphinidae</b>	
<i>Sotalia fluviatilis</i>	Santos et al., 1996; Present data
<i>Stenella coeruleoalba</i>	Kagei, 1967; Abril et al., 1986; Raga & Balbuena 1987; Present data
<i>Stenella frontalis</i>	Mignucci-Giannone et al., 1998;
<i>Tursiops truncatus</i>	Dollfus, 1968; Raga & Balbuena, 1987
<i>Delphinus delphis</i>	Diesing, 1860; Davey, 1971
<i>Globicephala maelena</i>	Dollfus, 1968; Stiplely, 1905
<i>Globicephala macrorhynchus</i>	Mignucci-Giannone et al., 1998
<i>Steno bredanensis</i>	Forrester & Robertson, 1975
<i>Feresa attenuata</i>	Conti & Frohlich, 1984; Zerbini & Santos, 1997
<i>Peponocephala electra</i>	Cannon, 1977
<i>Lagenorhynchus obscurus</i>	Baylis, 1929
<b>Phocoenidae</b>	
<i>Phocoena phocoena</i>	Still & Hassall, 1899; Davey, 1971
<b>Pontoporidae</b>	
<i>Pontoporia blainvillei</i>	Kagei et al., 1967; Praderi, 1984; Aznar et al., 1995; Andreade et al., 1997

Table 5. Allele frequencies at 21 enzyme loci in *Anisakis typica* samples.

Loci/Alleles	BRA	MAD	EMS	SOM	Loci/Alleles	BRA	MAD	EMS	SOM			
<i>Iddh</i>	95	1.00	1.00	1.00	<i>fEst-2</i>	130	0.20	0.16	–	–		
						140	0.80	0.84	1.00	1.00		
<i>Mdh-1</i>	79	1.00	1.00	1.00	<i>Lap-1</i>	90	1.00	1.00	1.00	1.00		
<i>Icdh</i>	93	0.92	0.93	1.00	1.00	<i>Lap-2</i>	85	1.00	1.00	1.00	1.00	
	100	0.08	0.07	–	–	<i>PepB</i>	70	0.97	1.00	1.00	1.00	
<i>6Pgdh</i>	86	0.40	0.50	0.37	0.28		80	0.03	–	–	–	
	93	0.60	0.42	0.39	0.56	<i>PepC-1</i>	98	1.00	1.00	1.00	1.00	
	97	–	0.08	0.24	0.18		<i>PepC-2</i>	96	1.00	1.00	1.00	1.00
<i>Gapdh</i>	100	1.00	1.00	1.00	1.00	<i>Mpi</i>	75	0.09	0.08	0.26	0.06	
<i>NADHdh</i>	100	1.00	1.00	1.00	1.00		83	0.29	0.30	0.26	0.12	
<i>Sod-1</i>	104	1.00	1.00	1.00	1.00		90	0.20	0.08	0.20	0.35	
							94	0.18	0.38	0.13	–	
<i>Sod-2</i>	100	1.00	1.00	1.00	1.00		97	0.18	0.12	0.04	0.32	
							104	0.03	0.04	0.04	0.15	
<i>Np</i>	90	0.96	1.00	0.86	0.92		118	–	–	0.07	–	
	100	0.04	–	0.14	0.08		130	0.03	–	–	–	
<i>Aat-2</i>	95	0.90	0.92	1.00	1.00	<i>Gpi</i>	80	–	0.03	–	0.05	
	110	0.10	0.08	–	–		88	1.00	0.92	0.96	0.90	
<i>Adk-2</i>	95	1.00	1.00	1.00	1.00		100	–	0.05	0.04	0.05	
						<i>Pgm-2</i>	98	0.02	–	–	–	
<i>cEst-1</i>	95	0.35	0.21	0.10	–		110	0.98	1.00	1.00	1.00	
	100	0.65	0.79	0.90	1.00							

BRA, Atlantic coast of Brazil; MAD, Atlantic Ocean, off Madeira; EMS, Eastern Mediterranean Sea; SOM, Somali coast of Indian Ocean.

Larval and adult samples from the same geographical area were pooled together.

### Genetic variation of *Anisakis typica*

Allele frequencies, observed at 21 enzyme loci in *A. typica* samples from different localities and hosts, are reported in Table 5. No significant differences were found among larval and adult samples from the same geographical area; accordingly, their allele frequencies were pooled together. No significant deviations from the Hardy-Weinberg equilibrium were observed at the polymorphic loci.

Parameters of genetic variability ( $A$ ,  $P_{99}$ ,  $P_{95}$ ,  $H_e$ ) found in *A. typica* samples are given in Table 6. The obtained values fall within the range reported for the other *Anisakis* species previously studied genetically (Mattiucci et al., 1997, 2001; Paggi et al., 1998).

*A. typica* populations were found to be genetically similar, despite being geographically quite distant.  $D_{Nei}$  ranged from 0.004 between Mediterranean and Somali samples to 0.010 between Madeiran and Somali samples, with an average value of  $D_{Nei} = 0.008$ . The average  $F_{st}$  value among all the populations was 0.04. The indirect estimate of gene flow among *A. typica* populations studied, given by the parameter  $Nm$  from  $F_{st}$  values (Wright, 1943, 1951), was, on average,  $Nm = 6.00$ . Similar low levels of population structuring were previously reported in other *Anisakis* species (Mattiucci et al., 1997).

Table 6. Parameters of genetic variability observed in *Anisakis typica* populations, and comparison of mean values observed in species of the genus so far studied genetically (range values in parentheses).

Populations	<i>n</i>	<i>A</i>	<i>P</i> <sub>99</sub>	<i>P</i> <sub>95</sub>	<i>He</i>
<i>A. typica</i> (Atlantic coast of Brazil)	86	1.7 ±0.3	0.45	0.30	0.12 ±0.05
<i>A. typica</i> (Atlantic Ocean, off Madeira)	19	1.6 ±0.3	0.35	0.35	0.12 ±0.04
<i>A. typica</i> (Somali coast of Indian Ocean)	25	1.5 ±0.2	0.25	0.25	0.09 ±0.05
<i>A. typica</i> (Eastern Mediterranean Sea)	40	1.6 ±0.3	0.30	0.25	0.11 ±0.05
Average		1.6	0.34	0.29	0.11
	<i>N</i>				
<i>A. simplex s.s.</i>	7	2.2 (1.7 - 2.8)	0.49 (0.42 - 0.67)	0.41 (0.29 - 0.54)	0.16 (0.10 - 0.22)
<i>A. pegreffii</i>	4	2.1 (1.6 - 2.4)	0.53 (0.40 - 0.67)	0.32 (0.21 - 0.42)	0.11 (0.07 - 0.12)
<i>A. simplex C</i>	4	2.5 (2.1 - 2.9)	0.59 (0.62 - 0.87)	0.59 (0.45 - 0.75)	0.21 (0.18 - 0.24)
<i>A. ziphidarum</i>	2	1.2 (1.1 - 1.3)	0.17 (0.08 - 0.25)	0.10 (0.04 - 0.17)	0.03 (0.02 - 0.04)
<i>A. physeteris</i>	2	1.6 (0.2-0.3)	0.34 (0.17-0.52)	0.30 (0.17-0.43)	0.09 (0.06-0.12)
<i>A. brevispiculata</i>	3	1.2 (1.1-1.3)	0.18 (0.13-0.26)	0.13 (0.04-0.26)	0.04 (0.01-0.07)

*A*, mean number alleles per locus, ±s.e.; *P*<sub>99</sub> and *P*<sub>95</sub>, proportion of polymorphic loci at the 0.99 and 0.95 criteria; *He*, expected mean heterozygosity per locus, ± s.e.; *n*, number of specimens tested; *N*, number of populations considered s.e., standard error.

Data from Mattiucci et al. (1997, 1998, 2001), Paggi et al. (1998) and unpublished.

#### Genetic relationships between *A. typica* and other *Anisakis* species

The values of genetic identity and distance among the *Anisakis* species so far investigated genetically are given in Tables 7 and 8. *D*<sub>Nei</sub> was, on average, 1.12 between *A. typica* and the members of the *A. simplex* complex (*A. simplex s.s.*, *A. pegreffii*, *A. simplex C*) and *D*<sub>Nei</sub> = 1.41 with respect to *A. ziphidarum*. Higher values were observed between *A. typica* and both *A. physeteris*: *D*<sub>Nei</sub> = 2.76 and *A. brevispiculata* *D*<sub>Nei</sub> = 2.54 (Table 7).

A spatial representation of the genetic relationships among *A. typica* and the other *Anisakis* species is given by Figures 1 and 2, which show the first two dimensions of MDS analyses based on Cavalli-Sforza & Edwards' (1967) chord distance values. Figure 1 shows that *A. typica*, the members of the *A. simplex* complex and *A. ziphidarum* cluster well apart from *A. physeteris* and *A. brevispiculata*. Figure 2 zooms

in the species group having larvae of morphotype I (*sensu* Berland, 1961) and shows that *A. typica* is well differentiated both from the members of the *A. simplex* complex and *A. ziphidarum*.

#### Discussion

Genetic markers, such as gene-enzyme systems, provide powerful tools for the molecular systematics of various nematode groups (e.g. Nascetti et al., 1986; Beveridge et al., 1995; Chilton et al., 1997; Mattiucci et al., 1997, 2001; Andrews & Chilton, 1999). Among other things, they enable the identification of anisakid species of both sexes and at any developmental stage. Using this approach, it has been demonstrated that the larval morphotype *Anisakis* Type I (*sensu* Berland, 1961) comprises several species: *A. typica* (present data), *A. simplex s.s.*, *A. pegreffii*, *A. simplex C* and *A. ziphidarum* (see Mattiucci et al., 1997; and unpub-

Table 7. Average and ranges of Nei's (1972) values of standard genetic identity ( $I_{Nei}$ , above the diagonal) and distance ( $D_{Nei}$ , below the diagonal) between *Anisakis typica* and the other species of *Anisakis* so far studied genetically. Intraspecific  $D_{Nei}$  values are given along the diagonal (ranges in parentheses).

	<i>N</i>	<i>A. typica</i>	<i>A. simplex s.s.</i>	<i>A. pegreffii</i>	<i>A. simplex C</i>	<i>A. ziphidarum</i>	<i>A. physeteris</i>	<i>A. brevispiculata</i>
<i>A. typica</i>	4	0.008 (0.004-0.010)	0.362 (0.339-0.384)	0.286 (0.273-0.293)	0.340 (0.316-0.377)	0.244 (0.229-0.254)	0.064 (0.060-0.066)	0.079 (0.074-0.086)
<i>A. simplex s.s.</i>	7	1.016 (0.958-1.082)	0.015 (0.002-0.040)	0.686 (0.663-0.702)	0.707 (0.682-0.723)	0.237 (0.225-0.255)	0.056 (0.047-0.060)	0.065 (0.052-0.078)
<i>A. pegreffii</i>	5	1.251 (1.228-1.297)	0.378 (0.353-0.410)	0.002 (0.001-0.003)	0.681 (0.658-0.718)	0.181 (0.179-0.184)	0.055 (0.055-0.057)	0.056 (0.054-0.059)
<i>A. simplex C</i>	4	1.081 (0.976-1.152)	0.347 (0.324-0.383)	0.384 (0.331-0.418)	0.050 (0.002-0.094)	0.223 (0.208-0.248)	0.075 (0.063-0.082)	0.075 (0.063-0.086)
<i>A. ziphidarum</i>	2	1.411 (1.370-1.473)	1.443 (1.366-1.490)	1.707 (1.695-1.720)	1.505 (1.395-1.568)	0.003 (0.003-0.003)	0.056 (0.055-0.058)	0.052 (0.051-0.053)
<i>A. physeteris</i>	2	2.757 (2.715-2.810)	2.885 (2.816-3.051)	2.893 (2.869-2.905)	2.593 (2.497-2.758)	2.877 (2.849-2.906)	0.007 (0.004-0.012)	0.420 (0.414-0.427)
<i>A. brevispiculata</i>	3	2.537 (2.450-2.606)	2.742 (2.557-2.960)	2.875 (2.823-2.916)	2.592 (2.450-2.764)	2.954 (2.932-2.972)	0.795 (0.764-0.820)	0.080 (0.066-0.090)

*N*, number of populations considered.



Table 8. Average and ranges of values of the standard genetic distance of Rogers (1972, modified by Wright, 1978) (*D<sub>r</sub>*, below the diagonal) and of Cavalli-Sforza & Edwards (1967) (*D<sub>c</sub>*, above the diagonal) between *Anisakis typica* and the other species of *Anisakis* so far studied genetically.

	<i>N</i>	<i>A. typica</i>	<i>A. simplex s.s.</i>	<i>A. pegreffii</i>	<i>A. simplex C</i>	<i>A. ziphidarum</i>	<i>A. physeteris</i>	<i>A. brevispiculata</i>
<i>A. typica</i>	4	–	0.712 (0.696-0.726)	0.752 (0.746-0.761)	0.722 (0.705-0.734)	0.777 (0.773-0.783)	0.866 (0.862-0.870)	0.860 (0.857-0.862)
<i>A. simplex s.s.</i>	7	0.747 (0.734-0.764)	–	0.494 (0.485-0.509)	0.496 (0.476-0.512)	0.777 (0.766-0.785)	0.873 (0.868-0.879)	0.865 (0.860-0.871)
<i>A. pegreffii</i>	5	0.801 (0.798-0.806)	0.527 (0.505-0.544)	–	0.518 (0.491-0.530)	0.806 (0.803-0.810)	0.875 (0.873-0.877)	0.871 (0.867-0.873)
<i>A. simplex C</i>	4	0.743 (0.728-0.752)	0.490 (0.476-0.504)	0.520 (0.492-0.540)	–	0.784 (0.781-0.786)	0.858 (0.851-0.869)	0.856 (0.848-0.863)
<i>A. ziphidarum</i>	2	0.837 (0.833-0.841)	0.833 (0.822-0.847)	0.875 (0.872-0.878)	0.823 (0.816-0.832)	–	0.872 (0.871-0.874)	0.878 (0.878-0.878)
<i>A. physeteris</i>	2	0.918 (0.912-0.926)	0.914 (0.896-0.929)	0.927 (0.921-0.932)	0.884 (0.870-0.898)	0.940 (0.937-0.942)	–	0.693 (0.688-0.698)
<i>A. brevispiculata</i>	3	0.922 (0.909-0.934)	0.921 (0.898-0.942)	0.938 (0.925-0.949)	0.896 (0.874-0.916)	0.953 (0.943-0.963)	0.715 (0.700-0.728)	–

*N*, number of populations considered.

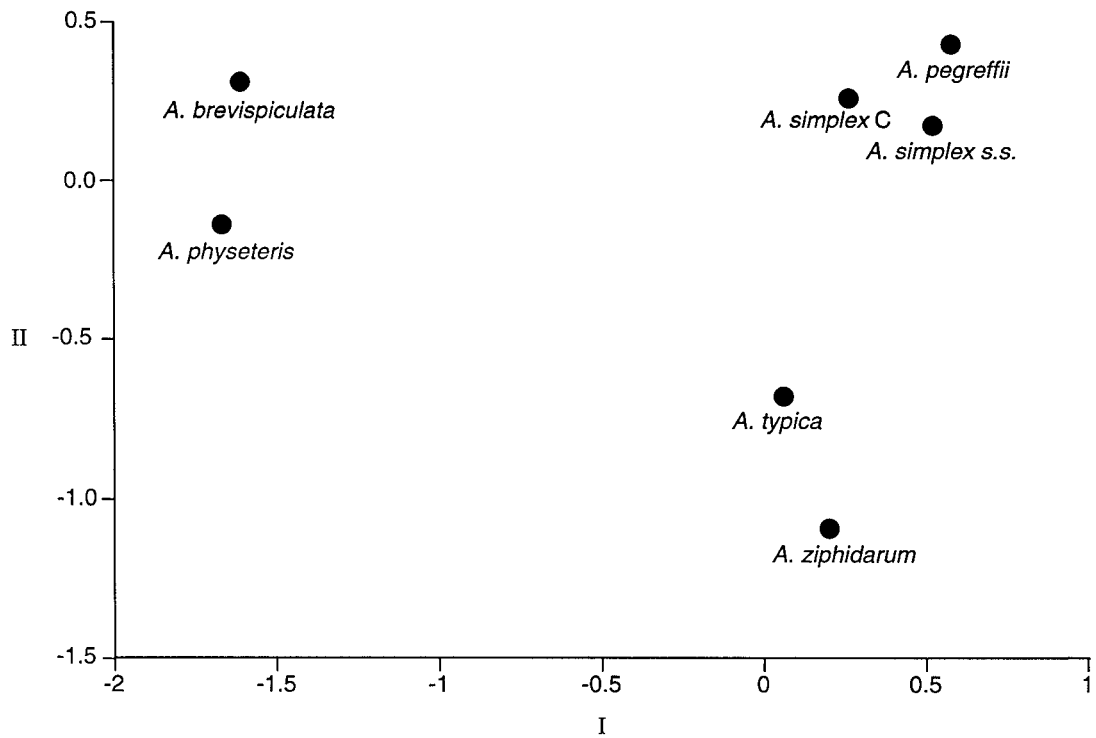


Figure 1. Plot of the first two dimensions of a MDS ordination (Guttman, 1964) from chord distance values ( $D_c$ , Cavalli-Sforza & Edwards, 1967), showing the genetic relationships between *A. typica* and other *Anisakis* species.

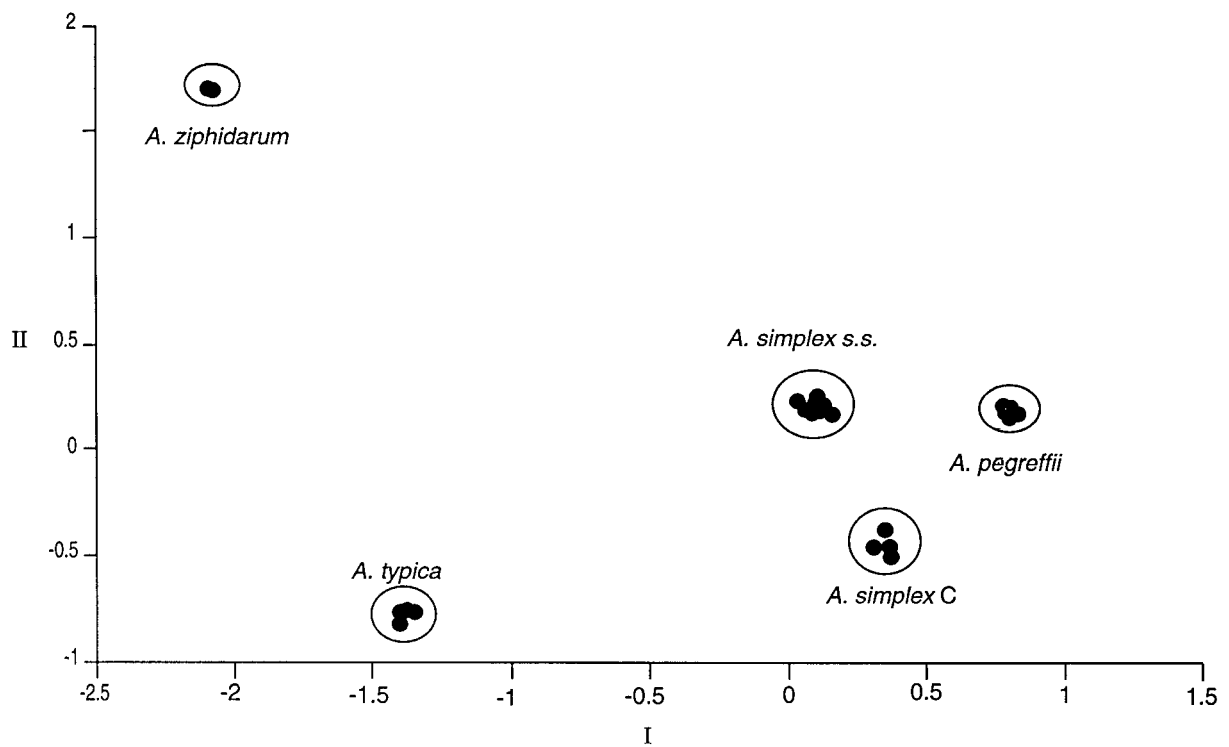


Figure 2. Plot of the first two dimensions of a MDS ordination (Guttman, 1964) from chord distance values ( $D_c$ , Cavalli-Sforza & Edwards, 1967), showing the genetic relationships between *Anisakis* species with Type I larvae (*sensu* Berland, 1961).

lished data), whereas, *Anisakis* Type II (*sensu* Berland, 1961) includes *A. physeteris* and *A. brevispiculata* (Mattiucci et al., 1986, 2001). Routine identification of *Anisakis* larvae by allozyme markers is particularly useful when different *Anisakis* species occur sympatrically in the same host and/or the same geographical area.

The fish species reported in the present paper represent the first record of intermediate/paratenic hosts of *A. typica*. Moreover, the present findings extend the geographical range of this species to include Somali waters of the Indian Ocean and the eastern Mediterranean Sea. As to the definitive hosts of *A. typica*, most of the cetaceans reported in the literature and in the present paper, belong to the Family Delphinidae. The only exceptions recorded so far are the phocoenid *Phocoena phocoena* and the pontoporiid *Pontoporia blainvillei*.

According to the data available, the range of *A. typica* extends from 30°S to 35°N in warmer temperate and tropical waters.

The populations of *A. typica* tested showed a remarkable genetic homogeneity (average  $D_{Nei} = 0.008$ ). Similar low values, at the intraspecific level, were frequently observed in other *Anisakis* species and even between populations sampled thousands of kilometres apart, e.g. *A. pegreffii* and *A. simplex s.s.* (see Mattiucci et al., 1997), *A. ziphidarum* (see Paggi et al., 1998), *A. physeteris* and *A. brevispiculata* (see Mattiucci et al., 2001). This indicates high levels of gene flow in these anisakid nematodes, which can be explained by the high vagility of the intermediate/paratenic and definitive hosts involved in their life-cycle. A possible exception is *A. simplex* C, whose populations from the Canadian Pacific and the Southern Oceans are genetically rather well differentiated: average  $D_{Nei} = 0.08$  (Mattiucci et al., 1997).

The genetic heterogeneity of the genus *Anisakis* is confirmed by the present data. Two groups of genetically well differentiated species (possibly two genera) are currently included in this genus: one group includes *A. typica*, the members of the *A. simplex* complex and *A. ziphidarum*; the second includes *A. physeteris* and *A. brevispiculata*. The two species groups are morphologically differentiated both at larval and adult stage: Type I larvae (*sensu* Berland, 1961) characterise the first group, Type II larvae the second one; at the adult stage, the two species groups show differences in the length and shape of the ventriculus and in spicule length (*sensu* Davey, 1971).

## Acknowledgements

The authors wish to express their gratitude to David I. Gibson (The Natural History Museum, London) for his helpful comments and suggestions. We thank Dr Andonis Magoulas, of the Genetics Department, Institute of Marine Biology of Crete (IMBC), for his helpful collaboration in providing fish samples from the Sea of Crete.

The research was carried out with financial support from the Commission of the European Communities, *Agriculture and Fisheries*: FAIR CT 97-3494 'Genhake'.

## References

- Abril, E., Almor, P., Raga, J.A. & Duguy, R. (1986) Parasitism by *Anisakis typica* (Diesing, 1860) in the striped dolphin (*Stenella coeruleoalba*) of the North-East Atlantic. *Bulletin de la Société Zoologique de France*, **111**, 131–133.
- Andreade, A., Pinedo, M.C. & Pereira, J., Jr (1997) The gastrointestinal helminths of the Franciscana, *Pontoporia blainvillei*, in southern Brazil. *Annual Report of the International Whaling Commission* **47**.
- Andrews, R.H. & Chilton, N.B. (1999) Multilocus enzyme electrophoresis: a valuable technique for providing answers to problems in parasite systematics. *International Journal for Parasitology*, **29**, 213–253.
- Aznar, F.J., Raga, J.A., Corcuera, J. & Monzon, F. (1995) Helminths as biological tags for franciscana (*Pontoporia blainvillei*) (Cetacea: Pontoporidae) in Argentinian and Uruguayan waters. *Mammalia*, **59**, 427–435.
- Bagrov, A.A. (1982) On morphological variability of larvae of nematodes of the genus *Anisakis* (Nematoda, Anisakidae). *Parazitologiya*, **16**, 469–475. (In Russian).
- Baylis, H.A. (1929) Parasitic Nematoda and Acanthocephala collected in 1925–27. *Discovery Reports*, **1**, 541–560.
- Berland, B. (1961) Nematodes from some Norwegian marine fishes. *Sarsia*, **2**, 1–50.
- Beveridge, I., Chilton, N.B. & Andrews, R.H. (1995) Relationships within the *Rugopharynx delta* species complex (Nematoda: Strongyloidea) from Australian marsupials inferred from allozyme electrophoresis. *Systematic Parasitology*, **32**, 149–156.
- Brewer, G.J. & Sing, C.F. (1970) *An introduction to isoenzyme techniques*. New York & London: Academic Press, 186 pp.
- Cannon, L.R.G. (1977) Some aspects of the biology of *Peponocephala electra* (Cetacea: Delphinidae). II. Parasites. *Australian Journal of Marine and Freshwater Research*, **28**, 717–722.
- Cavalli-Sforza, L.L. & Edwards, A.W.F. (1967) Phylogenetic analysis: models and estimation procedures. *American Journal of Human Genetics*, **19**, 233–257.
- Chilton, N.B., Beveridge I. & Andrews, R.H. (1997) An electrophoretic analysis of patterns of speciation in *Cloacina clarkae*, *C. communis*, *C. petrogale* and *C. similis* (Nematoda: Strongyloidea) from macropodid marsupials. *International Journal for Parasitology*, **27**, 483–493.
- Conti, J.A. & Frohlich, R.K. (1984) Gastrointestinal parasitism in pigmy killer whales. *Proceedings of the Helminthological Society of Washington*, **51**, 364–365.

- Cruz, H. (1946) Contributions to the helminthology of Ceylon. II. Notes on some parasitic nematodes, with a description of *Anisakis turstopis* sp. nov. *Ceylon Journal of Sciences, B, Zoology*, **23**, 57–66.
- Davey, J.T. (1971) A revision of the genus *Anisakis* Dujardin, 1845 (Nematoda: Ascaridida). *Journal of Helminthology*, **45**, 51–72.
- Diesing, K.M. (1860) Revision der Nematoden. *Akademie der Wissenschaften zu Wien, Sitzungsberichte, Mathematisch-naturwissenschaftliche Klasse*, **42**, 595–763.
- Dollfus, R. (1968) Nematodes des cetaces odontocetes (*Globicephalus et Tursiops*). Récoltes au large de la côte Méditerranéenne du Maroc par Herry Aloncl. *Bulletin de l'Institut des Pêches Maritimes du Maroc*, **16**, 35–53.
- Forrester, D.J. & Robertson, W.D. (1975) Helminths of rough-toothed dolphin, *Steno bredanensis* Lesson 1828, from Florida Waters. *Journal of Parasitology*, **61**, 922.
- Guttman, L.A. (1968) A general nonmetric technique for finding the smallest coordinate space for a configuration of points. *Psychometrika*, **33**, 469–506.
- Harris, H. (1966) Enzyme polymorphism in man. *Proceedings of the Royal Society Series B*, **164**, 298–310.
- Hillis, D.M., Moritz, C. & Mable, B.K. (1996) *Molecular systematics*. Sunderland, Mass.: Sinauer Associates Inc. Publishers, 655 pp.
- Kagei, N., Oshima, T. & Takemura, A. (1967) Survey of *Anisakis* spp. (Anisakidae: Nematoda) in marine mammals of the coast of Japan. *Japanese Journal of Parasitology*, **16**, 427–435.
- Kagei, N., Tobayama, T. & Nagasaki, Y. (1976) On the helminth of franciscana, *Pontoporia blainvillei*. *Science Report of The Whales Researches Institute*, **28**, 161–166.
- Mattiucci, S., Nascetti, G., Bullini, L., Orecchia, P. & Paggi, L. (1986) Genetic structure of *Anisakis physeteris* and its differentiation from the *Anisakis simplex* complex (Ascaridida: Anisakidae). *Parasitology*, **93**, 383–387.
- Mattiucci, S., Nascetti, G., Cianchi, R., Paggi, L., Arduino, P., Margolis, L., Bratley, J., Webb, S.C., 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.
- Mattiucci, S., Paggi, L., Nascetti, G., Ishikura, H., Kikuchi, K., Sato, N., Cianchi, R. & Bullini, L. (1998) Allozyme and morphological identification of *Anisakis*, *Contracaecum* and *Pseudoterranova* from Japanese waters (Nematoda, Ascaridoidea). *Systematic Parasitology*, **40**, 81–92.
- Mattiucci, S., Paggi L., Nascetti G., Abollo E., Webb, S.C., Pascual, S., Cianchi, R. & Bullini, L. (2001) Genetic divergence and reproductive isolation between *Anisakis brevispiculata* and *Anisakis physeteris* (Nematoda: Anisakidae). *International Journal for Parasitology*, **31**, 9–14.
- Mignucci-Giannone, A.A., Hoberg, H.P., Siegel-Causey, D. & Williams, E.H. Jr (1998) Metazoan parasites and other symbionts of cetaceans in the Caribbean. *Journal of Parasitology*, **84**, 939–946.
- Nascetti, G., Paggi, L., Orecchia, P., Smith, J.W., Mattiucci, S. & Bullini L. (1986) Electrophoretic studies on the *Anisakis simplex* complex (Ascaridida: Anisakidae) from the Mediterranean and North East Atlantic. *International Journal for Parasitology*, **16**, 633–640.
- Nei, M. (1972) Genetic distance between populations. *The American Naturalist*, **106**, 283–292.
- Paggi, L., Nascetti, G., Webb, S.C., Mattiucci, S., Cianchi, R. & Bullini, L. (1998) A new species of *Anisakis* Dujardin, 1845 (Nematoda, Anisakidae) from beaked whales (Ziphiidae): allozyme and morphological evidence. *Systematic Parasitology*, **40**, 161–174.
- Praderi, R. (1984) Mortalidad de franciscana, *Pontoporia blainvillei*, en pesquerías artesanales de tiburón de la costa Atlántica Uruguaya. *Revista del Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' e Instituto Nacional de Investigación de las Ciencias Naturales*, **13**, 259–272.
- Praderi, R. (1985). Incidental mortality of dolphins (*Pontoporia blainvillei*) in Uruguay. *National Geographic Society Research Report*, **21**, 395–403.
- Poulik, M.D. (1957) Starch gel electrophoresis in a discontinuous system of buffers. *Nature*, **180**, 1477.
- Raga, J.A. & Balbuena, J.A. (1987) Algunas características zoológicas de los helmintos de los cetáceos en el Mediterráneo, con especial referencia a la helmintofauna del delfín listado. In: Sans-Coma, V., Mas-Coma, S. & Gsalbez, J. (Eds) *Mamíferos y helmintos*. Barcelona: Ketres Editores, pp. 195–201.
- Rogers, J.S. (1972) Measures of genetic similarities and genetic distance. *Studies in Genetics VII, University of Texas Publications*, **7213**, 145–153.
- Richardson, B.J., Baverstock, P.R. & Adams, M. (1986) *Allozyme electrophoresis. A handbook for animal systematics and population studies*. Sydney: Academic Press, 410 pp.
- Santos, C.P., Rohde, K., Ramos, R., Di Benedetto, A.P. & Capistrano, L. (1996) Helminths of cetaceans on the southeastern coast of Brazil. *Journal of the Helminthological Society of Washington*, **63**, 149–152.
- Selander, R.K., Smith, M.H., Yang, S.Y. & Gentry, J.B. (1971) Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation of the old-field mouse (*Peromyscus polionotus*). *Studies in Genetics VI, University of Texas Publications*, **7103**, 49–90.
- Shiple, A.E. (1905) Notes on a collection of parasites belonging to the Museum of the University College, Dundee. *Proceedings of the Cambridge Philosophical Society. Biological Sciences. London*, **13**, 95–102.
- Smith, J.W. & Wootten, R. (1978) *Anisakis* and Anisakiasis. *Advances in Parasitology*, **16**, 93–163.
- Sokal, R.R. & Rohlf, F.J. (1981) *Biometry*. 2nd Edition. New York: W.H. Freeman and Company, 859 pp.
- Stiles, C.H. & Hassall, A. (1899) Internal parasites of the fur seal. In: *The fur seals and fur seal islands of the North Pacific Ocean*. Washington: Government Printing Office, Part III, pp. 99–107.
- Swofford, D.L. & Selander, R.B. (1989) *BYOSYS-1, a computer program for the analysis of allelic variation in population genetics and biochemical systematics*, version 1.7. Champaign: Illinois Natural History Survey, 31 pp.
- Wilkinson, L. & Leland, F.J. (1989) *SYSTAT: The system for statistics*. Evanston, Illinois: SYSTAT Inc.
- Wright, S. (1943) Isolation by distance. *Genetics*, **28**, 114–138.
- Wright, S. (1951) The genetical structure of populations. *Annals of Eugenics*, **15**, 323–354.
- Wright, S. (1978) *Evolution and the genetics of populations*. Vol. 4. Variability within and among natural populations. Chicago, Illinois: The University of Chicago Press, 580 pp.
- Zerbini, A.N. & Santos, M.C. (1997) First record of the pygmy killer whale *Feresa attenuata* (Gray, 1874) for the Brazilian coast. *Aquatic Mammals*, **23**, 105–109.