Genetic and Morphological Approaches Distinguish the Three Sibling Species of the *Anisakis simplex* Species Complex, with a Species Designation as *Anisakis berlandi* n. sp. for *A. simplex* sp. C (Nematoda: Anisakidae)

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GENETIC AND MORPHOLOGICAL APPROACHES DISTINGUISH THE THREE SIBLING SPECIES OF THE *ANISAKIS SIMPLEX* SPECIES COMPLEX, WITH A SPECIES DESIGNATION AS *ANISAKIS BERLANDI* N. SP. FOR *A. SIMPLEX* SP. C (NEMATODA: ANISAKIDAE)

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ABSTRACT: Numerous specimens of the 3 sibling species of the *Anisakis simplex* species complex (*A. pegreffii*, *A. simplex* (senso stricto)), and *A. simplex* sp. C) recovered from cetacean species stranded within the known geographical ranges of these nematodes were studied morphologically and genetically. The genetic characterization was performed on diagnostic allozymes and sequences analysis of nuclear (internal transcribed spacer [ITS] of ribosomal [r]DNA) and mitochondrial (mitochondrial [mt]DNA *cox2* and *rrnS*) genes. These markers showed (1) the occurrence of sympatry of the 2 sibling species *A. pegreffii* and *A. simplex* sp. C in the same individual host, the pilot whale, *Globicephala melas* Traill, from New Zealand waters; (2) the identification of specimens of *A. pegreffii* in the striped dolphin, *Stenella coeruleoalba* (Meyen), from the Mediterranean Sea; and (3) the presence of *A. simplex* (s.s.) in the pilot whale and the minke whale, *Balaenoptera acutorostrata* Lacépède, from the northeastern Atlantic waters. No F1 hybrids were detected among the 3 species using the nuclear markers. The phylogenetic inference, obtained by maximum parsimony (MP) analysis of separate nuclear (ITS rDNA region), combined mitochondrial (mtDNA *cox2* and *rrnS*) sequences datasets, and by concatenated analysis obtained at both MP and Bayesian inference (BI) of the sequences datasets at the 3 studied genes, resulted in a similar topology. They were congruent in depicting the existence of the 3 species as distinct phylogenetic lineages, and the tree topologies support the finding that *A. simplex* (s.s.), *A. pegreffii*, and *A. berlandi* n. sp. (=*A. simplex* sp. C) represent a monophyletic group. The morphological and morphometric analyses revealed the presence of morphological features that differed among the 3 biological species. Morphological analysis using principal component analysis, and Procrustes analysis, combining morphological and genetic datasets, showed the specimens clustering into 3 well-defined groups. Nomenclatural designation and formal description are given for *A. simplex* species C: the name *Anisakis berlandi* n. sp. is proposed. Key morphological diagnostic traits are as follows between *A. berlandi* n. sp. and *A. simplex* (s.s.): ventriculus length, tail shape, tail length/total body length ratio, and left spicule length/total body length ratio; between *A. berlandi* n. sp. and *A. pegreffii*: ventriculus length and plectane 1 width/plectane 3 width ratio; and between *A. simplex* (s.s.) and *A. pegreffii*: ventriculus length, left and right spicule length/total body length ratios, and tail length/total body length ratio. Ecological data pertaining to the geographical ranges and host distribution of the 3 species are updated.

The systematics and nomenclature of anisakid nematodes belonging to the genus *Anisakis* Dujardin, 1845 was controversial and confused until the application of genetic and molecular methodologies that have lead, during the past 20 yr, to an apparently stable and widely accepted taxonomy (Mattiucci and Nascetti, 2006, 2008). One of the main results has been the discovery of sibling species within 1 of the nominal species of the genus, *Anisakis simplex* (Rudolphi, 1809), that was believed to be a cosmopolitan species parasitic in a wide array of definitive hosts (Davey, 1971). Three biological species are currently included within the concept of *Anisakis simplex* (s.l.), i.e., the *A. simplex* species complex*: Anisakis pegreffii* Campana-Rouget & Biocca, 1955, *A. simplex* (s.s.), and *A. simplex* sp. C (see Mattiucci et al., 1997). The geographical distribution and hosts reported for these species have been reviewed and updated (Mattiucci and Nascetti, 2008; Mattiucci et al., 2009). Such species have been demonstrated to have distinct gene pools (inferred from allozyme analyses), to be characterized by distinct diagnostic genetic markers, and to be reproductively isolated, because F1 adult fertile hybrids have not been detected. Although a few larval F1 hybrids, detected by allozymes, have occasionally been identified between *A. pegreffii* and *A. simplex* (s.s.) in fish hosts (Mattiucci et al., 2004), these hybrids are therefore true biological species sensu Mayr (1970). Other genetic markers have confirmed the existence of these 3 cryptic species by using phylogenetic analyses of DNA sequences of mitochondrial (mitochondrial [mt]DNA *cox2*) and nuclear (internal transcribed spacer [ITS] of ribosomal [r]DNA) genes (Nadler et al., 2005; Mattiucci et al., 2009; Cavallero et al., 2011).

Morphological analyses of specimens of other species of *Anisakis*, previously assigned by molecular markers to their respective biological species, have provided sets of morphological characters whose combined use permits species recognition (Mattiucci et al., 2005, 2009). As part of the process of clarifying situations involving cryptic species, it is necessary, whenever possible, to assess their nomenclatural designation and to give a morphological description (Nadler and Pérez-Ponce de León, 2011). In this respect, the name *Anisakis pegreffii* Campana-Rouget & Biocca, 1955, belonging to a species originally described from the Mediterranean monk seal, *Phoca monaca*, was resurrected for *A. simplex* sp. A (see Nascetti et al., 1986) detected genetically in the Mediterranean Sea, within the geographical range of this named species. Instead, the name *A. simplex* (s.s.) was retained for the sibling species *A. simplex* sp. B of Nascetti et al. (1986) that occurs in the Arctic Boreal region of the Atlantic and Pacific oceans. An attempt to find morphological characters to distinguish the 2 sibling species *A. pegreffii* and *A. simplex* (s.s.) has been recently made in a morphological study (Quiazon et al., 2008) on larval and adult specimens (cultured in vitro) after genetic determination. Furthermore, a nomenclatural designation and morphological description is still lacking for the species *A. simplex* sp. C. The existence of *A. simplex* sp. C was demonstrated genetically for the first time using allozymes (Mattiucci et al.,

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TABLE I. Specimens of *Anisakis berlandi* n. sp. (=*A. simplex* sp. C), *A. pegreffii*, and *A. simplex* (s.s.) genetically identified and morphologically studied herein. N_H, code for the definitive host examined; N_{MAE}, N_{cox2}, N_{rrnS}, and N_{ITS} are number of specimens analyzed by allozymes and DNA sequence analyses of the mitochondrial cytochrome *c* oxidase subunit II (*cox2*), small subunit rRNA (*rrnS*), and ITS rDNA genes, respectively.

Species	Definitive host	Collecting locality	N_H	Life-history stage	N_{MAE}	N_{cox2}	N_{rrnS}	N_{ITS}	N_{morph}
A. berlandi n. sp.	*Globicephala melas*	New Zealand coast (southeastern Pacific Ocean)	GM1	Adult	5	10	10	10	8 males, 2 females
A. berlandi n. sp.	*G. melas*	New Zealand coast (southeastern Pacific Ocean)	GM2	Adult	5	5	5	5	3 males, 2 females
A. pegreffii	*G. melas*	New Zealand coast (southeastern Pacific Ocean)	GM1	Adult	3	3	3	3	3 males
A. pegreffii	*Stenella coeruleoalba*	Adriatic Sea (eastern Mediterranean Sea)	ST1	Adult	–	4	4	4	3 males, 1 female
A. pegreffii	*S. coeruleoalba*	Tyrrhenian Sea (western Mediterranean Sea)	ST2	Adult	10	10	10	10	4 males, 6 females
A. pegreffii	*S. coeruleoalba*	Tyrrhenian Sea (western Mediterranean Sea)	ST3	Adult	–	6	6	6	5 males, 1 female
A. simplex (s.s.)	*Balaenoptera acutorostrata*	Northeastern Atlantic Ocean (Norwegian coast)	BA1	Adult	8	8	8	8	6 males, 2 females
A. simplex (s.s.)	*G. melas*	Northeastern Atlantic Ocean (Norwegian coast)	GM3	Adult		6	6	6	6 males

1997). It has been found in sympatric association with *A. simplex* (s.s.) in the false killer whale, *Pseudorca crassidens*, in the northeastern Pacific Ocean (off Vancouver Island), and at its larval stage in fish hosts it has been reported from the southern Pacific Ocean (off New Zealand) and the southeastern Atlantic Ocean (off South Africa) (Mattiucci et al., 1997).

The aims of the present study, based on new material belonging to the 3 species of the *A. simplex* species complex collected from different cetacean definitive hosts in various localities, were to (1) identify, using allozymes and multiple genes sequence analyses of nuclear (ITS region of the rDNA) and 2 mitochondrial genes (mtDNA *cox2* and *rrnS*), the 3 sibling species of *A. simplex* (s.l.); (2) determine any diagnostic morphological characters, present at the adult stage, between these 3 sibling species; (3) provide a nomenclature designation and a formal description for the species *A. simplex* sp. C; and (4) look for further molecular markers and morphological characters useful for the recognition of these species.

MATERIALS AND METHODS

Parasite material

Details, including the sampling localities of the definitive hosts (cetaceans) of the 3 sibling species of the *A. simplex* (s.l.) species complex examined in this study, are presented in Table I. Although some of the nematodes studied were obtained from both the frozen and wet collections of anisakids stored in the Section of Parasitology, Department of Public Health and Infectious Diseases of Sapienza University in Rome, the collection of new samples from stranded cetaceans was undertaken during 2010–2012. Retained nematodes were collected from 3 specimens of pilot whale, *Globicephala melas* Traill, in the southern Pacific Ocean (off New Zealand), from *G. melas* and minke whale, *Balaenoptera acutorostrata* Lacépède, in the northeastern Atlantic Ocean (off Norway), and from 3 striped dolphins, *Stenella coeruleoalba* (Meyen), off the Italian coast. Details of the specimens analyzed are given in Table I. Nematodes collected from the stomach of their hosts were repeatedly washed in saline solution and preserved by freezing at –70 C in distilled water or stored in absolute alcohol until genetic analysis, morphological analysis, or both.

All frozen specimens of *Anisakis* spp. were identified to the species level by using allozyme markers. In addition, specimens stored in alcohol (Table I) and all specimens identified by allozymes were sequenced at 3 genes: the ITS region of the rDNA (comprising ITS-1, 5.8S, and ITS-2), the mitochondrial cytochrome *c* oxidase subunit II (*cox2*), and the small subunit rRNA (*rrnS* rRNA) (Table I). For genetic comparison, some specimens belonging to the other species of *Anisakis* previously identified and characterized genetically by allozymes (Mattiucci et al., 1997, 2001, 2002, 2005, 2009) were sequenced at the same genes: *A. typica* (Diesing, 1860) from the spotted dolphin, *Stenella attenuata* Gray, in the northwestern Atlantic Ocean (code AT01) and from the rough-toothed dolphin, *Steno bredanensis* Lesson (code AT11); *A. ziphidarum* Paggi, Nascetti, Webb, Mattiucci, Cianchi and Bullini, 1988 from Gray's beaked whale, *Mesoplodon grayi* von Haast (codes AZ01 and AZ02), and Cuvier's beaked whale, *Ziphius cavirostris* Cuvier, off the South African coast (code AZ03); *A. nascettii* Mattiucci, Paoletti and Webb, 2009 from *M. grayi* off the New Zealand coast (codes AN09, AN10, and AN11); *A. physeteris* Baylis, 1923 from the sperm whale, *Physeter macrocephalus* L. (Physeteridae), in the Mediterranean Sea (codes AP03 and AP04); *A. brevispiculata* Dollfus, 1966 from the pygmy sperm whale, *Kogia breviceps* de Blainville, in the northwestern Atlantic Ocean (codes AB3 and AB04); and *A. paggiae* Mattiucci, Nascetti, Dailey, Webb, Barros, Cianchi and Bullini, 2005 from pygmy sperm whale in the northwestern Atlantic Ocean (codes AK01 and AK03).

Multilocus allozyme electrophoresis

The genetic identification of the specimens of *A. simplex* (s.l.) examined during this survey (Table I) was first undertaken using multilocus allozyme electrophoresis (MAE) on the frozen samples. Standard horizontal starch gel electrophoresis was performed at enzyme loci that have proven to be diagnostic among the 3 sibling species (Mattiucci et al., 1997, 2009; Mattiucci and Nascetti, 2006): superoxide dismutase (*Sod-1*, EC 1.15.1.1), aspartate amino transferase (*Aat-2*, EC 2.6.1.1), adenylate kinase (*Adk-2*, EC 2.7.4.3), leucine-amino peptidase (*Lap-2*, EC 3.4.11.1), leucine-leucine peptidase (*Pep B*, EC 3.4.11), and leucine-alanine peptidase (*PepC-1*, *PepC-2*, EC 3.4.11). Their staining procedures are those reported in detail by Mattiucci et al. (1997). Isozymes were numbered in order of decreasing mobility from the most anodal isozyme. Allozymes were identified by numbers indicating their mobility (in millimeters, standardized conditions) relative to the most common allele, designated as 100, found in the reference population (i.e., *A. pegreffii* from the Mediterranean Sea). The statistical significance of departures from the Hardy–Weinberg equilibrium was estimated using the chi-square test. Genetic analysis was performed using BIOSYS-2 software (Swofford and Selander, 1997).

DNA extraction, amplification and sequencing

The same specimens first identified by allozymes and all samples stored in alcohol (Table I) were sequenced at 3 DNA genes. The total DNA was extracted from 2 mg of tissue from each single nematode by using the cetyltrithylammonium bromide method (Valentini et al., 2006).

The following 3 genes were sequenced in all the nematodes analyzed: 629 bp of the mitochondrial cytochrome *c* oxidase subunit II (*cox2*) gene; 522 bp of the partial gene of the small subunit of the mitochondrial

ribosomal RNA gene (*rrnS*); and 972 bp of the ITS region of the ribosomal DNA.

The mitochondrial cytochrome *c* oxidase subunit II (*cox2*) gene was amplified using the primers 211F (5′-ttt tct agt tat ata gat tgr tty at-3′) and 210R (5′-cac caa ctc tta aaa tta tc-3′) (Nadler and Hudspeth, 2000) spanning the mtDNA nucleotide position 10,639–11,248, as defined in *Ascaris suum* (GenBank X54253). Polymerase chain reaction (PCR) was carried out using the following conditions: 94 C for 3 min (initial denaturation), followed by 34 cycles at 94 C for 30 sec (denaturation), 46 C for 60 sec (annealing), and 72 C for 90 sec (extension), followed by post-amplification at 72 C for 10 min.

The amplification of the small subunit of rRNA (*rrnS*) was carried out according to the procedures reported for anisakid nematodes (Garbin et al., 2011) by using the forward primer MH3 (5′-TTCCAGAA TAATCGGCTAGACTT-3′) and the reverse MH4.5 (5′-CTACTTTAC TACAACTTACTCC-3′) (Zhu et al., 2000) under the following conditions: 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.

Finally, for the internal transcribed spacer (ITS rDNA) region sequencing, PCR amplification was performed using the primers NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′), according to the procedure reported in Zhu et al. (2000). PCR amplification conditions were 94 C for 5 min (initial denaturation), followed by 30 cycles at 94 C for 30 sec (denaturation), 55 C for 30 sec (annealing), 72 C for 30 sec (extension), and a final elongation step at 72 C for 5 min (Zhu et al., 2000).

The reference specimens and the isolated DNA samples presented here are stored at the Section of Parasitology of the Department of Public Health and Infectious Diseases, Sapienza University of Rome.

Sequences analysis

The sequences obtained at the mtDNA *cox2* for all specimens of the *Anisakis* spp. analyzed in this study were compared with those already obtained for the same gene in previous studies (Valentini et al., 2006; Mattiucci et al., 2009). The following sequences of all 9 species of *Anisakis*, retrievable from GenBank, were used for the identification of all the specimens examined: *A. simplex* (s.s.) (DQ116426), *A. pegreffii* (DQ116428), *A. simplex* sp. C (DQ116429), *A. typica* (DQ116427), *A. ziphidarum* (DQ116430), *A. nascettii* (FJ685642), *A. physeteris* (DQ116432), *A. brevispiculata* (DQ116433), and *A. paggiae* (DQ116434) (Valentini et al., 2006; Mattiucci et al., 2009).

Both separated and combined phylogenetic trees for nuclear and mitochondrial genes were inferred using 2 inference methods: maximum parsimony (MP) and BI, by using PAUP* (Swofford, 2003) and MrBayes3.1 (Huelsenbeck and Ronquist, 2005), respectively. MP analysis was performed using the heuristic search with the tree-bisection-reconnection branch-swapping algorithm; the reliabilities of the phylogenetic relationships were evaluated using nonparametric bootstrap analysis on 1,000 pseudoreplicates (Felsenstein, 1985). Bootstrap values $\geq$70 were considered well supported (Hillis and Bull, 1993).

The mitochondrial (*cox2* and *rrnS*) and the nuclear (ITS rDNA) sequences datasets, obtained from the same individuals of *Anisakis* studied here, were combined by using the Concatenator software program (Pina-Martins and Paulo, 2008) to perform the phylogenetic analysis. The sequences datasets were first determined to be combinable by using the partition homogeneity test (Farris et al., 1994), as executed by PAUP* (Swofford, 2003). JModeltest (Posada, 2008) was used to determine the best-fit model for each gene considered in the study, as implemented in the Akaike Information Criterion (AIC) (Posada and Buckley, 2004). The best-fit jML models and parameters were used for the BI analysis; therefore, BI was performed using Trn+I+G model (I = 0.089 and G = 0.182) for mtDNA*cox2*, HKY+G (G = 0.01) for *rrnS*, and K80+G (G = 0.12) for the ITS region of rDNA. The Bayesian posterior probability analysis was performed using the MCMC algorithm: the number of chains was 4; the temperature of heated chains was 0.2; the number of generations was 1,000,000; and the subsampling frequency was 100, with a burn-in fraction of 0.25. Posterior probabilities were estimated and used to assess support for each branch in the inferred phylogeny with probabilities where $P = 95\%$ is indicative of significant support (Reeder, 2003). Phylogenetic trees were rooted using the ascarids *A. suum* and *Toxocara canis* as outgroups, as also used in other studies (Cavallero et al., 2011) in similar phylogenetic analyses.

Morphological analysis

From each genetically studied adult specimen, the anterior and the posterior parts of the body were preserved and cleared in lactic acid-phenol (1:1) for the morphological study; the central part of the worm's body was used for the molecular studies. Morphological and morphometric analyses were carried out with a microscope equipped with a drawing apparatus at a total magnification of ×100–400, except for the overall total length that was measured directly and spicule length that was measured at ×35. All measurements are in millimeters, except where indicated. Several morphological and morphometric features considered of diagnostic value, such as those given for anisakid nematodes by Fagerholm (1989) and Paggi et al. (1998) and for *Anisakis* spp. by Mattiucci et al. (2005, 2009), were examined, including the length and shape of the ventriculus, the distribution pattern of the male caudal papillae (labeled according to the nomenclature used by Fagerholm [1989], Paggi et al. [1998], and Mattiucci et al. [2009]), spicule length, and the size and shape of the caudal plates (plectanes [PLs]). Furthermore, the ratios between the longer and shorter spicules and between the width of first (WPL1) and third plectanes (WPL3) were calculated. In addition, to consider allometric variation, the lengths of the ventriculus, male tail length, and spicules were related to the total body length, whereas the distance between the paracloacal papilla (pc) and the distal papilla 1 (d1), and the maximum width of the largest PL, were related to tail length (TL).

Statistical analysis of morphological and morphometric data

Student's *t*-test (pairwise) was used to detect significant differences in absolute and relative morphometric variables between specimens belonging to the 3 taxa of the *A. simplex* species complex identified by molecular markers. A principal component analysis (PCA) was used to uncover patterns in specimens ordination based on the presence of specific morphometric traits (Jolliffe, 1986; Lessa, 1990). Because PCA requires linearity in the variation among data (Lessa, 1990), the morphometric data obtained were transformed into natural logarithms to allow for comparison between morphometric traits in different specimens and to eliminate the scale difference between variables.

To allow for and standardize comparison between different datasets, 2 distance matrices were derived from the molecular and morphometric characterization of specimens. An Euclidean distance matrix was used to measure the degree of morphometric similarity between the studied specimens. Euclidean distance allows a measurement of specimen similarity/dissimilarity in a multi-traits space (i.e., the measured morphometric characteristics), enabling the hierarchical partitioning of the variance, and thereby allowing calculation of the standardized differentiation estimated for morphological characters. The genetic distance matrix was obtained using the Tamura-Nei (TrN) model, based on mtDNA *cox2*, *rrnS*, and ITS rDNA sequence datasets obtained from the specimens studied morphologically, as implemented in jModeltest (Posada, 2008) using AIC (Posada and Buckley, 2004) performed using PAUP* (Swofford, 2003). Two separate PCAs were then performed on morphological and genetic distance matrices, providing an optimal approximation of the total multidimensional variation in fewer dimensions and maximizing the linear correlation between distance measurements in the ordination. The new information obtained was then used to reorganize the data into axes, each of them describing the intercorrelations among specific groups. A generalized Procrustes rotation (PR) was used to compare different ordinations of specimens based on both morphometric and genetic data. PR is a multivariate technique developed to simultaneously compare several datasets. It is based on a traditional singular value decomposition to decompose a matrix into principal components. The main idea of PR has been to compare 2 or more spaces, where the same variables (in the present case, the individuals of different species) are measured, by calculating a new set of factors (i.e., dimensions) that resemble all scores subspaces. The first 2 axes of the PCA ordinations were used to compare the morphometric and genetic traits of the sampled specimens. A Procrustes test (also known as analysis of congruence) was used to estimate the significances of the Procrustes statistics, assessing similarities between different ordinations. The significance was measured by a permutation test with 1,000 bootstrap replicates, measuring the Procrustes correlation *r* derived from the symmetric Procrustes residual. All the statistical analyses were performed by the program R (R Development Core Team, 2012).

RESULTS

Genetic identification of sibling species of *Anisakis* using MAE and sequences analysis at multiple genes

The adult specimens of *A. simplex* (s.l.) collected from the striped dolphin in the Mediterranean Sea (Table I) were genetically homogeneous at the allozyme level, belonged to the same gene pool, and were characterized by distinct alleles at the same diagnostic loci, as reported in previous studies (e.g., Mattiucci et al., 1997). Indeed, according to the alleles observed at the diagnostic loci, i.e., $Sod\text{-}1^{100}$, $Adk\text{-}2^{100}$, $PepB^{100}$, $PepC\text{-}1^{100}$, and $PepC2^{100}$, these specimens were assigned to the species *A. pegreffii*. Similarly, 3 nematodes recovered from pilot whales from off the New Zealand coast were also identified, based on the same alleles, as corresponding to *A. pegreffii*.

Likewise, all the adult specimens collected from minke whales and pilot whales from the northeastern Atlantic Ocean (Table I) were genetically homogeneous at the Hardy–Weinberg equilibrium and, according to the diagnostic alleles $Sod\text{-}1^{105}$, $Adk\text{-}2^{105}$, $PepB^{70}$, $PepC\text{-}1^{90}$ and $PepC2^{96}$ (Mattiucci et al., 1997), corresponded to the species *A. simplex* (s.s.).

Finally, 10 specimens collected from pilot whales in New Zealand waters (Table I) corresponded to *Anisakis simplex* sp. C, according to alleles $Adk\text{-}2^{100}$, $PepB^{70}$, $PepC\text{-}1^{92}$, $PepC2^{100}$, and $Aat\text{-}2^{105,110}$, with the latter locus being a unique allele (Mattiucci et al., 1997; Mattiucci and Nascetti, 2006). In addition, the 2 species, *A. pegreffii* and *A simplex* sp. C, were detected in sympatry and syntopy in the same individual hosts, i.e., pilot whales stranded on the New Zealand coast. No F1 hybrids were detected genetically, at the allozyme level, among the 3 sibling species.

The same specimens of *A. simplex* sp. C, *A. pegreffii*, and *A. simplex* (s.s.) identified by allozymes (MAE), plus those stored in alcohol (the latter does not permit the use of MAE), were sequenced at the 3 genes: the mitochondrial *cox2* and *rrnS* and the nuclear ITS region of rDNA. The sequence analysis was completed on the same 52 adult specimens studied (Table I). This confirmed the results achieved by allozymes, regarding the identification to the specific level of *A. simplex* (s.s.), *A. pegreffii*, and *A. simplex* sp. C, and it allowed the identification of all the specimens stored in alcohol (Table I).

The sequences obtained at the ITS rDNA region (908 bp) for specimens of the 3 *Anisakis* spp. collected from pilot whales in New Zealand waters and 20 specimens from striped dolphins in the Mediterranean Sea (Table I) were identical and matched 100% the sequence deposited in GenBank for the species *A. pegreffii*. Analogously, the 15 specimens of *Anisakis* spp. from hosts in the southeastern Pacific Ocean had identical ITS sequences that were a 99% match with the sequence deposited in GenBank (AY821736), corresponding to the species *A. simplex* sp. C. Finally, identical ITS sequences were found between 8 specimens from minke whales and the 6 specimens collected from pilot whales (including those individuals identified by MAE and those stored in alcohol) (Table I); they were found to correspond (99%) to the sequence in GenBank indicated as *A. simplex* (s.s.). These findings support the MAE in relation to the identification at the species level of the *Anisakis* specimens studied as belonging to *A. simplex* sp. C, *A. pegreffii*, and *A. simplex* (s.s.). The sequences obtained in the present study at the ITS region of the 3 species have been submitted to GenBank: their accession numbers for the ITS gene are as follows: *A. berlandi* (=*A. simplex* sp. C) (JX535519), *A. pegreffii* (JX535520), and *A. simplex* (s.s.) (JX535521) (Table I). In addition, some specimens of the other species of *Anisakis* used in the present study to infer phylogenetic relationships based on ITS rDNA sequences were sequenced and deposited in GenBank under the following accession numbers: *A. typica* (JQ912690), *A. ziphidarum* (JQ912691), *A. nascettii* (JQ912692), *A. physeteris* (JQ912693), *A. brevispiculata* (JQ912694), and *A. paggiae* (JQ912695). The sequence alignment of the ITS region of rDNA in the specimens of *A. simplex* (s.s.), *A. pegreffii*, and *A. berlandi* n. sp. (=*A. simplex* sp. C) studied in the present work, in comparison with that observed for other species of *Anisakis*, is given in Figure 1.

The same specimens of the 3 *Anisakis* taxa sequenced at the ITS rDNA region were also sequenced at the mitochondrial *cox2* gene. The sequences obtained at the mtDNA *cox2* gene (629 bp) of the 20 specimens of *A. pegreffii*, 14 individuals of *A. simplex* (s.s.), and 15 specimens of *A. simplex* C matched, respectively, the sequences deposited in GenBank for those species in our previous analysis (Mattiucci et al., 2009). The new sequences for the 3 species of the *A. simplex* complex have been submitted to GenBank for the mtDNA *cox2* gene, and their accession numbers are as follows: *A. berlandi* n. sp. (=*A. simplex* sp. C) (KC809999–KC810001), *A. pegreffii* (KC809996–KC809998) and *A. simplex* (s.s.) (KC810002–KC810004). Sequences of the mtDNA *cox2* gene revealed them to be highly polymorphic in the 3 species of the *A. simplex* complex with a high number of haplotypes detected in the *cox2* mitochondrial gene in all the 3 taxa. For *A. pegreffii*, 19 haplotypes were detected as occurring in the 2 definitive hosts from different geographical areas, whereas *A. simplex* (s.s.) exhibited 12 different haplotypes and 14 haplotypes were found in *A. berlandi* n. sp. (=*A. simplex* sp. C) identified from the same definitive host in southeastern Pacific waters.

Finally, the same specimens of the 3 species of *Anisakis* taxa were sequenced at the mitochondrial *rrnS* gene. The sequences obtained at this gene (519 bp) in the specimens of *A. pegreffii* collected from the different hosts and geographical areas were identical. Similarly, the 14 specimens of *A. simplex* (s.s.) recovered from the 2 different definitive hosts species were identical. *rrnS* sequences were found to be identical among the 15 specimens corresponding to the species *A. simplex* sp. C recovered from different individuals of *G. melas* in the southeastern Pacific Ocean. The *rrnS* sequences of *A. simplex* (s.s.), *A. pegreffii*, and *A. berlandi* n. sp. (=*A. simplex* sp. C) studied in the present work, and those for the other species of *Anisakis* used for comparison, were deposited in GenBank as follows: *A. berlandi* n. sp. (=*A. simplex* sp. C) (JX500049), *A. pegreffii* (JX500050), *A. simplex* (s.s.) (JX500051), *A. typica* (JX500052), *A. ziphidarum* (JX500053), *A. nascettii* (JX500054), *A. physeteris* (JX500055), *A. brevispiculata* (JX500056), and *A. paggiae* (JX500057). The sequence alignment of the *rrnS* in the specimens of *A. simplex* (s.s.), *A. pegreffii* and *A. simplex* sp. C studied in the present work, in comparison with that observed for the other species of *Anisakis*, is given in Figure 2.

Phylogenetic relationships

The MP inference obtained at the ITS ribosomal DNA region sequence analysis (Fig. 3) and the MP based on the combined sequences of the 2 mitochondrial genes mtDNA *cox2* and *rrnS* (Fig. 4) generated 2 trees having the same topology, depicting that all of the specimens collected from *S. coeruleoalba* off the Italian

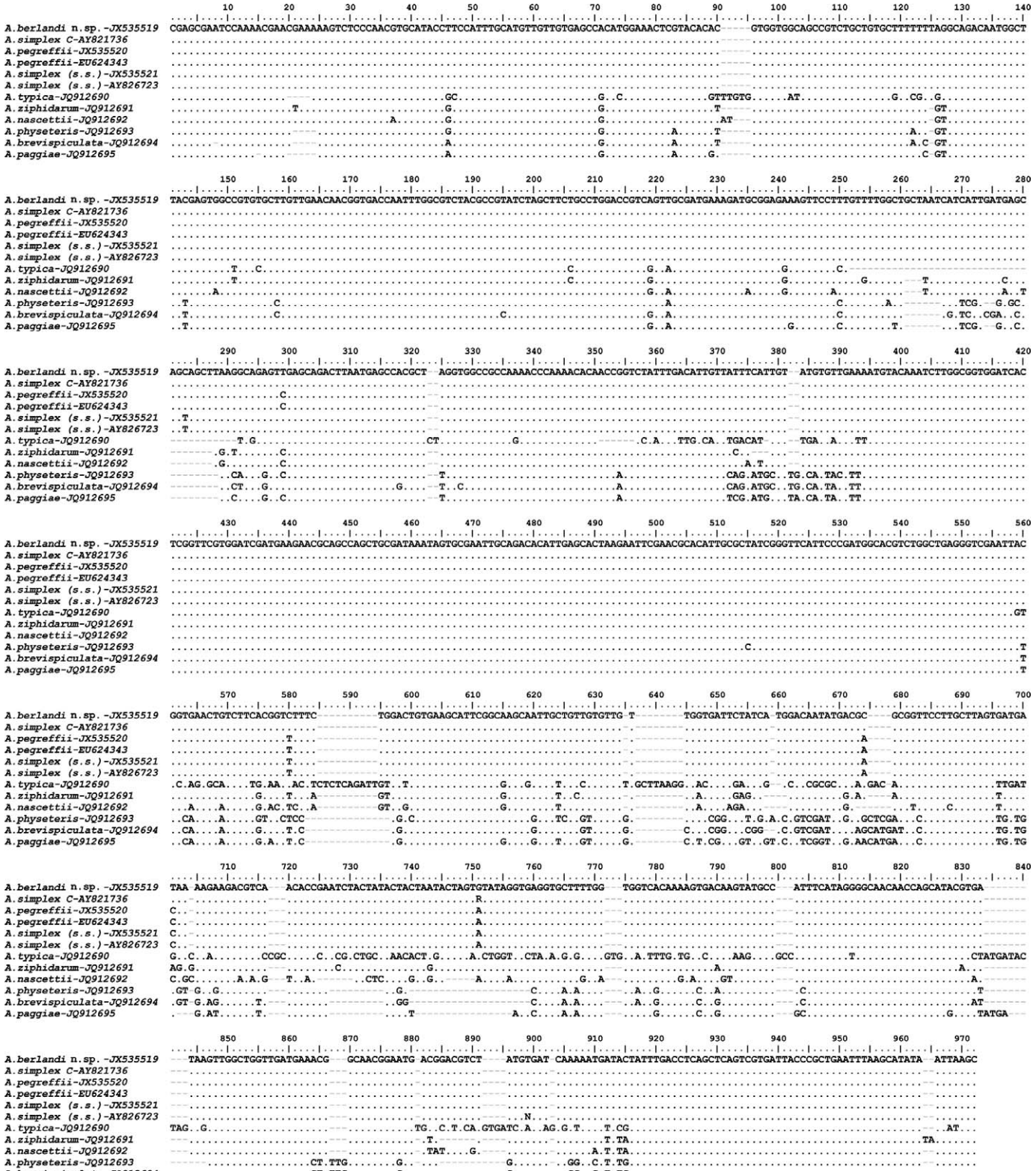

FIGURE 1. Alignment performed by Bioedit software (Hall, 1999) of the ITS region of the ribosomal DNA sequences in *Anisakis berlandi* n. sp. (=*A. simplex* sp. C), *A. simplex* (s.s.), and *A. pegreffii*, with respect to those of the same parasite species previously deposited in GenBank, and with respect to other *Anisakis* spp. sequenced at the same gene. One ITS rDNA sequence for each *Anisakis* species considered is reported with its GenBank accession number. Dots indicate identity and dashes indicate the gaps.

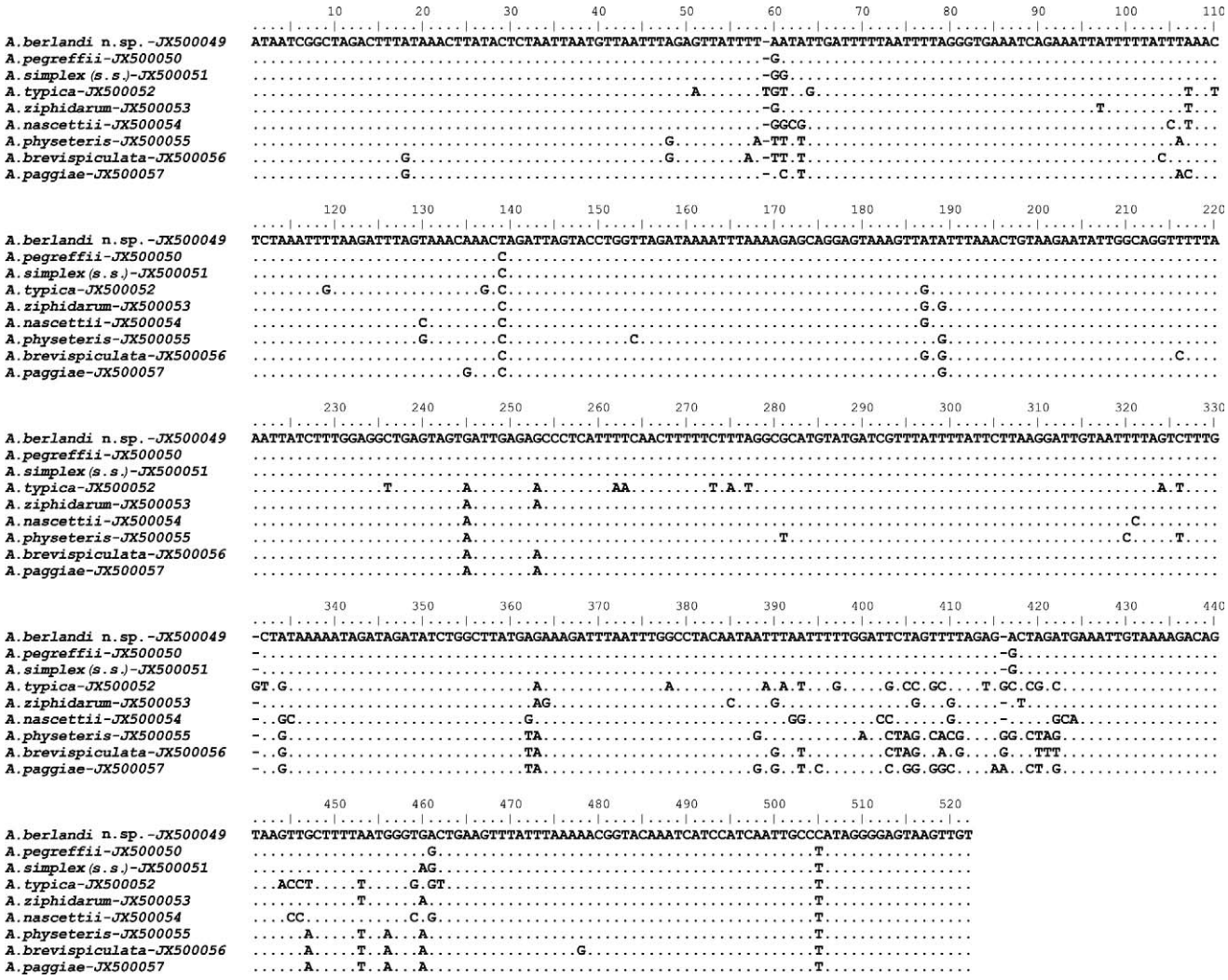

FIGURE 2. Alignment performed by Bioedit software (Hall, 1999) for the mitochondrial *rrnS* gene sequences of *Anisakis berlandi* n. sp. (=*A. simplex* sp. C), *A. simplex* (s.s.), and *A. pegreffii*, with respect to those of other *Anisakis* spp. One *rrnS* sequence for each *Anisakis* species considered is reported with its GenBank accession number. Dots indicate identity and dashes indicate the gaps.

coast and some of those collected from pilot whales off the New Zealand coast clustered together in a single and well-supported clade formed by specimens belonging to *A. pegreffii* (Figs. 3, 4). Similarly, the 14 specimens collected from minke and pilot whales in the northeastern Atlantic clustered in a unique clade, well supported by a high bootstrap value at both nuclear and mitochondrial levels (Figs. 3, 4), belonging to the species *A. simplex* (s.s.). Finally, 15 specimens collected from pilot whales on the New Zealand coast clustered in a third very distinct clade, again supported by high bootstrap values for the ITS rDNA region and at the 2 mitochondrial genes (Figs. 3, 4). This last clade corresponded to the species *A. berlandi* n. sp. (=*A. simplex* sp. C).

In addition, the MP analysis, using ITS sequence datasets (228 informative characters; consistency index [CI] = 0.87), delineated a tree showing 2 main clades. One clade was formed by the species of the *A. simplex* complex ((*A. berlandi* n. sp. (*A. pegreffii* and *A. simplex* (s.s.)) and ((*A. ziphidarum* and *A. nascettii*) and *A. typica*); however, the second clade did not receive high bootstrap value (Fig. 3). The second clade included (*A. paggiae* (*A. physeteris* and *A. brevispiculata*)) and it received a bootstrap value of 100.

Similarly, the combined MP tree inferred from the 2 mitochondrial sequences datasets produced a tree (490 informative characters; CI = 0.69) showing the following: 1 clade formed by the species of *A. simplex* (s.l.) complex (*A. berlandi* n. sp. (*A. pegreffii* and *A. simplex* (s.s.)), a second clade comprising the 2 species from ziphiids (*A. ziphidarum* and *A. nascettii*), and a third clade including (*A. paggiae* (*A. physeteris* and *A. brevispiculata*)) (Fig. 4). Finally, *A. typica* formed a separated clade, whose position as sister taxon to the 2 first clades seems to be not highly supported by a high bootstrap value (<70) using MP based on the combined mitochondrial genes (Fig. 4).

The concatenated inference, obtained from both the BI and the MP analyses based on the 3 combined nuclear (ITS rDNA) and mitochondrial (mtDNA *cox2* and *rrnS*) sequence datasets, resulted in identical topologies in supporting, with high posterior probability and bootstrap values (Fig. 5), the existence of the 3 species *A. simplex* (s.s.), *A. pegreffii*, and *A. berlandi* n. sp. (=*A. simplex* sp. C) as distinct phylogenetic lineages. The topology of the strict consensus tree (MP) (Fig. 5) and the Bayesian tree showed 4 main clades: 1 clade formed by (*A. berlandi* n. sp. (*A.

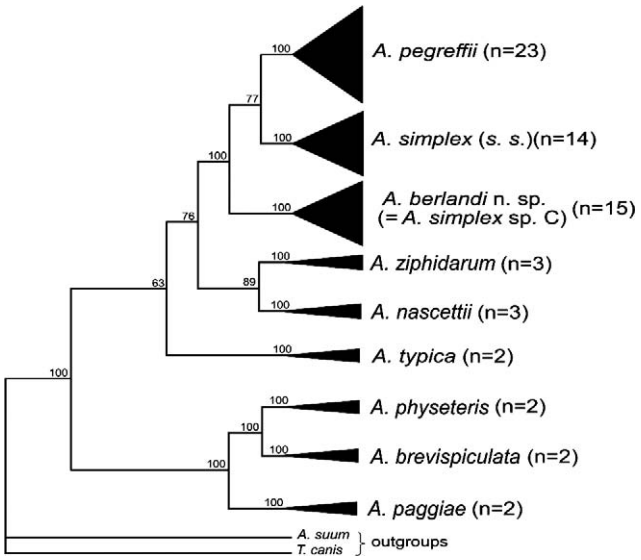

FIGURE 3. MP bootstrap consensus tree inferred by PAUP*4.0 (bootstrap method with heuristic search) (Swofford, 2003) from the sequences of the ITS rDNA region (228 parsimony informative characters; CI = 0.86) in the specimens of *Anisakis berlandi* n. sp. (=*A. simplex* sp. C) (n = 15), *A. simplex* (s.s.) (n = 14), and *A. pegreffii* (n = 23) analyzed herein (Table I), with respect to the other species of *Anisakis* sequenced for the same gene and deposited in GenBank. Numbers in brackets correspond to the individuals of each *Anisakis* species showing an identical ITS rDNA sequence. The vertical height of black triangles is proportional to the number of specimens sequenced. The analysis was run on 1,000 pseudoreplicates. Bootstrap values of clades as inferred by MP are shown at the nodes. *Toxocara canis* and *Ascaris suum* were used as outgroups.

pegreffii and *A. simplex* (s.s.)), with the support of 100% posterior probability in BI analysis and a bootstrap value of 100 at the MP inference; a second clade formed by the 2 species *A. ziphidarum* and *A. nascettii*, with the support of 100% at the BI and a bootstrap value of 77 at the MP inference; and a third clade formed by the species *A. physeteris*, *A. brevispiculata*, and *A. paggiae*, with a support of 100% posterior probability at the BI and a bootstrap value of 100 at the MP inference (Fig. 5). Finally, both concatenated phylogenetic trees (MP and BI) obtained from the combined mtDNA *cox2*, *rrnS*, and ITS rDNA sequences (Fig. 5) depicted *A. typica* as a separate lineage; its position as the sister group to the other main clades received a reliable bootstrap support at the MP analysis (81) and a posterior probability value of 100% at the BI analysis (Fig. 5).

Morphological and morphometric analysis of the 3 sibling species of the *A. simplex* complex

The absolute measurements and allometric data obtained on the same specimens of the 3 cryptic species are reported in Tables II and III. Apart for the total body length (TBL) and the ventriculus length (VL), the morphometric data were taken from male worms.

Some absolute measurements proved to be different in the 3 species. For example, VL measured 1.11 (0.80–1.35) in *A. simplex* sp. C, compared with 0.76 (0.55–0.90) in *A pegreffii* and 1.23 (1.08–1.44) in *A. simplex* (s.s.) (Fig. 6; Table II). However, although VL measurement was statistically significantly different among all 3 species (Fig. 6; Tables II, III) when allometry was taken into consideration, i.e., VL/TBL, statistically significant differences were observed only between *A. berlandi* n. sp. (=*A. simplex* sp. C) and both *A. pegreffii* and *A. simplex* (s.s.) and not between *A. pegreffii* and *A. simplex* (s.s.) (Table III).

The ratio between the right and left spicule lengths (R/L) exhibited a significant variation in *A. simplex* sp. C (0.49–0.70) with respect to *A. simplex* (s.s.) (0.46–0.83); furthermore, the ratio between both the right and left spicules with respect to the total body length (RS/TBL and LS/TBL) differed significantly for all 3 species (Table III). The length of the left spicule (LS) of *A. simplex* sp. C (2.10–2.90) was significantly different from that of both *A. simplex* (s.s.) (1.70–2.80) and *A. pegreffii* (1.50–2.65). Similarly, the ratio between right spicule (RS) size and TBL (RS/TBL) resulted in differences between *A. simplex* sp. C (0.024–0.027), *A. pegreffii* (0.026–0.039), and *A. simplex* (s.s.) (0.016–0.027). However, LS size in relation to TBL (LS/TBL) exhibited a significant difference only between *A. simplex* sp. C (0.039–0.058) and *A. simplex* (s.s.) (0.029–0.036) (Table III).

In the species *A. simplex* sp. C, the caudal region bearing the caudal papillae and other structures of the male nematode appears raised to resemble a "platform" clearly distinct from the remaining part of the caudal end of the nematode (Fig. 6); conversely, in *A. pegreffii* and *A. simplex* (s.s.) the topography of this same region blends more closely with the body and is often larger. Allometric characters, such as the TL with respect to the TBL (TL/TBL), were significantly different among all 3 species (Table III). Some allometric characters also support the overall differences observed in tail shape between the 3 species: in *A. simplex* sp. C the TL/TBL (0.0048–0.0060) differed significantly from both *A. simplex* (s.s.) (0.0030–0.0049) and *A. pegreffii* (0.0043–0.0080) (Tables II, III).

In addition, the distance between distal papillae d1 and paracloacal papillae pc (dpc-d1), when related to TL (dpc-d1/TL) showed different values between *A. simplex* sp. C (0.19–0.33) and *A. simplex* (s.s.) (0.25–0.31) (Fig. 6; Tables II, III). The male caudal PLs (PL1, PL2, and PL3) differed in shape and width among the 3 species (Fig. 6; Tables II, III). Indeed, in *A. simplex* (s.s.) the lateral extremities of the PL structures are rounded; conversely, they are more flattened and pointed in *A. pegreffii*. In addition, the ratio between the width of PL1 and PL3 (WPL1/WPL3) was significantly greater in *A. simplex* sp. C than in *A. pegreffii* (Fig. 6; Table III). Finally, in *A. simplex* sp. C, TBL (26–62 mm) was different from *A. simplex* (s.s.) (49–112), when all of the specimens of the latter species parasitic in different hosts, i.e., pilot and minke whales, were considered, whereas there were no differences in TBL among the 3 species *A. simplex* (s.s.) and *A. simplex* sp. C obtained from the same definitive host, a pilot whale.

The PCA carried out on the morphometric and allometric data from the 3 species showed a clear differentiation of sampled specimens corresponding to the species *A. pegreffii*, *A. simplex* (s.s.), and *A. simplex* sp. C, with regard to specific traits, with the first axis of the PCA (PCA1) explaining approximately 73% of the total variance and the second axis approximately 18% (Fig. 6). Therefore, the variation between specimens along the PCA1 axis was correlated mostly with the VL trait ($r = 0.789$), to a lesser extent with the WPL1/WPL3 trait ($r = 0.48$), and lastly with the VL/TBL trait ($r = 0.37$) (Fig. 6). The WPL1/WPL3 trait exhibited the highest correlation on the second axis ($r = 0.74$). For the VL trait (Fig. 6), the specimens belonging to *A. simplex* sp. C and *A.*

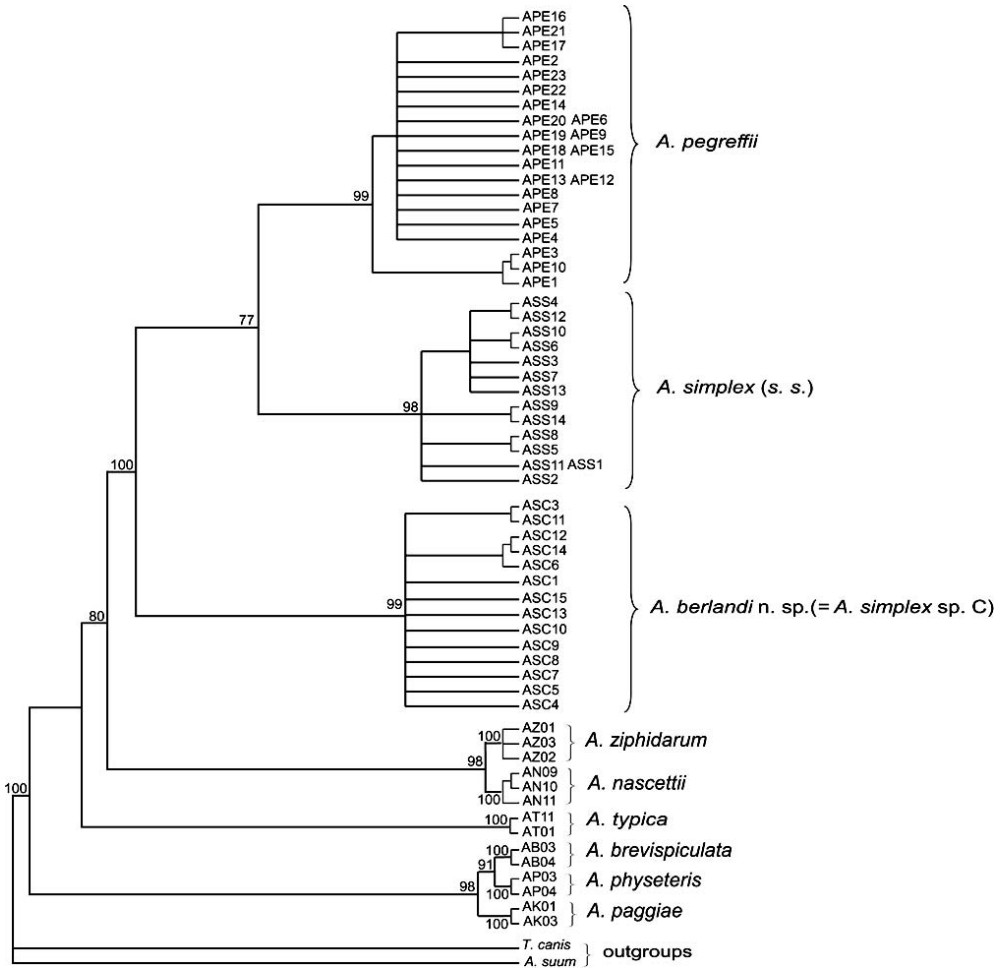

FIGURE 4. MP bootstrap consensus tree inferred by PAUP*4.0 (bootstrap method with heuristic search) (Swofford, 2003) based on the combined mitochondrial *cox2* and *rrnS* genes sequences (490 parsimony informative characters; CI = 0.69) in the 52 specimens of *Anisakis berlandi* n. sp. (=*A. simplex* sp. C), *A. simplex* (s.s.), and *A. pegreffii* analyzed herein (see Table I for specimens code), with respect to the other species of *Anisakis* sequenced for the same gene and deposited in GenBank. The sequences datasets were first determined to be combinable by using the partition homogeneity test (Farris et al., 1994), as executed in PAUP* (Swofford, 2003). Bootstrap values (≥70), reported at the nodes, were obtained over 1,000 pseudoreplicates. *Toxocara canis* and *Ascaris suum* were used as outgroups.

simplex (s.s.) followed a similar trend, whereas specimens attributed to *A. pegreffii* showed the opposite trend (Fig. 6). The WPL1/WPL3 trait exhibited intraspecific variation across specimens of *A. pegreffii* (Fig. 6; Tables II, III).

When constructing the PCA performed on both molecular and morphometric distance matrices, the first axis based on the molecular/genetic distance matrix (data not shown) accounted for most of the variation (approximately 57%). A similar trend was observed in the ordination of the specimens based on the morphometric distance matrix, with the first axis of the PCA explaining 40% of the total ordination (data not shown). However, the intra-group similarity based on morphometric variables was less pronounced than the genetic similarity. This could be explained by a high intraspecific variation in specific morphological traits, as shown in Figure 6 (i.e., the WPL1/WPL3 trait). The PR (Fig. 7) showed convergence between different ordinations based on the distance values obtained from both genetic and morphometric characteristics among specimens of the 3 species. This resulted in a pattern characterized by a clustering of the specimens in 3 well-defined groups, each representing a different member of the *A. simplex* species complex (Fig. 7). These results suggest that both the morphometric and genetic traits (explained by the first dimension of the PR) convey similar information (Procrustes $r = 0.87$, $P < 0.001$), thus providing interchangeable information useful for the assessment of morphological and genetic variation between specimens of different species.

DESCRIPTION

Anisakis berlandi n. sp.

(Fig. 8; Table IV)

General: Body stout. Cuticular annulations present on entire body, except for lips. Dorsal and ventrolateral lips with low anterior projection bearing dentigerous ridges on its inner surface. Dorsal lip with 2 double papillae; each ventrolateral lip with 1 double papilla, 1 single papilla and amphid. Interlabia absent. Excretory pore opens between ventrolateral lips. Mouth triangular in apical view. Esophagus muscular; ventriculus often curved and S-shaped (sigmoid), thin-walled, oblong, connects with intestine slightly obliquely.

Male (measurements given for holotype and 10 paratypes): TBL 51.23 (42.00–60.00); body width (BW) 1.60 (1.52–1.73). Esophagus 4.59 (4.10–

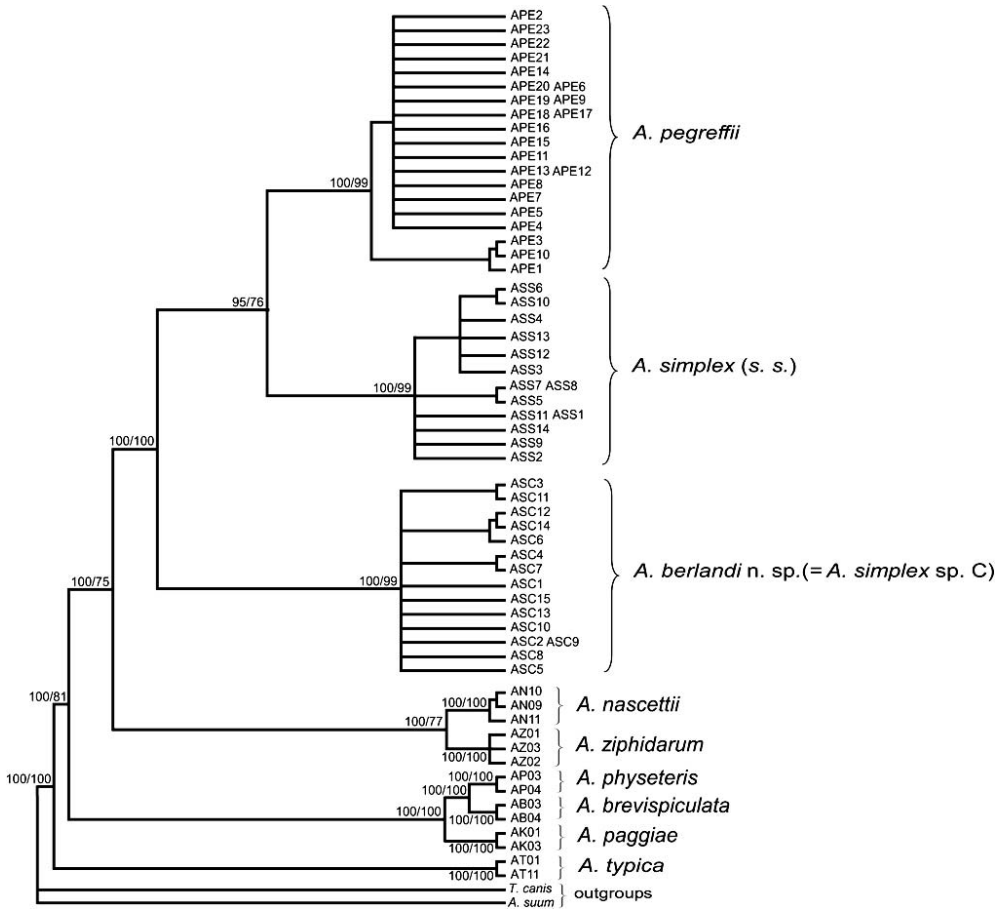

FIGURE 5. BI tree, performed by MrBayes3.1 (Huelsenbeck and Ronquist, 2005) on the combined mitochondrial *cox2*, *rrnS*, and nuclear ITS rDNA sequences datasets of the 52 specimens of *Anisakis berlandi* n. sp. (=*Anisakis simplex* sp. C), *A. simplex* (s.s.), and *Anisakis pegreffii* analyzed herein (see Table I for specimen codes), with respect to the other species of *Anisakis* sequenced for the same genes. The sequences datasets were first determined to be combinable by using the partition homogeneity test (Farris et al., 1994), as executed in PAUP* (Swofford, 2003). JModeltest (Posada, 2008) was used to determine the best-fit model for each gene considered in the study, as implemented in the AIC (Posada and Buckley, 2004). BI was performed using Trn+I+G model (I = 0.089 and G= 0.182) for mtDNA *cox2*, HKY+G (G = 0.01) for *rrnS*, and K80+G (G = 0.12) for the ITS region of rDNA. The Bayesian posterior probability analysis was performed using the Markov chain Monte Carlo algorithm: the number of chains was 4; the temperature of heated chains was 0.2; the number of generations was 1,000,000; and the subsampling frequency was 100, with a burn-in fraction of 0.25. Posterior probabilities were estimated and used to assess support for each branch in the inferred phylogeny, with probabilities $P = 95\%$ indicative of significant support (Reeder, 2003). Posterior probability values listed first, followed by the bootstrap MP values. The MP bootstrap consensus tree, inferred by PAUP*4.0 (bootstrap method with heuristic search) (Swofford, 2003) based on the combined mitochondrial (*cox2* and *rrnS* genes) and nuclear (ITS rDNA) sequences in the same 52 specimens of *Anisakis* spp. studied, depicted the same tree topology as BI inference. Bootstrap values ($\geq$70), reported at the nodes, were obtained over 1,000 pseudoreplicates. Trees rooted by *Ascaris suum* and *Toxocara canis* sequences at the same nuclear and mitochondrial genes analyzed.

5.80) long. Ventriculus 1.05 (0.90–1.12) long. VL/TBL 0.022 (0.019–0.026). Spicules unequal; distal end with rounded point; LS 2.45 (2.10–2.90), RS 1.34 (1.10–1.58); R/L 0.58 (0.49–0.70); LS/TBL 0.047 (0.039–0.058); RS/TBL 0.026 (0.024–0.027). TL 0.271 (0.220–0.290), with ventral surface raised to form pyriform platform bearing most of postcloacal papillae and PLs. TL/TBL 0.0050 (0.0048–0.0058). Three PLs (caudal plates) with spinose margins; first plectane (pl1) closest to cloaca, 0.085 (0.068–0.110) in width (WPL1); second plectane (pl2) slightly narrower than pl1, 0.083 (0.075–0.102) in width (WPL2); distal plectane (pl3) 0.089 (0.076–0.114) in width (WPL3).

Caudal papillae (nomenclature according to Fagerholm, 1989, Paggi et al., 1998, and Mattiucci et al., 2005): proximal papillae numerous and disposed in single ventrolateral row on each side; 1 median papilla; 1 pair of proximal papillae (p) posterior to cloaca, lateral to PLs; 1 pair of double paracloacal papillae (pc); and 4 pairs of distal papillae (d1, d2, d3, and d4). Measurements of paracloacal papillae: pc = 0.027 (0.023–0.035), d2–d4 = 0.015 (0.013–0.016), d1 = 0.018 (0.015–0.021); distance between pc and d1 (dpc-d1) = 0.074 (0.060–0.107); ratio with respect to TL (dpc-d1/TL) = 0.27 (0.19–0.33). One pair of very small papilla-like phasmids situated laterally and posterior to last pair of distal papillae.

Female (measurements given for 5 specimens): TBL 48.25 (26.0–62.0). Esophagus 5.36 (5.15–5.57) long. Ventriculus 1.15 (0.80–1.35) long. VL/TBL 0.026 (0.021–0.030). TL 0.28 (0.26–0.30). Vulva in first third of body length. Eggs 49 (45–53) × 54 (49–56) μm.

Taxonomic summary

Synonym: Anisakis simplex sp. C of Mattiucci et al. (1997).

Type host: Globicephala melas Traill (Cetacea: Delphinidae).

Type locality: Southern Pacific Ocean (off the New Zealand coast); other geographical locality: northern Pacific Ocean.

Other definitive hosts: Pseudorca crassidens (Owen) (Cetacea: Delphinidae) from the northeastern Pacific Ocean (off Vancouver Island).

Site: Stomach.

Collector: Stephen Webb.

Type material: Holoype: 1 male (ASC1) collected from New Zealand pilot whale on the New Zealand coast. Anterior and posterior regions of

TABLE II. Morphometric data of adults of *Anisakis berlandi* n. sp. (=*A. simplex* sp. C), *A. pegreffii*, and *A. simplex* (s.s.) studied herein. The data are presented in comparison with those of *A. simplex* (s.s.) and *A. pegreffii* given by Quiazon et al. (2008) and with respect to *A. ivanizkii* and *A. kukenthalii*. n, number of specimens studied; L, length; W, width. Ratio values are reported as a percentage. All measurements are given in millimeters.

Parasite species	*A. berlandi* n. sp.	*A. pegreffii*	*A. simplex* (s.s.)
Reference	This study	This study	This study
Host	*G. melas*	*G. melas*, *S. coeruleoalba*	*G. melas*, *B. acutorostrata*
Specimen (n)	15 (11 males, 4 females)	23 (15 males, 8 females)	14 (12 males, 2 females)
TBL (mm)	50.0 (26.0–62.0)	41.6 (33.0–55.0)	70.0 (49.0–11.2)
Esophagus length	4.69 (4.10–5.80)	4.30 (3.36–5.10)	–
VL	1.11 (0.80–1.35)	0.76 (0.55–0.90)	1.29 (1.08–1.44)
VL/TBL	0.023 (0.016–0.030)	0.018 (0.013–0.023)	0.018 (0.011–0.028)
Ventriculus shape	Sigmoid	Sigmoid	Sigmoid
LS	2.50 (2.10–2.90)	2.10 (1.50–2.65)	2.17 (1.70–2.80)
RS	1.35 (1.20–1.60)	1.30 (1.00–1.60)	1.44 (1.16–1.82)
R/L	0.58 (0.49–0.70)	0.64 (0.54–0.77)	0.68 (0.46–0.83)
LS/TBL	0.047 (0.039–0.058)	0.047 (0.037–0.056)	0.033 (0.029–0.036)
RL/TBL	0.026 (0.024–0.027)	0.029 (0.026–0.039)	0.023 (0.016–0.027)
TL	0.27 (0.22–0.29)	0.25 (0.19–0.32)	0.28 (0.22–0.37)
TL/TBL	0.005 (0.005–0.006)	0.006 (0.004–0.008)	0.004 (0.003–0.005)
WPL1	0.086 (0.068–0.11)	0.082 (0.077–0.087)	0.077 (0.066–0.093)
WPL2	0.083 (0.075–0.102)	0.081 (0.076–0.086)	0.076 (0.065–0.092)
WPL3	0.089 (0.076–0.114)	0.081 (0.074–0.089)	0.074 (0.061–0.088)
WPL1/WPL3	1.04 (1.01–1.11)	0.94 (0.87–1.03)	0.97 (0.94–1.00)
Largest PLW/TL	0.33 (0.26–0.39)	0.32 (0.27–0.41)	0.33 (0.26–0.50)

the body are deposited in the Natural History Museum (NHM), London, under NHMUK 2013.6.10.1; mtDNA *cox2* sequence of the same specimen is deposited in GenBank with the following accession numbers: DQ116429 for the mtDNA *cox2*, JX535519 for ITS rDNA, and JX500049 for the *rrnS*. Paratypes: 1 male (ASC6) and 2 females (ASC10 and ASC11) from the same host and locality as the holotype; anterior and posterior regions are deposited, at NHM, under NHMUK 2013.6.10.2–4. Other material examined: 7 males and 3 females from the same host and locality as the holotype.

Etymology: The species *A. simplex* sp. C is affectionately named herein as *A. berlandi* n. sp., dedicated to Emeritus Professor Bjørn Berland of the Zoological Institute of Bergen University, Bergen, Norway, whose scientific intuition with regard to the systematics and ecology of anisakid nematodes during the last century anticipated much of the findings obtained in the past 20 yr by molecular systematics and phylogenetic approaches. Without his passionate interest in these worms, some of the studies of some of the authors of this paper could never have existed.

Remarks

According to the genetic data gathered using allozymes and the DNA sequencing at multiple loci (ITS rDNA, mtDNA *cox2*, and *rrnS*), *A. berlandi* n. sp. (formerly indicated as *A. simplex* sp. C) represents a distinct phylogenetic lineage, with respect to the species *A. pegreffii* and *A. simplex* (s.s.). Indeed, in both the MP and the BI phylogenetic concatenated analyses performed on 3 combined genes, i.e., mtDNA *cox2*, *rrnS* rRNA, and ITS rDNA, all the specimens belonging to the species *A. berlandi* n. sp. that were analyzed at the morphological level formed a very well-supported clade with respect not only to *A. pegreffii* and *A. simplex* (s.s.) but also to the other species of *Anisakis*.

At the statistical level, some morphological characters, taken as absolute measurements, do permit the recognition of adult males of *A. berlandi* n. sp., and a reliable identification can be obtained by using a combined set of morphological and morphometric characters with respect to the other sibling species of the *A. simplex* species complex (Table III). This species has so far been reported from oceanic dolphins in the Pacific Ocean (Mattiucci et al., 1997; this study).

A search for an existing name for *A. berlandi* n. sp. from the list of synonyms of *A. simplex* (s.l.) provided by Davey (1971) enabled a morphological comparison of *A. berlandi* n. sp. with only 2 taxa with a similarly shaped male caudal region: *A. kuekenthalii* (see Cobb, 1889) and *A. ivanizkii* Mozgovoi, 1949. However, in a morphological and morphometric comparison, *A. berlandi* n. sp. differs from *A. ivanizkii* in VL (0.80–1.35 vs. 1.54 mm in *A. ivanizkii*) and in the measurement of LSs and RSs (2.10–2.90 and 1.10–1.58 vs. 3.16–3.70 and 2.20–2.56 in *A. ivanizkii*, respectively); consequently, the RS/LS are also different. Unfortunately, several other measurements and features (such as PL shape and width, and the distribution pattern of the caudal papillae) were not reported in the original description. The host and geographical distribution of *A. ivanizkii* are also quite different, as it was originally described from the sperm whale off the Commander Islands in the North Pacific (Mozgovoi, 1953). With respect to *A. kuekenthalii*, *A. berlandi* n. sp. exhibits a different ratio between the short and long spicules (0.58 vs. 0.75 in *A. kuekenthalii*) and has a different LS/TBL (0.047 vs. 0.035 in *A. kuekenthalii*). Moreover, the host, the beluga whale, *Delphinapterus leucas* (Monodontidae), and type locality, the northeastern Arctic Ocean, of *A. kuekenthalii* (see Cobb, 1889) are not in accordance with the known data for *A. berlandi* n. sp. Therefore, we consider it appropriate to erect *Anisakis berlandi* n. sp. as a new specific name for *A. simplex* sp. C (see Mattiucci et al., 1997).

Genetic differentiation at inter- and intraspecific levels

The interspecific sequence differentiation among the 3 sibling species, as described by the TrN value implemented using JModeltest on the sequence datasets, ranged from a TrN of 0.01 observed at the *rrnS* gene level between the species *A. berlandi* n. sp. and *A. simplex* (s.s.) to a TrN of 0.06 found at the *cox2* level between, for example, *A. berlandi* n. sp. and *A. simplex* (s.s.). Indeed, a higher level of genetic differentiation among the 3 sibling species was observed at the mtDNA *cox2* gene level, where the TrN value was 0.05 between *A. berlandi* n. sp. and *A. simplex* (s.s.) but 0.06 between *A. berlandi* n. sp. and *A. pegreffii* and between *A. pegreffii* and *A. simplex* (s.s.). Lower values of genetic differentiation were observed at both ITS rDNA and at the *rrnS* levels, where pairwise genetic differentiation resulted in a TrN value of 0.01 among these 3 *Anisakis* species.

At the intraspecific level, a lower genetic differentiation estimation of TrN of 0.008 for all 3 species was observed for the mitochondrial gene mtDNA *cox2*.

DISCUSSION

The multigene molecular approach based on allozymes and sequence analyses of nuclear and mitochondrial genes used herein demonstrated the presence and distinction of the 3 former sibling

TABLE II. Extended.

A. pegreffii	*A. simplex* (s.s.)	*A. ivanizkii*	*A. kuekenthalii*	*A. kuekenthalii*
Quiazon et al. (2008)	Quiazon et al. (2008)	Mosgovoy (1949)	Mosgovov (1949)	Davey (1971)
In vitro cultured	In vitro cultured	–	–	–
8 (males)	11 (males)	–	–	24
14.30–20.55	17.68–29.65	(56–85)	(45–65)	
2.00–2.95	2.33–2.95	–	–	–
0.50–0.70	1.10–1.40	1.54	0.95	
–	–			
Sigmoid	Sigmoid			
–	–	3.17–3.72	2.30	1.80–2.25
–	–	2.20–2.56	1.70	1.05–1.80
–	–	0.81–0.59	0.75	0.86–0.50
–	–	0.043	0.035	
–	–			
–	–			
–	–	–	–	–
–	–	–	–	–
–	–	–	–	–
–	–	–	–	–

species of the *A. simplex* species complex as 3 independent evolutionary lineages (i.e., species). It also provided further evidence for their reproductive isolation, even in sympatric situations. Indeed, further support in relation to the existence of the sibling species *A. berlandi* n. sp., as a biological species distinct from *A. pegreffii* and *A. simplex* (s.s.), was demonstrated by the finding of adults belonging to this species in sympatry with specimens of *A. pegreffii* in the same definitive host, a pilot whale, in New Zealand waters (this study), and with *A. simplex* (s.s.) in a false killer whale from North Pacific waters (Mattiucci et al., 1997). No F1 hybrids and/or introgressed individuals at the adult stage among the 3 species have been detected even in syntopic and sympatric situations at the studied genes.

Furthermore, the concatenated phylogenetic analysis, carried out on mtDNA *cox2*, *rrnS* rRNA, and ITS rDNA sequence datasets (Fig. 5) was concordant in showing that *A. simplex* (s.s.), *A. pegreffii*, and *A. berlandi* n. sp. are 3 independent lineages and that *A. simplex* (s.s.) and *A. pegreffii* are sister taxa. The same tree topology was obtained at the MP inference based on the ITS rDNA sequences analysis and by the same analysis obtained on the sequence data of the 2 combined mitochondrial genes. According to the tree topologies obtained, *A. pegreffii*, *A. simplex* (s.s.), and *A. berlandi* n. sp. are phylogenetically very closely related, despite representing 3 clearly distinct clades well supported by bootstrap analysis. However, the phylogenetic analysis (MP) of the ITS rDNA sequences dataset has shown that the 3 species of the *A. simplex* complex form a monophyletic group with the 2 species from ziphiids (*A. ziphidarum* and *A. nascettii*) and *A. typica*, even if this main clade did not receive high support. However, the precise positioning of *A. typica* is not clearly resolved, although it consistently represents a sister taxon to the other clades in the phylogenetic analyses based upon concatenated MP and BI.

Alternatively, with the exception of the position of *A. typica*, an overall general congruence was found between the tree topologies obtained from both separate and combined datasets of phylogenies based on 3 genes and the phenetic clustering gathered from

TABLE III. *P* values indicating the significance in the Student's *t*-test of morphometric and allometric measurements between pairs of cryptic species of the *A. simplex* species complex. NS, not significant.

	A. berlandi n. sp. (=*A. simplex* sp. C) versus *A. pegreffii*	*A. berlandi* n. sp (=*A. simplex* sp. C) versus *A. simplex* (s.s.)	*A. pegreffii* versus *A. simplex* (s.s.)
VL	$P < 0.01$	$P < 0.01$	$P < 0.01$
VL/TBL	$P < 0.01$	$P < 0.05$	NS
R/L	NS	$P < 0.05$	NS
LS/TBL	NS	$P < 0.01$	$P < 0.01$
RS/TBL	$P < 0.05$	$P < 0.05$	$P < 0.01$
TL/TBL	$P < 0.05$	$P < 0.01$	$P < 0.01$
dpc-d1/TL	NS	NS	$P < 0.05$
WPL1/WPL3	$P < 0.05$	NS	NS

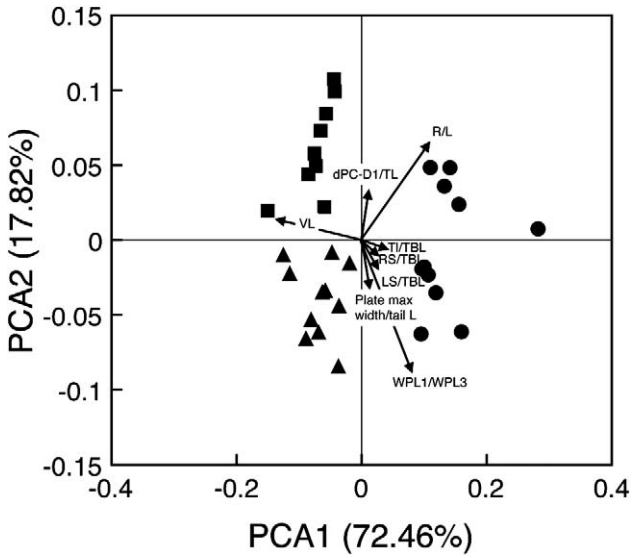

FIGURE 6. PCA analysis performed on specimens of *Anisakis berlandi* n. sp. (=*Anisakis simplex* sp. C), *A. pegreffii*, and *Anisakis simplex* (s.s.) by using morphological variables. Morphological ordination of specimens is based on the standardized differentiation estimated from their ordination along the Euclidean space. PCA ordination shows the clustering of sampled specimens in 3 well-defined groups on the basis of their relationship with the measured morphometric traits, with the first PC1 axis explaining 72.46% of total variance in the ordination. Triangles, *A. berlandi* n. sp. (=*A. simplex* sp. C); squares, *A. simplex* (s.s.); circles, *A. pegreffii*. Trait acronyms are the same as Table II.

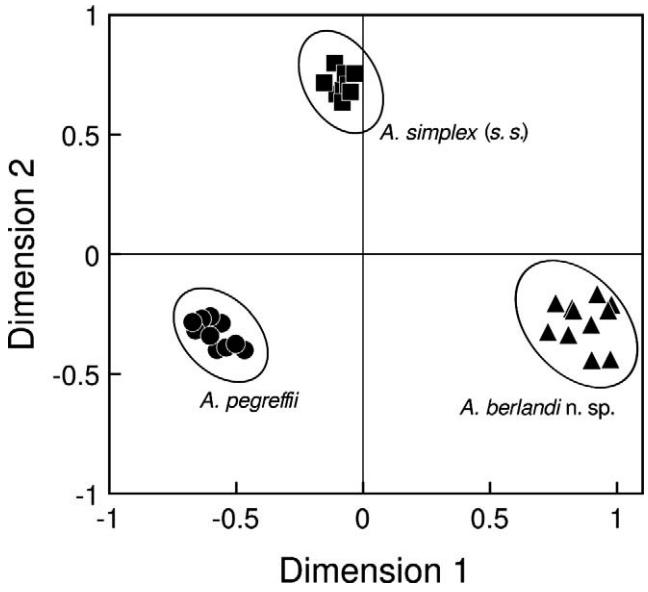

FIGURE 7. Procrustes ordination of specimens based on combined morphological and genetic datasets of *Anisakis simplex* (s.s.), *A. pegreffii*, and *Anisakis berlandi* n. sp. (=*A. simplex* sp. C). Procrustes analysis was performed by comparing the morphometric and molecular ordination (based on the 3 genes studied) of the specimens. Analysis exhibited a significant correlation (Procrustes $r = 0.89$, $P < 0.001$) between morphological and molecular traits, showing the clustering of the sampled specimens in 3 well-defined clusters corresponding to the 3 sibling species. Triangles, *A. berlandi* n. sp. (=*A. simplex* sp. C); squares, *A. simplex* (s.s.); circles, *A. pegreffii*.

allozyme data generated previously (Valentini et al., 2006; Mattiucci et al., 2009).

The data from our study represent the first finding of the adult stage of *A. berlandi* n. sp. in dolphins from South Pacific waters; previously, in this area, this species was reported (Mattiucci et al., 1997) at the larval 3 stage in the New Zealand teleosts *Parapercis colias*, *Pseudophycis bachus*, and *Thyrsites atun*. Larval stages of the species *A. simplex* sp. C were previously recognized genetically (sequences analysis of the ITS1 and ITS2 genes of the rDNA) in a dwarf sperm whale, *Kogia sima*, from Australian waters (Sahmsi et al., 2012).

Moreover, herein, a formal description of *A. simplex* sp. C is presented under the name *A. berlandi* n. sp., which is proposed for this taxon. Indeed, Nadler and Pérez-Ponce de León (2011) suggested that "cryptic" sister-taxa detected by genetic data are always "provisionally" cryptic, until additional morphological study reveals diagnostic structural differences that permit a morphological diagnosis and a formal species description. The molecular clades depicted by the phylogenetic trees for the 3 species of *Anisakis* provided a basis that enabled the investigation of several morphological structures, such as VL, PL structure and dimensions (e.g., WPL), spicule lengths (LS and RS), and male caudal papillae arrangement and associated ratios. Key morphological traits diagnostic for the 3 species are VL, tail shape, TL/TBL, and LS/TBL for *A. berlandi* n. sp. versus *A. simplex* (s.s.); VL and WPL1/WPL3 for *A. berlandi* n. sp. versus *A. pegreffii*; and VL, LS/TBL, RS/TBL, and TL/TBL for *A. simplex* (s.s.) and *A. pegreffii*. In contrast, VL and shape, PL shape and PLW, and spicule lengths have been previously found to be of diagnostic value for other sister-species of *Anisakis*, such as between *A. nascettii* and *A. ziphidarum* (see Mattiucci et al., 2009) and between *A. brevispiculata* and *A. paggiae* (see Mattiucci et al., 2005) as well as between sister-taxa of other anisakid genera, such as between *Contracaecum gibsoni* and *C. overstreeti* (see Mattiucci et al., 2010).

The measurement of the ventriculus and distal papillae distribution pattern has been reported as a distinctive feature distinguishing adults, cultured in vitro, belonging to *A. pegreffii* and *A. simplex* (s.s.) (Quiazon et al., 2008); however, the same study did not mention other morphometric characters differentiating the 2 taxa. They reported that VL does not increase with body length; in agreement with this, measurements of the ventriculus of *A. pegreffii* and *A. simplex* (s.s.) reported herein proved to be constant, between a fixed range, despite differences in the TBL of the worms. Furthermore, Quiazon et al. (2008) reported a different distribution pattern for the distal papillae between *A. pegreffii* and *A. simplex* (s.s.) "... having the 3rd pair of distal papillae located inside the 4th pair in *A. simplex* (s.s.), while, in *A. pegreffii* the 3rd pair were located outside the 4th pair." Nevertheless, the nomenclature for the distal papillae used by Quiazon et al. (2008) is not in accordance with that proposed by Fagerholm (1989) and used herein. Regardless, the specimens of *A. pegreffii* observed herein exhibited a distribution pattern for the distal papillae (Fig. 5) that is in agreement with the illustrations of Quiazon et al. (2008), although our specimens of *A. simplex* (s.s.) showed a higher variability in the distribution of the distal papillae. We found a close morphological similarity in the distribution pattern of the distal papillae between *A. pegreffii* and *A. berlandi* n. sp. (Fig. 6) and, therefore, it seems that this character cannot be considered a constant diagnostic feature for distinguishing the 3 sibling species.

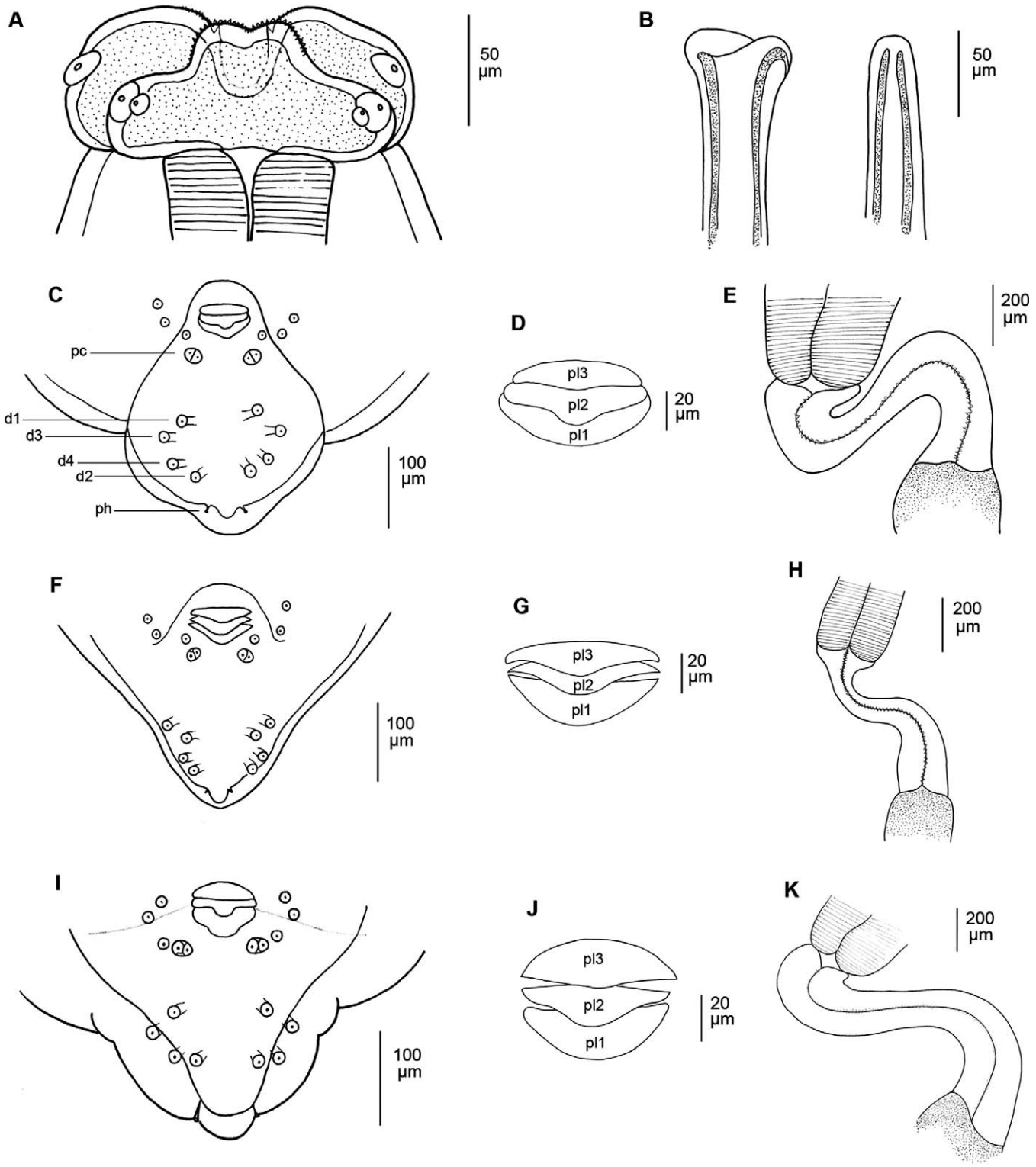

FIGURE 8. *Anisakis berlandi* n. sp. (**A**) Anterior end, dorsal view. (**B**) Proximal and distal ends of the spicules. (**C**) Posterior end of the male, ventral view. (**D**) PLs. (**E**) Ventriculus, anterior end. *Anisakis pegreffii*. (**F**) Posterior end of the male, ventral view. (**G**) PLs; (**H**) Ventriculus, anterior end. *Anisakis simplex* (s.s.). (**I**) Posterior end of the male, ventral view. (**J**) PLs. (**K**) Ventriculus, anterior end.

TABLE IV. Morphometric data from *A. berlandi* n. sp. (=*A. simplex* sp. C). Ratio values are reported as a percentage. All the measurements are expressed in millimeters.

Morphological character	Minimum–maximum	Average	SD
TBL	26.00–62.00	50.53	9.26
BW	1.60–1.73	1.61	0.01
Esophagus length (EL)	4.10–5.80	4.69	0.61
Esophagus width	0.30–042	0.36	0.05
VL	0.80–1.35	1.09	0.18
VL/TBL	1.90–3.00	2.30	0.40
VL/EL	16.00–29.00	23.30	4.50
LS	2.10–2.90	2.45	0.29
RS	1.10–1.58	1.34	0.17
R/L	49.00–70.00	58.40	6.30
LS/TBL	3.90–5.80	4.70	0.60
RS/TBL	2.40–2.70	2.60	0.10
TL	0.22–0.29	0.27	0.02
TL/TBL	0.48–0.58	0.53	0.04
dpc-d1	0.06–0.11	0.07	0.01
dpc-d1/TL	19.00–33.00	27.38	3.39
WPL1	0.076–0.114	0.089	0.02
WPL2	0.075–0.102	0.083	0.01
Plectane 3 width (WPL3)	0.068–0.110	0.086	0.01
WPL1/TL	23.00–39.00	32.74	5.70
WPL1/WPL3	1.01–1.11	1.04	0.05
Diameter of distal papillae D2–D4	0.013–0.016	0.015	0.002
D1 papilla diameter	0.015–0.021	0.017	0.003
Paracloacal papilla diameter	0.023–0.035	0.027	0.014

In the present study, the application of morphological and genetic datasets combined in a multivariate discriminate analysis, such as the PR (Fig. 7), was used for the first time for the molecular systematics of anisakid nematodes. The discriminant analysis of principal components inferred from both genetic and morphological datasets based on distance methods demonstrated that the 3 biological species *A. simplex* (s.s.), *A. pegreffii*, and *A. berlandi* n. sp. clustered in 3 distinct groups. Both molecular and morphological data provided evidence for the separation of these 3 distinct groups into the 3 distinct species. The versatility of the application of discriminant analysis in population genetics and the molecular systematics of animal populations, including parasites, has been emphasized previously (De Meeus et al., 2007; Jombart et al., 2010).

Furthermore, in general, the morphology of the species studied, at both adult and larval stages, seems to support the existence of the 3 major well-resolved clades found in the phylogenetic elaborations: (*A. berlandi* n. sp. (*A. simplex* (s.s.) and *A. pegreffii*)), (*A. ziphidarum* and *A. nascettii*), and (*A. paggiae* (*A. physeteris* and *A. brevispiculata*)). At the adult stage, in the *Anisakis* species included in the first clade, the spicules are long, thin, and unequal, and the ventriculus is longer than broad and sometimes sigmoid (Quiazon et al., 2008; Shamsi et al., 2012; data presented above); the second clade encompasses *Anisakis* spp. with long, thin spicules that are equal or very slightly subequal and with a ventriculus that is long but not sigmoidal (Paggi et al., 1998; Mattiucci et al., 2009); and the third major clade includes species that exhibit stout, equal spicules and a ventriculus that is short and broader than it is long (Mattiucci et al., 2005). In addition, the Type I larval morphology (sensu Berland, 1961) is characteristic of *Anisakis* spp. forming the first and second major clades, whereas the Type II morphology is typical of the species included in the third clade (i.e., *A. physeteris*, *A. brevispiculata*, and *A. paggiae*) (Mattiucci et al., 2005).

The mitochondrial DNA *cox2* gene region sequence has proved to be highly polymorphic in the 3 species of *Anisakis* studied; this finding supports the possible use of this gene in further studies of the population genetics and phylogeography of species belonging to the genus *Anisakis*, as previously suggested (Baldwin et al., 2011). For the mtDNA *cox2* variable gene region, nucleotide variation was mainly related to changes in the position of the third codon, consistent with that previously observed in species of both *Anisakis* (Kim et al., 2006; Valentini et al., 2006), *Contracaecum* (Mattiucci, Paoletti et al., 2008; Mattiucci et al., 2010; Garbin et al., 2011), and *Pseudoterranova* (Timi et al., 2013). Moreover, in future applications, the possible use of gene sequencing of a large number of these parasites could provide information on the possible existence of the population structure of their definitive and intermediate/paratenic fish hosts.

Our study also provides support for the existence of host specificity among *A. simplex* (s.s.), *A. pegreffii*, and *A. berlandi* n. sp. for "oceanic dolphins" and whales, as suggested by Mattiucci and Nascetti (2008) and others (e.g., Cavallero et al., 2011). Indeed, the 3 species have been identified as the only species of *Anisakis* parasitizing striped dolphins, pilot whales, and minke whales. Interestingly, *G. melas* was found to host all 3 species, depending on its locality in relation to the geographical ranges reported for *A. pegreffii*, *A. simplex* (s.s.), and *A. berlandi* n. sp. Notably, for pilot whales in South Pacific waters, *A. pegreffii* and *A. berlandi* n. sp. (=*A. simplex* sp. C) were detected sympatrically and syntopically in the same individual host; this cetacean species represents a new host record for *A. berlandi* n. sp. In contrast, *A. pegreffii* has been identified based on molecular markers (sequences data of the ITS1 and ITS-2 rDNA) and described morphologically from the short-beaked common dolphin, *Delphinus delphis*, and the common bottlenose dolphin, *Tursiops truncatus*, from southeastern Australian waters; similarly, larval stages belonging to the species *A. simplex* sp. C were found in dwarf sperm whale from the same geographical area (Shamsi et al., 2012).

All these findings appear to confirm that the 3 species share, in different geographical areas, the same definitive hosts and involve in their life cycles different pelagic and demersal fish hosts in their respective ranges (Mattiucci and Nascetti, 2008). In contrast, the same definitive host, the pilot whale, has previously been found in Spanish Atlantic waters to be parasitized by adults of *A. pegreffii* and *A. simplex* (s.s.) in sympatry (S. Mattiucci and G. Nascetti, unpubl. data); these 2 species are already known to occur sympatrically in this region (Mattiucci et al., 1997; Abollo et al., 2001; Mattiucci et al., 2004). Interestingly, it has been suggested that 2 subspecies of pilot whales exist (Rice, 1998), with subspecies *G. melas melas* in the Boreal region and subspecies *G. melas edwardii* (Smith) in the Austral region. The occurrence of *A. simplex* (s.s.) in boreal individuals of pilot whales (see Mattiucci and Nascetti, 2008), and the detection of *A. berlandi* n. sp. in austral specimens of pilot whales in the present study, seem to support this hypothesis and the possible use of *Anisakis* spp. for gathering information on the migration routes and population structure of their definitive hosts. The larvae of *Anisakis* spp. have been widely used as biological markers of

several fish populations (e.g., Mattiucci et al., 2004, 2007; Mattiucci, Farina 2008; Baldwin et al., 2011).

In our study, we emphasized that the use of genetic and molecular markers in distinguishing the 3 species of *Anisakis* is fundamental not only for the definition of the taxonomic status of these species but also for their recognition, even after a morphological reconciliation. Furthermore, the use of molecular markers has proved to be necessary for an epidemiological understanding of human cases of anisakiasis worldwide. Indeed, among the 3 species of *Anisakis* studied herein, cases of gastric and intestinal anisakisiasis are reported to be caused by *A. pegreffii* (see D'Amelio et al., 1999; Umehara et al., 2007; Fumarola et al., 2009; Mattiucci et al., 2011, 2013) and by *A. simplex* (s.s.) (see Umehara et al., 2007). In addition, *A. pegreffii* has been demonstrated to cause gastric-allergic anisakiasis (Daschner et al., 2012) in humans in Italy, when the live larval stage penetrates the gastric mucosal wall (Mattiucci et al., 2013). So far, no data are reported for *A. berlandi* n. sp. as an etiological agent of human anisakiasis in countries bordering the South Pacific.

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