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Cover Figure Caption:
Anisakis sp. from deep ulcerations in the forestomach of a bottlenose dolphin (*Tursiops truncatus*). Image courtesy of Tomislav Gomerčić, University of Zagreb, Croatia.

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Three *Anisakis* spp. isolated from toothed whales stranded along the eastern Adriatic Sea coast ☆

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ABSTRACT

Knowledge concerning cetacean ecology in the Mediterranean is limited but important for sustainable planning and enforcement of appropriate conservation measures. Any information that might help to elucidate their ecology is essential. We explored the population and genetic structures of *Anisakis* spp. nematodes isolated from four toothed whale species – bottlenose dolphins (*Tursiops truncatus*), striped dolphins (*Stenella coeruleoalba*), Risso's dolphins (*Grampus griseus*) and Cuvier's beaked whales (*Ziphius cavirostris*) – stranded along the eastern Adriatic Sea coast (1990–2012) to reveal more information on host ecological patterns. Lower parasite prevalence was observed in resident dolphin species compared with occasionally occurring species, as well as in young compared with adult dolphins, indicating different feeding habits related to age. No unequivocal relationship between the biological traits of a host (age, body length, body mass and blubber depth) and *Anisakis* population parameters was observed. Phylogenetic analysis revealed a new geographical record of *Anisakis simplex* sensu stricto (1.96%) and *Anisakis physeteris* (1.31%) in the Adriatic Sea in addition to resident *Anisakis pegreffii* (96.73%). In an assessment of the Adriatic Sea and oceans worldwide, the genetic structure of *Anisakis* revealed that *A. pegreffii* populations do not differ among various final host species but do differ with respect to geographical location in contrast to previously accepted *Anisakis* panmixia.

1. Introduction

Toothed whales constitute 10 different families, of which two – Delphinidae and Ziphiidae – contain the majority of the 72 living species. These whales have a wide distribution range, extending from river dolphins inhabiting small specific areas to sperm and killer whales with global distributions. Several toothed whale species undertake seasonal migrations over great distances otherwise common in baleen whales, while other species migrate on a smaller scale following the migration of their prey (Hooker, 2002). To date, cetacean biodiversity data for the Adriatic Sea record the presence of three species of baleen and eight species of toothed whales (Gomerčić, H., Huber, Đ., 1989. Research and protection of marine mammals in the Adriatic. In: Grgić, P. (Ed.), Plenarni referati i izvodi saopštenja četvrte konferencije o zaštiti Jadrana. SSRNBiH, Neum, October 19–20, p. 191. See http://www.vef.unizg.hr/dolphins/radovi/pdf/gomercic%20huber%201989,%20morski%20sisavci%20jadrana,%20neum.pdf (in Croatian)). Although the bottlenose dolphin (*Tursiops truncatus*) is the most frequently observed and considered to be the only resident species living and reproducing in the area of the Adriatic Sea (Bearzi and Notarbartolo di Sciara, 1995), other observed species – striped dolphins (*Stenella coeruleoalba*) and Risso's dolphins (*Grampus griseus*) – are most likely migrating from the Mediterranean Sea. Other species, such as the common dolphin (*Delphinus delphis*) and Cuvier's beaked whale (*Ziphius cavirostris*), are rarely noted in the Adriatic Sea. Knowledge of cetacean ecology in the Adriatic and Mediterranean Seas is still limited, even for such general aspects as population size, migrational patterns, feeding habits, habitat use or health status. Therefore, any information that might contribute to the current knowledge and enable sustainable planning and enforcement of appropriate conservation measures is essential. Due to the aquatic, highly mobile, legally protected and often inconspicuous life-style of cetaceans, most available data have been acquired from the carcasses of stranded animals. In addition to this sampling bottleneck, the slow mutation rate of marine mammalian DNA may prevent the early discovery of population isolation and therefore cause a delay in implementation of proper conservation measures. A possible solution to this problem might be the use of

☆ *Note:* The nucleotide sequence data reported in this paper are available in the GenBank database under accession numbers KC479891–KC480043.

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parasites as biomarkers or biological tags, which has been successfully conducted in fish stock analysis (MacKenzie, 2002, 2005; Mattiucci et al., 2008) but has been mostly neglected in cetacean research. Specific parasite species have been considered good biomarkers due to their close evolutionary relationship with a host revealing many ecological traits, as well as the history or migrational patterns of the host.

Genus *Anisakis* Dujardin, 1845 consists of nematodes with an indirect life cycle that takes place in the marine environment and relies on moving through different life stages through trophic webs of different marine hosts. Currently, 10 *Anisakis* spp., all inhabiting the alimentary tracts of their marine final hosts, are recognised (Mattiucci and Nascetti, 2008). Crustaceans, mostly euphausiids, represent the first intermediate hosts; fish and cephalopods are paratenic hosts; while cetaceans and pinnipeds act as final hosts in which nematodes reach the adult stage and reproduce (Marcogliese, 1995). Although such an indirect life cycle depends on at least three different host taxa, the close connection of the *Anisakis* life cycle with the stability of the marine food web enables a generalist character and ubiquitous distribution of the nematode from temperate to polar waters, limited only by low salinity environments where intermediate hosts cannot propagate. Thus, hundreds of fish species have been registered as paratenic hosts and 23 cetacean and 11 pinniped species as final hosts, demonstrating a wide ecological niche for genus *Anisakis*, which is attributable to the highly mobile and migratory nature of its hosts (McClelland, 2005). Additionally, humans can become accidental hosts by consumption of raw or inadequately thermally treated fishery products that are contaminated with live *Anisakis* larvae and that represent a public health risk for a zoonotic disease known as anisakidosis or anisakiasis. Although infective larvae cannot complete their life cycle in humans, they penetrate the gastrointestinal tract, thereby causing severe symptoms of disease (for reviews see Chai et al., 2005; Audicana and Kennedy, 2008; Hochberg and Hamer, 2010) with potentially carcinogenic consequences (Yoo et al., 2008). Although considered one of the most significant emerging food-borne zoonoses, anisakiasis is still misdiagnosed or underestimated in many Mediterranean countries (European Food Safety Authority (EFSA), 2010).

The aim of this study was to determine the characteristics of the *Anisakis* population within the stranded final hosts during the period from 1990 to 2012, which represents the largest cetacean sample studied to date. Furthermore, we assessed the genetic structure of *Anisakis* spp. parasitising the Adriatic cetacean population to better understand the ecological and migrational traits of the latter and consequently improve conservation of their declining stocks. Additionally, we tested the hypothesis of *Anisakis* populations' panmixia in oceans at a global level, analysing available data stored in a public repository such as GenBank.

2. Materials and methods

2.1. Parasite sampling

Anisakid nematodes were isolated from intact gastrointestinal tracts of toothed whales stranded between 1990 and 2012 (total sample of whales, $n = 181$) in the Croatian part of the Adriatic Sea (Fig. 1): 35 bottlenose dolphins (*T. truncatus*), 13 striped dolphins (*S. coeruleoalba*), three Risso's dolphins (*G. griseus*) and one Cuvier's beaked whale (*Z. cavirostris*). For each toothed whale, data on the stranding site and date found, determination of species, sex, age, body mass, external measurements and decomposition condition code were noted as previously described (Đuras Gomerčić et al., 2008). Pathoanatomical dissection was performed according to standard protocols (Kuiken, T., Hartmann, M.G., 1991. Standard protocol for the basic postmortem examination and tissue sampling of small cetaceans. In: Kuiken, T., Hartmann, M.G.

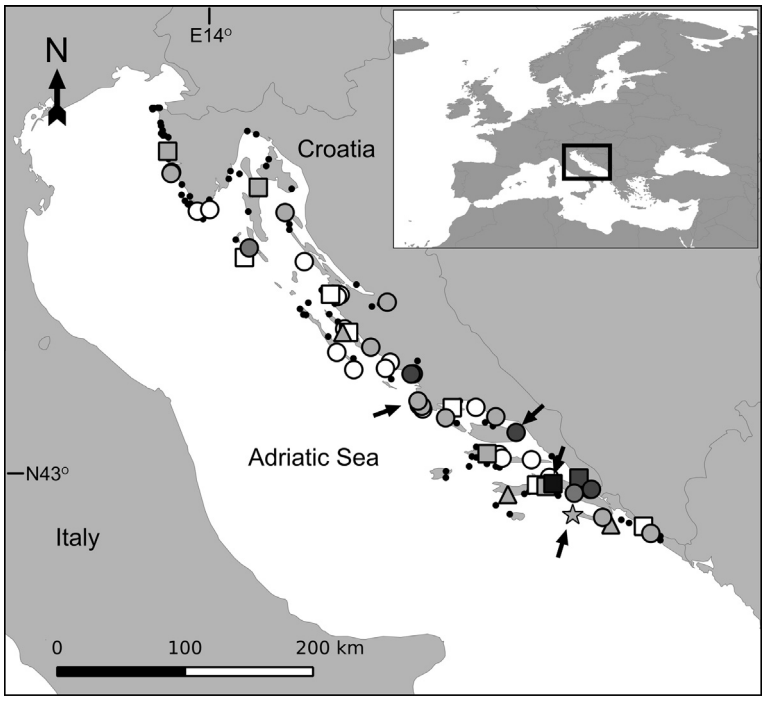

Fig. 1. Geographical representation of the Adriatic Sea with marked locations of stranding sites of genus *Anisakis*' final hosts (toothed whales) ($n = 163$) in the Croatian region, from October 1990 to April 2012. Different symbols depict whale species: circle, bottlenose dolphin (*Tursiops truncatus*); triangle, Risso's dolphin (*Grampus griseus*); square, striped dolphin (*Stenella coeruleoalba*); star, Cuvier's beaked whale (*Ziphius cavirostris*). Small black dots represent uninfected hosts and white and grey-coloured symbols represent infected individuals (31.90%). Grey-coloured marks correspond to toothed whale stranding sites, where *Anisakis* spp. have been identified by molecular methods and the colour scale differentiates number of identified *Anisakis pegreffii* haplotypes from lightest (one haplotype) to darkest grey (10 haplotypes). Arrows indicate stranded toothed whales with mixed *A. pegreffii* and *Anisakis simplex* sensu stricto infection or *A. pegreffii* and *Anisakis physeteris* infection.

(Eds.), Proceedings of the First ECS Workshop on Cetacean Pathology: Dissection Techniques and Tissue Sampling. ECS, Leiden, ECS newsletter No. 17 (Special Issue), pp. 26–39), noting the presence/absence of gastric lesions and decomposition condition code and using one of the following categories:

1. Fresh (as if it had just died, no bloating) – Code F (fresh).
2. Moderate decomposition (bloating, skin peeling, penis may be extended in males, organs still intact, excluding postmortem damage) – Code MD (moderate decomposition).
3. Advanced decomposition (major bloating, skin peeling, penis extended in males, organs beyond recognition, bones exposed due to decomposition) – Code AD (advanced decomposition).
4. Mummified or just skeletal remains – Code M/SR (mummified/skeletal remains).

Geographical locations for host stranding sites were visualised using ArcView GIS 3.2 (Environmental Systems Research Institute, 1992. ArcView GIS: Release 3.2. Redlands, California) (Fig. 1).

Nematodes were identified as reported in Mladineo et al. (2012) and stored in buffered 4% formaldehyde for morphological analysis or 70% ethyl alcohol for molecular identification. Morphological characteristics of sampled anisakids were analysed at the genus level under a stereomicroscope Nikon SMZ-U and light microscope Nikon Microphot FXA. For each host, nematodes were classified into four life-stage groups according to Grabda (1976): males, females, L3s and pre-adults (sexually immature adults and L4s).

2.2. Molecular identification of Anisakis spp.

In total, 165 anisakids (1–15 parasite specimens per host) were isolated from 26 toothed whales (Supplementary Table S1) for molecular analysis. Genomic DNA was isolated and amplified at the mitochondrial cytochrome oxidase 2 (*cox2*) locus (~600 bp) as previously described (Petrić et al., 2011). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced in both directions on an ABI 3100 automatic DNA sequencer (Applied Biosystems, USA) using the ABI PRISMBigDye Terminator Cycle Sequencing Kit. Sequences were aligned with other anisakid sequences stored in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html): *Anisakis simplex* sensu stricto (s. s.) (DQ116426), *Anisakis pegreffii* (DQ116428), *Anisakis simplex C* (DQ116429), *Anisakis typica* (DQ116427), *Anisakis ziphidarum* (DQ116430), *Anisakis physeteris* (DQ116432), *Anisakis brevispiculata* (DQ116433), *Anisakis paggiae* (DQ116434) and *Anisakis nascettii* (DQ116431) (as reported in Mattiucci et al., 2009), by Clustal X implemented in MEGA 5.05 software (Tamura et al., 2011) using default parameters and further verified by GBlocks (http://molevol.cmima.csic.es/castresana/Gblocks.html). Sequences were added to GenBank and accession numbers were obtained (KC479891–KC480043).

2.3. Anisakis spp. population data analysis

Parasite population parameters were calculated from the sample of 181 toothed whales using Quantitative Parasitology 3.0 software (Rózsa et al., 2000; Reiczigel, J., Rózsa, L., 2005; Quantitative Parasitology 3.0, Budapest, distributed by the authors, see www.zoologia.hu/qp/qp.html). Prevalence, mean abundance and intensity of nematodes were determined according to Bush et al. (1997) and supported with Sterne's exact 95% confidence interval (CI) for prevalence and bootstrap 95% CI (number of bootstrap replications = 2,000) for mean abundance and intensity. The common parasite distribution in a host population is asymmetrical and aggregated (right-skewed), therefore the best theoretical model for comparison by the maximum likelihood method (level of significance = 0.05) is a negative binomial model (Bliss and Fisher, 1953). Asymmetry of distribution was estimated with three aggregation indices: variance to mean ratio as a measure of overdispersion, exponent k of the negative binomial for the distribution asymmetry and discrepancy index D, ranging from 0 at uniform distribution to 1, for maximum discrepancy (Poulin, 1993).

The above-mentioned parameters were calculated for two data sets: one consisting of all host species of all ages (n = 181), and the second including only animals older than 1 year of age (n = 163). Because *Anisakis* can only be acquired through the food chain and not propagated by vertical transmission from mother to calf, all suckling toothed whales younger than 1 year were excluded from the analyses, except in the case of age group analyses. Prevalence was further separately calculated for datasets based on host species, decomposition stage of the carcass (F/MD, AD), sex, and the two most abundant host species by sex and age (*T. truncatus* and *S. coeruleoalba*). Age groups were defined based on changes in dolphin growth, development and life-style (earliest beginning of sexual maturation, latest ending of sexual and physical maturation) according to Archer and Perrin (1999) and Jagar (Jagar, I., 2005. Sexual maturity in female bottlenose dolphins (*Tursiops truncatus*) from the Adriatic Sea. Original scientific student paper. Faculty of Veterinary Medicine University of Zagreb, 13pp. See http://www.vef.unizg.hr/dolphins/radovi/sazeci%20eng/jagar%20student%202005.htm (in Croatian)): age group I (calves), age group II (juveniles), age group III (adults 15–22 years), age group IV (adults 23–30 years). Analysis of age groups was not possible in Risso's dolphins and Cuvier's beaked whales due to insufficient numbers.

Mean abundance and intensity of *Anisakis* spp. were calculated separately for bottlenose and striped dolphins, and results were compared using a bootstrap two-sample t-test. Differences in parasite prevalence for the two dolphin species were tested with exact unconditional tests (Reiczigel et al., 2008). Statistical significance of differences in percentages (infected bottlenose dolphins with gastric lesions versus infected striped dolphins with gastric lesions) and differences in age (infected versus non-infected dolphins for two species separately) were determined using t-tests (StatSoft Inc., 2011; Statistica: A system for statistical data analysis, including a wide range of analytical procedures and methods; see https://www.statsoft.com).

Shapiro–Wilk (Shapiro and Wilk, 1965) test results for datasets showed non-parametric, non-normal and non-linear distributions (P = 0.00001), therefore Spearman's correlation (StatSoft Inc., 2011) was tested between parasite abundance (total number, adults and L3s) and the following host variables: age, body length, body mass and dorsal/ventral blubber depth (for the total number of hosts, and separately for bottlenose and striped dolphins, excluding carcasses of code AD). Differences in nematode loads per host sex were tested with Mann–Whitney U tests (StatSoft Inc., 2011). $P < 0.05$ was considered significant.

2.4. Anisakis pegreffii genetic diversity and population structure

Parameters of genetic diversity, including numbers of haplotypes (H) and polymorphic sites (S), haplotype diversity (h; Nei, 1987), nucleotide diversity (p; Nei, 1987) and the average numbers of pairwise nucleotide differences (k; Tajima, 1983), were determined using DnaSP 5.0 (Librado and Rozas, 2009) and Arlequin 3.5 (Excoffier et al., 2005). Pairwise and overall distances among haplotype sequences and pairs of global populations were calculated using MEGA 5.05 (Tamura et al., 2011). Statistical selection of the best substitution model and gamma distribution shape parameter for the rate of heterogeneity among sites in analysed sequences was performed in jModelTest 0.1.1 (Posada, 2008) using Bayesian information criteria (BIC; Schwarz, 1978). The selected

evolution model TrN+G (Tamura and Nei, 1993) with a gamma parameter (0.063) was utilised for analysis of molecular variances (AMOVAs) and phylogenetic analysis.

Genetic diversity and population structure of *A. pegreffii* were estimated from parasite sequences grouped by: (i) all final host species in the Adriatic Sea ($n = 148$; sequences from this research); and (ii) worldwide geographical origin ($n = 342$; *A. pegreffii* sequences available in GenBank including 148 Adriatic Sea final host sequences). In the first case, *A. pegreffii* isolated from the gastrointestinal tract of bottlenose dolphins were marked as a *Tursiops* population (Tt; $n = 76$), from striped dolphins as a *Stenella* population (Sc; $n = 41$), from Risso's dolphins as a *Grampus* population (Gg; $n = 25$) and from Cuvier's beaked whale as a *Ziphius* population (Zc; $n = 6$). Secondary analyses ($n = 342$) included all available *A. pegreffii* sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html) with a matching fragment ($n = 194$) of the *cox2* locus and sequences obtained in the current research from toothed whales in the Adriatic Sea ($n = 148$). In this case, sequences from the Adriatic Sea were marked as AS (Adriatic Sea population; $n = 233$, 148 isolated from final and 85 from paratenic hosts), from the western Pacific Ocean as WP (western Pacific Ocean population; $n = 70$, isolated from paratenic hosts only), from the Mediterranean Sea as MS (Mediterranean Sea population; $n = 6$, one isolated from a final host and five from paratenic hosts) and from the eastern Pacific Ocean as EP (eastern Pacific Ocean population; $n = 33$, all isolated from paratenic hosts). Assessment of a population's hypothesised pattern of spatial genetic structure was carried out through a hierarchical AMOVA, which is based on partitioning of variance components attributable to population variance and to individuals within the populations. Significance of pairwise population comparison was tested with 10,000 permutations. Genetic differentiation between pairwise populations was determined using a fixation index, F_{ST}, and tested for significance with 10,000 permutations. AMOVA and F_{ST} analyses were performed in Arlequin 3.5.

A null hypothesis of population panmixia was tested with an exact test of the differentiation of haplotypes among populations implemented in Arlequin 3.5 software. Two neutrality tests, Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997), were calculated (on 20,000 simulated samples) to verify the null hypothesis of selective neutrality, which would be expected with population expansion. Mismatch distribution (Harpending, 1994) was used for estimation of sudden population expansion or balance and tested in Arlequin 3.5. The fit between the observed and expected distributions was tested using the Harpending raggedness index (*HRI*; Harpending, 1994) and the sum of squared deviations (*SSD*) for the estimated models of stepwise expansion (Schneider and Excoffier, 1999) in Arlequin 3.5. Statistical significance was estimated based on the parameters with 10,000 permutation tests under the null hypothesis that sudden population expansion cannot be rejected.

2.5. Phylogenetic analysis

Phylogenetic relationships and distributions of *A. pegreffii* haplotypes in the Adriatic Sea and at the global level were reconstructed with median-joining networks of mutations using Network v4.5.1.6 software (available at http://www.fluxus-engineering.com/sharenet.htm). Bayesian inference (BI; Larget and Simon, 1999) analysis was performed in MRBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) using a TrN+G (Tamura and Nei, 1993) evolutionary model of nucleotide substitution with a gamma parameter (0.063) selected in jModelTest 0.1.1 (Posada, 2008) using only the Adriatic Sea data set and reference GenBank sequences. Four incremented "heated" Markov chains were carried through 2,000,000 generations while sampling every thousandth generation, and 500 samples were discarded as burnin. Markov chain Monte Carlo (MCMC) parameters were calculated with default properties. A consensus tree with the 50% majority rule was constructed from the tree output file produced in the BI analysis and visualised using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

3.1. Anisakis spp. populations in toothed whales in the Adriatic Sea

Anisakid nematodes were found in 52 of 181 toothed whales (prevalence 28.73%; 95% CI, 22.5–35.9) stranded in the Adriatic Sea (Fig. 1). A logarithm of the number of *Anisakis* spp. nematodes per host and year of host stranding is shown in Fig. 2. Excluding 1-year-olds ($n = 18$) from the sample, the prevalence was 31.9% ($n = 163$, 95% CI, 25.1–39.5), with a mean abundance of 1,209.96 (95% CI, 510.4–2,764.9) and intensity of 3,781.13 (95% CI, 1,778.9–7,128.4) nematodes per host. The nematode variance/mean abundance ratio was 14,228.12; discrepancy index $D = 0.911$; and exponent k of the negative binomial $k = 0.048$, statistically fitting the negative binomial distribution ($\chi^2 = 24.6769$, $P > 0.05$). With respect to different host species, the highest prevalence was recorded in striped dolphins (52%). Three of six stranded Risso's dolphins were infected and one of two stranded Cuvier's beaked whales, while bottlenose dolphins had the lowest prevalence (26.9%). The parasite prevalence and other population dynamics parameters for the two most abundant host species (bottlenose and striped dolphins) are shown in Table 1. The nematode prevalence was significantly higher ($P = 0.015$; $P < 0.05$) in striped dolphins (52%) than in bottlenose dolphins (26.9%), in contrast to their intensities. In both species, non-infected animals (average age 11.63 and 12.92 years, respectively) were significantly younger ($P = 0.011$; $P = 0.0237$) than animals infected with *Anisakis* nematodes (average age 15.43 and 18.15 years, respectively). Variations in prevalences in age groups of bottlenose and striped dolphins are shown in Supplementary Fig. S1. The results of testing the differences in prevalences between different age groups for each host species are shown in Supplementary Tables S2 and S3. In both species, calves (age group I) and juveniles (age group II) showed the lowest prevalence values, while sexually and physically mature adults (age groups III and IV) had the highest parasite prevalence values. However, decreasing prevalence was observed in the oldest dolphins of both species. The youngest infected hosts were bottlenose dolphins aged 3 years. The parasite abundance (adults and larvae) increased significantly with host age (Spearman's

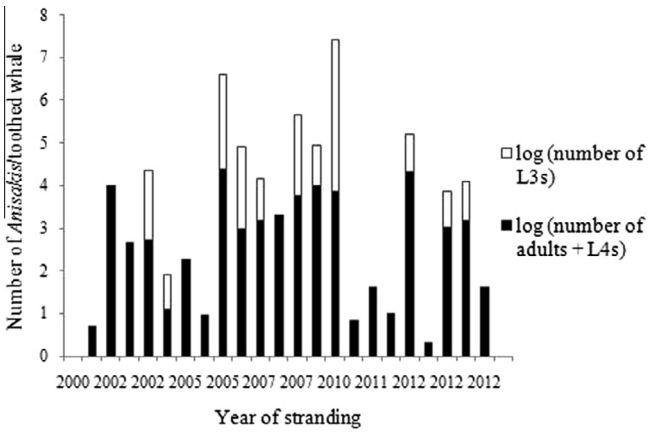

Fig. 2. A logarithm of the number of *Anisakis* spp. nematodes per host and year of host stranding. Black bars in the histogram represent the number of adults and L4s (pre-adult), and white bars numbers of L3s.

Table 1
Population of *Anisakis* spp. nematodes isolated from bottlenose (*Tursiops truncates*) and striped (*Stenella coeruleoalba*) dolphins from the Adriatic Sea. Prevalence is given with Stern's exact 95% confidence intervals (CI), mean intensity and abundance values with bootstrap 95% CI, and variance-to-mean ratio as a measure of overdispersion. Discrepancy index value (D) = 0–1.

Host species (number of hosts)	Prevalence (95% CI)	Mean intensity (95% CI)	Mean abundance (95% CI)	Variance/mean abundance	Discrepancy index D
Bottlenose dolphin (130)	26.92% (19.94–35.34)	4,381.46 (1,777.54–9,706.38)	1,186.65 (406.63–3,047.19)	13,935.31	0.901
Striped dolphin (25)	52.00% (31.71–70.41)	3,378.30 (379.30–9,546.20)	1,778.05 (201.74 – 5,590.42)	14,980.42	0.849

r = 0.37–0.42; P < 0.01) (Table 2) in all tested models, except in striped dolphins. The largest number of *Anisakis* spp. (n = 24,032) per host was found in an adult 23-year-old male bottlenose dolphin; similarly, the greatest number of nematode larvae (n = 3,561) was found in an adult 20-year-old male bottlenose dolphin. In general, the number of anisakid nematodes increased with the body length of the host and, to the contrary, *Anisakis* abundance decreased with body mass and blubber depth.

Significantly higher parasite prevalence (51.35%, 95% CI, 21.3–48.2; P = 0.0004) was observed in fresh and moderately decomposed toothed whale carcasses (n = 74) compared to a prevalence of 15.73% in carcasses in an advanced stage of decomposition (n = 89). No significant difference in prevalence was observed in all males (37.93%) and all females (25.33%) (Supplementary Table S4).

The gastric chambers were the most frequent sites of infection with *Anisakis* spp. in the hosts, although parasites were also found in the oesophagus (Supplementary Fig. S2) and the cranial part of the small intestine. In bottlenose and striped dolphins, anisakids were regularly found in the forestomach and less frequently in the fundic chamber. In Risso's dolphins, anisakids were more frequently present in the fundic chamber compared with the forestomach, while in the Cuvier's beaked whale, which is missing a forestomach, the site of anisakids was the fundic chamber. In 71% of all infected toothed whales, lesions ranging from superficial erosions to deep ulcerations with perforation of all gastric mucosal layers were observed (Supplementary Fig. S3). The percentage of infected striped dolphins bearing gastric lesions (92.31%) was significantly higher (P = 0.04, 95% CI, 1.18–48.42) compared with the infected bottlenose dolphin population (61.76%).

Table 2
Results for Spearman's correlation (R) with associated P values between specific biological traits of the host species (*Tursiops truncatus* or *Stenella coeruleoalba*) stranded along the Adriatic Sea coast, and *Anisakis* spp. abundance.

Host biological trait	*Anisakis* abundance		
	Total	Adults	L3s
Age			
All hosts	R = 0.4241[a]	R = 0.3915[a]	R = 0.3665[a]
	P = 0.0003	P = 0.0011	P = 0.0023
T. truncatus	R = 0.3666[a]	R = 0.3145[a]	R = 0.3365[a]
	P = 0.0104	P = 0.0295	P = 0.0194
S. coeruleoalba	R = 0.4542[a]	R = 0.4542[a]	R = 0.3002
	P = 0.0443	P = 0.0443	P = 0.1984
Body length			
T. truncatus	R = 0.3703[a]	R = 0.3548[a]	R = 0.2709
	P = 0.0096	P = 0.0134	P = 0.0626
S. coeruleoalba	R = 0.2681	R = 0.2681	R = 0.2773
	P = 0.2531	P = 0.2531	P = 0.2365
Body mass			
T. truncatus	R = 0.2796	R = 0.2775	R = 0.2126
	P = 0.0892	P = 0.0916	P = 0.2001
S. coeruleoalba	R = −0.5891[a]	R = −0.5891[a]	R = −0.5437[a]
	P = 0.0266	P = 0.0266	P = 0.0445
Blubber depth on back			
T. truncatus	R = −0.1735	R = −0.1735	R = −0.1688
	P = 0.3045	P = 0.3045	P = 0.3179
S. coeruleoalba	R = −0.0854	R = −0.0854	R = 0.3731
	P = 0.7716	P = 0.7716	P = 0.1888
Blubber depth on abdomen			
T. truncatus	R = −0.3047	R = −0.3016	R = −0.3320[a]
	P = 0.0667	P = 0.0697	P = 0.0447
S. coeruleoalba	R = 0.0660	R = 0.0660	R = 0.5043
	P = 0.8226	P = 0.8226	P = 0.0659

[a] P < 0.05 was statistically significant.

3.2. Molecular identification of genus Anisakis specimens from the Adriatic Sea

Comparison of analysed sequences (n = 153) and known GenBank sequences using the BLASTn tool indicated the presence of three *Anisakis* spp. in the Adriatic Sea. The majority of nematodes (n = 148, 96.73%) belonged to *A. pegreffii*, while *A. simplex* s. s. and *A. physeteris* were represented in only a few individuals (n = 3, 1.96% and n = 2, 1.31%, respectively). The host species and the geographical locations of their stranding sites, including identified nematodes species, are shown in Fig. 1. Mixed infections were present in the bottlenose dolphins (*A. pegreffii* 97.44% and *A. simplex* s. s. 2.56%), striped dolphin (*A. pegreffii* 97.62% and *A. simplex* s. s. 2.38%) and Cuvier's beaked whale (*A. pegreffii* 75.00% and *A. physeteris* 25.00%), but not in Risso's dolphins (*A. pegreffii* 100%).

3.3. Genetic diversity of A. pegreffii cox2 gene fragment at local (Adriatic Sea) and global levels

Anisakis pegreffii (n = 148) infecting the final hosts that were stranded in the Adriatic Sea had 47 variable (polymorphic) sites and 38 haplotypes. Among 47 polymorphic sites in the Adriatic sequences, 19 were singletons with two possible variants and 28 were parsimony informative. Unique haplotypes represented within one individual (n = 25) constituted the majority (66%) of defined haplotypes (n = 38). The greatest proportion of unique haplotypes (72%) was in the *Tursiops* population, while the rest were in the *Stenella* population. *Tursiops* and *Stenella* populations shared the most haplotypes (n = 9). Haplotype 1 had the highest total (60%) and intra-population frequency, and it was the only one shared among all four populations. The sequence divergences (Tamura's and Nei's distances) ranged from 0% (0.2%) to 9.1% (average value 0.7%). The genetic diversity indices suggested high haplotype diversity (h) 0.6360 ± 0.0471 and low nucleotide diversity (π) 0.004520 ± 0.002706. The average number of nucleotide differences was 2.53576 ± 1.37166. The genetic diversity indices for individual Adriatic host populations are presented in Table 3.

Analysis of *A. pegreffii* genetic diversity at a global level, inferred from a *cox2* fragment (429 bp) included 342 sequences. *Anisakis pegreffii* had 75 polymorphic sites and 95 haplotypes. Among the 75 polymorphic sites, 26 were singleton variable sites (24 with two possible variants) and 49 were parsimony informative. Unique haplotypes represented within one individual (n = 59) comprised the majority (62%) of the defined haplotypes (n = 95). The highest proportion of unique haplotypes (44%) was in the Adriatic Sea population, and the rest were in the western Pacific Ocean (27%), eastern Pacific Ocean (27%) and Mediterranean Sea (2%) populations. Populations of the western and the eastern Pacific Ocean shared

Table 3
Anisakis pegreffii genetic diversity values for populations grouped by final host species in the Adriatic Sea, inferred by mitochondrial cytochrome oxidase 2 (*cox2*) gene sequence data.

Host species	Nematode population	*n*	*H*	*S*	*h*	π	*k*
Tursiops truncatus	Tt	76	30	42	0.7467 ± 0.0547	0.006226 ± 0.003556	3.492982 ± 1.800191
Stenella coeruleoalba	Sc	41	17	27	0.6622 ± 0.0864	0.004256 ± 0.002625	2.387805 ± 1.325746
Grampus griseus	Gg	25	3	2	0.2900 ± 0.1095	0.000535 ± 0.000643	0.300000 ± 0.323671
Ziphius cavirostris	Zc	6	1	0	0.0000 ± 0.0000	0.000000 ± 0.000000	0.000000 ± 0.000000
	Total	148	38	47	0.6360 ± 0.0471	0.004520 ± 0.002706	2.535760 ± 1.371660

Tt, *Tursiops* population; Sc, *Stenella* population; Gg, *Grampus* population; Zc, *Ziphius* population; *H*, number of haplotypes; *S*, number of segregating sites; *h*, haplotype diversity (±S.D.); π, nucleotide diversity (±S.D.); *k*, mean pairwise difference (±S.D.).

most of the haplotypes ($n = 6$), while both shared the lowest number with the Mediterranean Sea population. The Adriatic Sea population shared less haplotypes ($n = 3$) with the Mediterranean Sea than with the western ($n = 4$) or the eastern Pacific Ocean ($n = 5$). Haplotype 1 had the highest frequency in total (38%) and within two populations (AS 48% and MS 33%), and it was the only haplotype shared between all four populations. Divergence (Tamura's and Nei's distances) between sequences at a global level ranged from 0% (0.2%) to 14.3% (average 1.9%). Estimates of evolutionary divergence over sequence pairs between global populations are shown in Table 4. Values for all known *A. pegreffii* sequences showed high haplotype diversity (*h*) 0.8442 ± 0.0193 and low nucleotide diversity (π) 0.0103 ± 0.005613. The average number of nucleotide differences was high at a global level (4.397764 ± 2.176807). The genetic diversity indices for individual global *A. pegreffii* populations are presented in Table 5.

3.4. Anisakis pegreffii population genetic structure

The estimation of genetic differentiation between parasite populations inferred by a fixation index (F_{ST}) is shown in Tables 6 and 7. The overall F_{ST} value for *Anisakis* nematodes isolated from the Adriatic Sea samples was −0.0221 with a non-significant *P* value (0.9784), indicating the absence of a genetic structure between *Anisakis* populations in different stranded toothed whale species. The fixation index results were confirmed by a distribution of genetic variance AMOVA (Table 8) showing that −2.21% of genetic variance emerges among populations and 102.21% within *Anisakis* populations in each host species. Overall, the Adriatic Sea non-differentiation exact *P* values were not significant (0.985), thereby not rejecting the hypothesis that populations of *A. pegreffii* are panmictic in different whales species in this area (Table 6).

The *A. pegreffii* global population F_{ST} value was high (0.41) and revealed significant differentiation of genetic structure ($P = 0.000$) between nematode populations grouped by distant worldwide geographical areas. The pairwise F_{ST} values were high and significant for all population pairs except between the Adriatic and the Mediterranean Seas. The fixation index results were confirmed by AMOVA (Table 9), which attributed 41% of genetic variation to variability among populations from different seas worldwide and 59% of genetic variance within populations. In addition, a significant non-differentiation exact *P* value (0.000) rejected the hypothesis of global panmixia of *A. pegreffii* between distant geographical locations. The pairwise values of a non-differentiation exact test showed non-significant values between the Mediterranean and the Adriatic Seas, and the western and the eastern Pacific Ocean regions (Table 7). Further, *P* values between the Adriatic Sea and both Pacific Ocean populations, as well as between the two Pacific Ocean areas were significant, therefore rejecting panmixia among these areas.

Table 5
Estimates of evolutionary divergence over sequence pairs between *Anisakis pegreffii* global populations.

	AS	MS	WP	EP
AS		0.003	0.021	0.015
MS	0.008		0.024	0.017
WP	0.028	0.031		0.021
EP	0.027	0.029	0.032	

AS, *A. pegreffii* sequences sampled from the Adriatic Sea (Adriatic Sea population); MS, Mediterranean Sea population; WP, western Pacific Ocean population; EP, eastern Pacific Ocean population. Tamura and Nei distances are shown below the diagonal and S.E. estimate(s) above the diagonal.

Table 6
Pairwise F_{ST} (below diagonal) and *P* values for an exact test of population differentiation (above diagonal) among final host populations of *Anisakis pegreffii* from the Adriatic Sea, inferred by mitochondrial cytochrome oxidase 2 (*cox2*) gene sequence data.

	Tt	Sc	Gg	Zc
Tt	a	0.91605	0.89752	1.00000
Sc	−0.01630	a	0.69521	1.00000
Gg	−0.00973	−0.00159	a	1.00000
Zc	−0.08700	−0.09004	−0.04178	a

Tt, *Tursiops* population; Sc, *Stenella* population; Gg, *Grampus* population; Zc, *Ziphius* population.
[a] Significance was tested with 10,000 permutations.

Table 4
Anisakis pegreffii genetic diversity values for populations grouped by geographic location worldwide, inferred by available GenBank mitochondrial cytochrome oxidase 2 (*cox2*) gene sequence data.

Sampling location	Nematode population	*n*	*H*	*S*	*h*	π	*k*
Adriatic Sea	AS	233	54	56	0.7623 ± 0.0303	0.006007 ± 0.003577	2.576957 ± 1.386978
Mediterranean Sea	MS	6	5	7	0.9333 ± 0.1217	0.005439 ± 0.003972	2.333333 ± 1.475730
Western Pacific Ocean	WP	70	26	29	0.8542 ± 0.0314	0.011320 ± 0.006194	4.856315 ± 2.397252
Eastern Pacific Ocean	EP	33	27	32	0.9830 ± 0.0138	0.017050 ± 0.009105	7.314394 ± 3.512249
	Total	342	95	75	0.8442 ± 0.0193	0.010251 ± 0.005613	4.397764 ± 2.176807

AS, *A. pegreffii* sequences sampled from the Adriatic Sea (Adriatic Sea population); MS, Mediterranean Sea population; WP, western Pacific Ocean population; EP, eastern Pacific Ocean population; *H*, number of haplotypes; *S*, number of segregating sites; *h*, haplotype diversity (±S.D.); π, nucleotide diversity (±S.D.); *k*, mean pairwise difference (±S.D.).

Table 7
Pairwise F_{ST} (below diagonal) and P values for an exact test of population differentiation (above diagonal) among populations of *Anisakis pegreffii* divided by geographic location in seas worldwide, inferred from available GenBank mitochondrial cytochrome oxidase 2 (*cox2*) gene sequence data.

	AS	MS	WP	EP
AS		0.27219	0.000	0.0000
MS	−0.01737		0.41008[a]	0.6788
WP	0.52510[a]	0.09147		0.0000
EP	0.34264[a]	0.17877[a]	0.13788[a]	

AS, *A. pegreffii* sequences sampled from the Adriatic Sea (Adriatic Sea population); MS, Mediterranean Sea population; WP, western Pacific Ocean population; EP, eastern Pacific Ocean population.
[a] Significance was tested with 10,000 permutations.

3.5. Anisakis pegreffii demographic history

The overall average number of sequence pairwise differences was higher than the number of polymorphic sites that resulted in a significant (0.001) and negative Tajima's D value (−2.127) for *A. pegreffii* isolated from toothed whales from the Adriatic Sea. The results of Tajima's D and Fu's F_S statistics with associated P values are presented in Tables 10 and 11. The Tajima's D test results were negative for all of the Adriatic Sea whale populations and significantly deviated from the model of neutral evolution, except for the *Grampus* population (Gg). The second test of mutation neutrality, Fu's F_S, also had significant negative values, overall and among the Adriatic Sea toothed whales populations, except for the *Grampus* population. Thus, rejection of a neutral evolution null hypothesis for all populations, except for the *Grampus* parasite population, was confirmed.

Mismatch distribution analysis was used to investigate *A. pegreffii* demographic history in the Adriatic Sea. The goodness of fit for *A. pegreffii* showed ambiguous results and deviation from a predicted model of sudden population expansion. Low and non-significant values of *HRI* (Table 12) indicated a good fit between the observed and the expected values of the sudden expansion model by Rogers and Harpending (1992). However, overall and for *Tursiops* (Tt; $n = 76$) and *Stenella* (Sc; $n = 41$) populations, *SSD* results significantly deviated from the predicted model of sudden population expansion, thereby indicating a departure from the null hypothesis in these cases. *Grampus* (Gg; $n = 25$) *SSD* and *HRI* values were both low and non-significant, supporting its sudden expansion. Large differences in initial populations before (parameter θ_0) and after (parameter θ_1) expansion indicated a sudden expansion of *Tursiops* (Tt) and *Stenella* (Sc) populations, while the tau (τ), expansion divergence time parameter with value 0 showed

Table 10
Tajima's D and Fu's Fs statistics with corresponding P values based on cytochrome oxidase 2 (*cox2*) gene sequence data for *Anisakis pegreffii* isolated from toothed whales of the Adriatic Sea.

Population	Tajima's D		Fu's Fs	
	D	P	Fs	P
Tt	−1.91723	0.00585	−18.92957	0.00000
Sc	−2.11169	0.00365	−9.43973	0.00005
Gg	−0.94066	0.19395	−1.00397	0.16450
Total	−2.12690	0.00110	−26.77220	0.00000

Tt: *Tursiops* population; Sc: *Stenella* population; Gg: *Grampus* population.

Table 11
Tajima's D and Fu's Fs statistics with corresponding P values based on available GenBank cytochrome oxidase 2 (*cox2*) gene sequence data for *Anisakis pegreffii* isolated worldwide.

Population	Tajima's D		Fu's Fs	
	D	P	Fs	P
AS	−2.12425	0.00040	−26.55171	0.00000
MS	−1.39031	0.04345	−1.78570	0.05890
WP	−0.61151	0.30855	−9.03727	0.00655
EP	−0.25826	0.45855	−16.40184	0.00000
Total	−1.83039	0.00555	−25.18193	0.00010

AS, *A. pegreffii* sequences sampled from the Adriatic Sea (Adriatic Sea population); MS, Mediterranean Sea population; WP, west Pacific Ocean population; EP, eastern Pacific Ocean population.

an absence of sudden population growth. The different divergence time of the *Grampus* population with respect to others suggested that population expansion might date to a different period in history.

At the global level, the average number of sequence pairwise differences was higher than the number of polymorphic sites, which resulted in significant (0.006) and negative (−1.83) Tajima's D values for *A. pegreffii* (Table 11). The Tajima's D test was negative for all populations but significantly deviated from the model of neutral evolution for the Adriatic ($n = 233$) and the Mediterranean Sea populations ($n = 6$), while the western Pacific ($n = 70$) and the eastern Pacific ($n = 33$) Ocean population values were not significant. Fu's F_S test, based on the distribution of haplotypes, had significant negative values, overall and among global populations, except in the Mediterranean Sea population, where it was negative but not significant (0.05890). In general, results of Tajima's D and Fu's F_S for *A. pegreffii* at a global level indicated an excess of rare haplotypes over what would be expected under a neutral model of evolution and, consequently, the rejection of the null hypothesis for all populations except for the Mediterranean Sea *A. pegreffii*.

Table 8
Molecular variance analysis (AMOVA) of *Anisakis pegreffii* populations in different final hosts from the Adriatic Sea, inferred by mitochondrial cytochrome oxidase 2 (*cox2*) gene sequence data.

Source of variation	Degrees of freedom ($n - 1$)	Sum of squares	Variance components	Percentage of variation	F_{ST}	$P(F_{ST})$
Among populations	33	1.966	−0.04301 Va	−2.21	−0.02208	0.97842
Within populations	1444	286.701	1.99098 Vb	102.21		
Total	1477	288.668	1.94797			

Table 9
Molecular variance analysis (AMOVA) for *Anisakis pegreffii* populations in different geographic locations worldwide, inferred from available GenBank mitochondrial cytochrome oxidase 2 (*cox2*) gene sequence data.

Source of variation	Degrees of freedom ($n - 1$)	Sum of squares	Variance components	Percentage of variation	F_{ST}	$P(F_{ST})$
Among populations	3	355.476	2.09244 Va	41.42	0.41421	0.00000
Within populations	338	1,000.215	2.95922 Vb	58.58		
Total	341	1,355.691	5.05165			

Table 12
Mismatch distribution parameter estimates with corresponding *P* values based on cytochrome oxidase 2 (*cox2*) gene sequence data for *Anisakis pegreffii* from final hosts in the Adriatic Sea.

Population	Mismatch distribution			Goodness-of-fit tests			
	τ	θ_0	θ_1	*SSD*	*P*	*HRI*	*P*
Tt	0.000	0.000	99 999.000	0.63482	0.00000	0.03360	1.00000
Sc	0.000	0.000	93.600	0.51884	0.00000	0.04168	1.00000
Gg	3.000	0.000	0.430	0.00781	0.39970	0.25790	0.59830
Total	0.000	0.000	99 999.000	0.47206	0.00010	0.05418	0.99990

Tt: *Tursiops* population; Sc: *Stenella* population; Gg: *Grampus* population; *SSD*: sum of squared differences; *HRI*: Harpending's raggedness index.

Table 13
Mismatch distribution parameter estimates with corresponding *P* values based on all available GenBank cytochrome oxidase 2 (*cox2*) gene sequence data for *Anisakis pegreffii*.

Population	Mismatch distribution			Goodness-of-fit tests			
	τ	θ_0	θ_1	*SSD*	*P*	*HRI*	*P*
AS	2.2	1.12852	3.96973	0.01171	0.52790	0.03944	0.65660
MS	2.5	0.00000	99999.000	0.00997	0.84530	0.07556	0.82990
WP	9.7	0.00352	6.69736	0.04635	0.16150	0.04384	0.37120
EP	9.5	0.00176	25.82031	0.01412	0.18410	0.01690	0.45060
Total	9.8	0.00176	5.53008	0.01530	0.66930	0.01852	0.85210

AS, *A. pegreffii* sequences sampled from the Adriatic Sea (Adriatic Sea population); MS, Mediterranean Sea population; WP, western Pacific Ocean population; EP, eastern Pacific Ocean population; *SSD*, sum of squared differences; *HRI*, Harpending's raggedness index.

At a global level, overall and for each population, mismatch distribution analysis of *A. pegreffii* sequences had low and non-significant ($P > 0.05$) *HRI* and *SSD* results (Table 13), providing evidence for species demographic population expansion in the past. Similarly, the initial populations (θ_0) were overall and in all cases smaller than the final populations (θ_1), thereby confirming a historical sudden expansion of species. The divergence time (τ) had similar values for pairs of populations (AS and MS; WP and EP), indicating that the Adriatic and Mediterranean Sea *A. pegreffii* populations had gone through sudden expansions in close historical periods but different from *A. pegreffii* populations from the Pacific Ocean region.

3.6. Network analysis and phylogenetic relationships of A. pegreffii haplotypes

The reconstruction of phylogenetic relationships between 38 haplotypes belonging to *A. pegreffii* sp. isolated from stranded toothed whales in the Adriatic Sea showed a radial shape of the net, which suggests demographic expansion of the species during

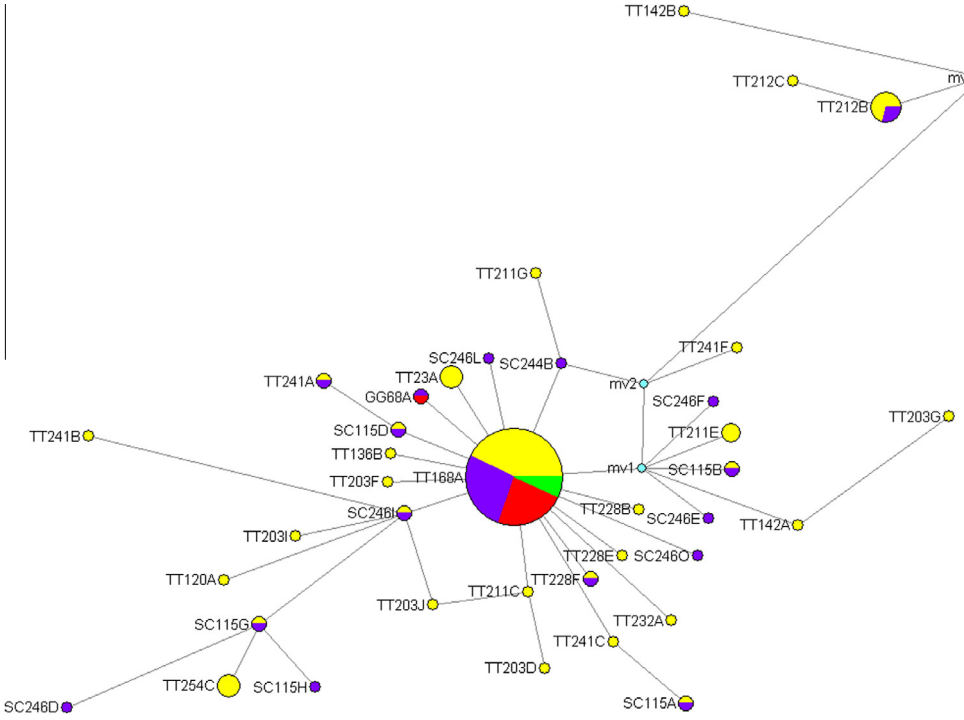

Fig. 3. Phylogenetic network of 38 haplotypes from *Anisakis pegreffii* species infecting stranded toothed whales in the Adriatic Sea. Network lines mark relationships between individual haplotypes (circles). Circle sizes correspond to numbers of sequences that belong to certain haplotypes. Colours of circles represent species of the toothed whale from which particular *A. pegreffii* haplotypes were isolated: red, Risso's dolphin (*Grampus griseus*); pink, striped dolphin (*Stenella coeruleoalba*); yellow, bottlenose dolphin (*Tursiops truncatus*); and green, Cuvier's beaked whale (*Ziphius cavirostris*). Light blue nodes marked with mv1, 2 and 3 represent hypothetical haplotypes necessary for emergence of sampled haplotypes.

some period in history (Fig. 3). The founder or predecessor of all haplotypes is the most common haplotype (H1 – TT168A) that is centrally located, containing 89 *A. pegreffii* sequences from all four toothed whale species. The furthest haplotypes by evolution are also the newest and were isolated from bottlenose and striped dolphins: TT212B, TT212C and TT142B with 10, 11 and 13 nucleotide differences from the ancestral haplotype.

A reconstructed network for 95 haplotypes of all available *A. pegreffii* sequences at a global level ($n = 342$) confirmed a radial shaped net of the haplotypes (Fig. 4). An ancestral haplotype (131 sequences; H1 – TT168A) placed in the centre of the net contained sequences from all four geographically distant populations (Adriatic Sea, Mediterranean Sea, western Pacific Ocean and eastern Pacific Ocean) in an unequal ratio. Nearly half of the investigated sequences from the Adriatic Sea (48%) belonged to the founder haplotype. The majority of the rest of the Adriatic Sea samples formed haplotypes closely related to the ancestral one, with the exception of some new and evolutionarily distant haplotypes (Fig. 4) in groups: (1) TT212B, TT212C and TT142B (from striped and bottlenose dolphin) with P-1 (from whiting, *Merlangius merlangus*) and ANI-2 (tuna, *Thunnus thynnus*); (2) 15ADRH (from short-finned squid, *Illex coindetii*) and (3) 16ADRH (from *I. coindetii*). Most of the western Pacific Ocean sequences (from paratenic hosts) formed divergent haplotypes placed in the separate part of the phylogenetic web, while only 21% belonged to the founder haplotype. The eastern Pacific Ocean sequences (from Pacific sardine, *Sardinops sagax*) were the least represented in the ancestral haplotype (6%), and in most cases formed new and distant evolutionary haplotypes placed in two different parts of the network. Few of the most divergent eastern Pacific Ocean haplotypes – 412EPA, 47EPA, 48EPA and 49EPA (from Pacific sardine) – are closely related to the most divergent Adriatic Sea haplotypes (bottlenose and striped dolphin *A. pegreffii* haplotypes TT212B, TT212C, TT142B, and haplotypes from fish: P-1 and ANI-2). The other eastern Pacific Ocean haplotypes are closer to the western Pacific Ocean haplotypes.

A consensus tree inferred by BI analysis shows phylogenetic relationships for nematodes of toothed whales in the Adriatic Sea (Fig. 5). Two main branches of the tree separated *Anisakis* spp. into two main clades and therefore supported the previously determined topology of this genus. The rest of the tree, belonging to *A. pegreffii* sequences, was shallow and unresolved, with an absence of well-supported groups. Populations were scattered through the entire tree and grouping was evident in only three clusters of *A. pegreffii* sequences (atop the tree), which are composed of two or more haplotypes (cluster 1: H21 and H12; cluster 2: H38, H7, H8 and H32; cluster 3: H4 i H29), and in clusters of species *A. simplex* and *A. physeteris*.

4. Discussion

The overall value of *Anisakis* spp. prevalence (31.90%) in aquatic mammals from the Adriatic Sea is significantly lower compared with observed values in fish hosts from the same area (Mladineo, 2003; Mladineo et al., 2012). In the paratenic hosts, oscillations in *Anisakis* dynamics have been attributed to seasonal fluctuation of biotic and abiotic environmental conditions that indirectly influence the migration of aquatic mammal final hosts, the quantity of parasite eggs laid and the availability of zooplankton as intermediate *Anisakis* hosts (Strømnes and Andersen, 2000). A significant difference was also observed between the two most abundant dolphin species, the bottlenose (26.9%) and striped (52.00%) dolphins, most

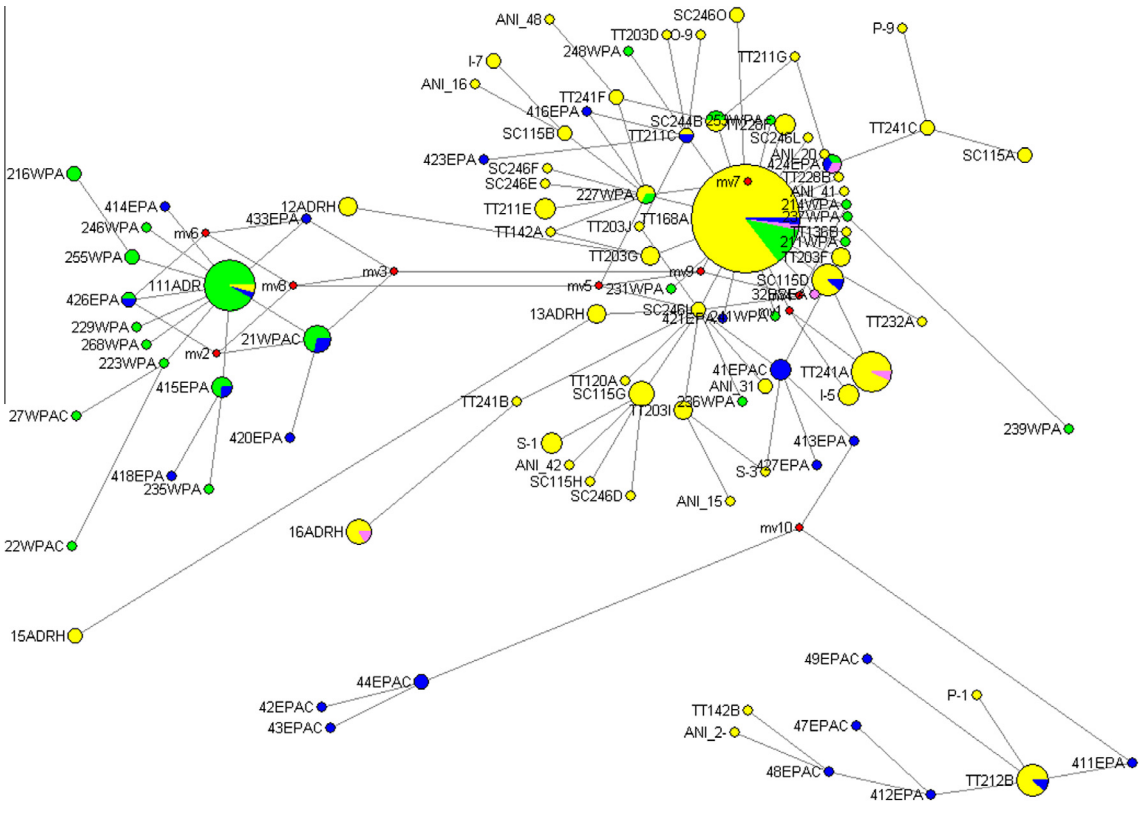

Fig. 4. Phylogenetic network of 95 haplotypes from *Anisakis pegreffii* species inferred from all available GenBank sequences. Network lines mark relationships between individual haplotypes (circles). Circle sizes correspond to numbers of sequences that belong to certain haplotypes. Colours of circles represent geographical origins from which particular nematode haplotypes were sampled: yellow, Adriatic Sea; pink, Mediterranean Sea; green, western Pacific Ocean; and blue, eastern Pacific Ocean. Red nodes marked with mv1–mv10 represent hypothetical haplotypes necessary for emergence of sampled haplotypes.

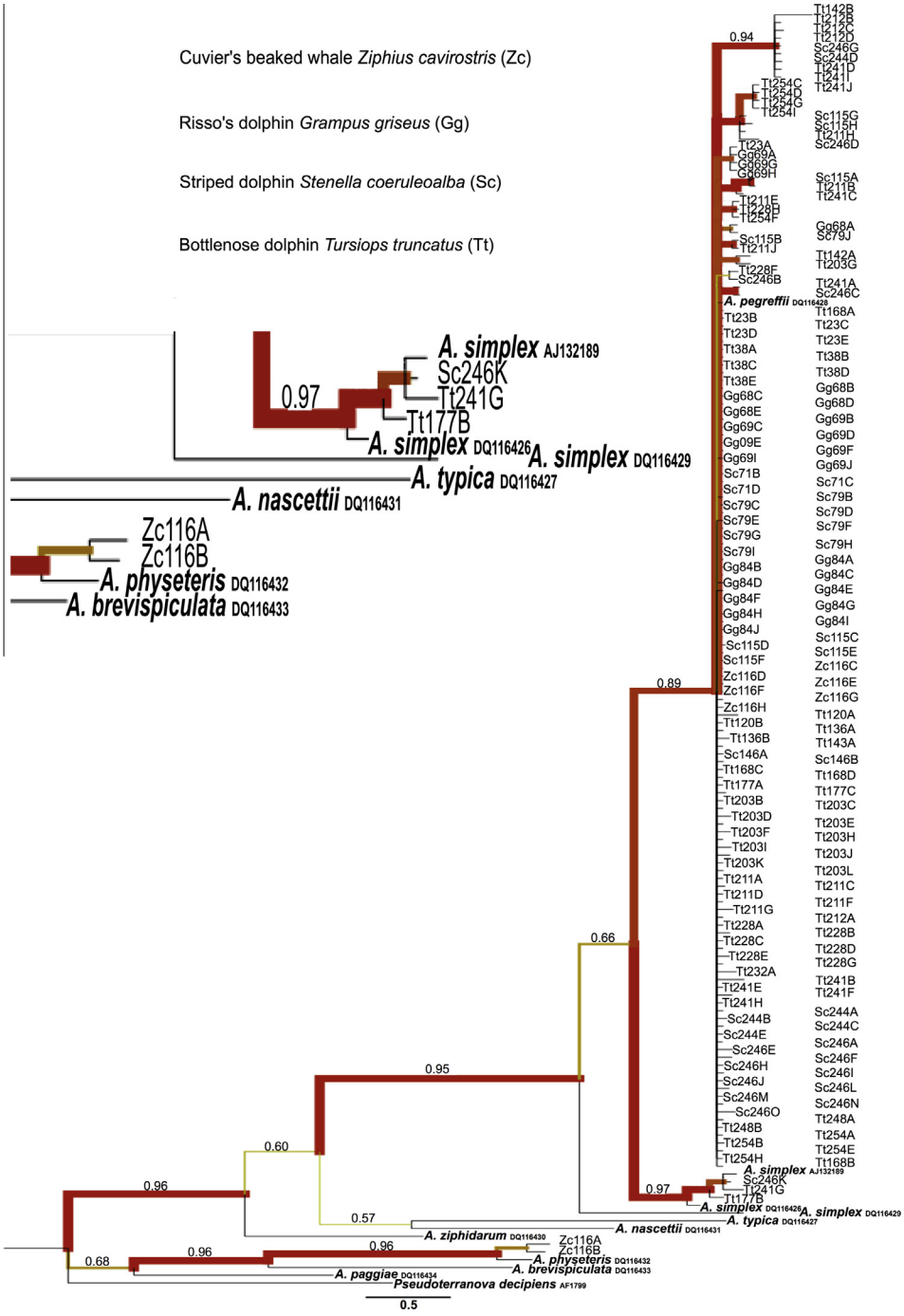

Fig. 5. Rooted phylogenetic tree inferred by Bayesian analysis of mitochondrial cytochrome oxidase 2 (*cox2*) gene locus fragments of *Anisakis* spp. isolated from toothed whales in the Adriatic Sea stranded between 1999 and 2011. Posterior probability values are shown in different colours (the thickest red line 0.9–1; thick orange line 0.8–0.9; thin coral line 0.7–0.8; the thinnest yellow line 0.6–0.7). *Anisakis pegreffii* isolated from toothed whales from the Adriatic Sea is represented by 148 isolates forming a sister clade with *A. pegreffii* (DQ116428), while three isolates (from bottlenose dolphins, *Tursiops truncates*, and striped dolphins, *Stenella coeruleoalba*) branched from *Anisakis simplex* sensu stricto (s. s.) (DQ116426), apart from the *A. pegreffii* group (blue circle). Two isolates from Cuvier's beaked whale, *Ziphius cavirostris*, branched from *Anisakis physeteris* (DQ116432) (green circle). For tree rooting, *Pseudoterranova decipiens* s. s. (AF179920) was used.

likely a result of their different ecological habits and behavioural patterns, while the small sample size of Risso's dolphins and Cuvier's beaked whales impeded statistically supportive analysis. In general, the presence of *Anisakis* in the final hosts still lacks a statistically balanced sample size that would give bases for adequate population determination, and to the best of our knowledge, this study represents the largest sampling effort in that direction. A more accurate knowledge of abundance of toothed whales present in the Adriatic Sea would also be very helpful, but such knowledge is rather insufficient (Galov et al., 2011) to draw conclusions on the final host availability. The most referred record approximated the cetacean population in the Croatian part of the Adriatic Sea to only 250 individuals of the bottlenose dolphin as the only resident species (Gomerčić, H., Huber, Đ., 1989. Research and protection of marine mammals in the Adriatic. In: Grgić, P. (Ed.), Plenarni referati i izvodi saopštenja četvrte konferencije o zaštiti Jadrana. SSRNBiH, Neum, October 19–20, p. 191. See http://www.vef.unizg.hr/dolphins/radovi/pdf/gomercic%20huber%201989,%20morski%20

sisavci%20jadrana,%20neum.pdf (in Croatian); Bearzi and Notarbartolo di Sciara, 1995). This suggested that high prevalence of *Anisakis* in the paratenic hosts originated from the striped dolphins as an occasionally occurring host coming in large groups from the Mediterranean Sea (Notarbartolo Di Sciara and Demma, 1994; Archer, 2002) rather than from the residential bottlenose dolphin, which is less infected by the nematode and thus contributes to a lesser degree to the number of shed *Anisakis* eggs. Additionally, differences in prevalences can be partially attributed to the types of host foraging grounds. While both striped and bottlenose dolphins feed on a variety of pelagic and benthopelagic fish and squid (Archer, 2002; Wells and Scott, 2002) the former dolphin also exploits coastal habitats (Spitz et al., 2006) and undertakes wide trans-Mediterranean migrations that might influence its higher *Anisakis* prevalence in contrast to the latter.

Mattiucci and Nascetti (2007) referred to an even lower *A. pegreffii* population dynamic in the bottlenose and striped dolphins (10.00% and 2.00%, respectively) in the Mediterranean Sea, which was probably influenced by the sampling effort (sample size and period) and/or the sampling ground (its ecological and oceanological characteristics).

In contrast, bottlenose dolphins from the Atlantic Ocean along the north western coast of Spain (Abollo et al., 1998) and the coasts of England and Wales (Gibson et al., 1998) had higher *Anisakis* prevalences (60.00% and 67.00%) compared with striped dolphins (37.50% and 57.00%) from the same areas, although the sample sizes in both studies were very small (bottlenose dolphins: $n = 10$ and $n = 3$; striped dolphins: $n = 8$ and $n = 14$).

The influence of geographical area on *Anisakis* population dynamics is best depicted by the comparison of two extreme locations: Antarctic/sub-Antarctic versus Arctic/sub-Arctic regions (Mattiucci and Nascetti, 2007). In the former, typically more than 10^5 parasites were isolated per final host in contrast to less than 10^2 parasites per host in the latter region. The Adriatic Sea values of overall mean abundance (1,210) and intensity (3,781) lie somewhere between these two extremes.

Anisakis spp. in toothed whales from the Adriatic Sea confirmed the typical negative binomial distribution of parasites within host populations (Rohde, 1993), where the majority of final hosts have low numbers of parasites and a small number of toothed whales carry the largest proportion of the total number of nematodes.

We have not observed an unequivocal, uniform relationship between a whale's biological trait (age, body length, body mass or blubber depth) and *Anisakis* population parameters. There was a significant increase in *Anisakis* abundance in bottlenose and striped dolphins with age, although a relatively low value of Spearman's coefficient indicates that the link between these two variables is also influenced by other unknown factors. The highest *Anisakis* abundance and prevalence were observed in age group III of bottlenose and striped dolphins (15–22 years old), while they decreased in age group IV (23–30 years old). According to Hudson and Dobson (1995), this pattern, in which interaction is described by a convex curve (the nematode number does not reach an asymptotic value but falls after an initial increase), is categorised as type 3. Usually there is more than one mechanism that influences this type of interaction (Wilson et al., 2002), but in our case there are two plausible explanations. Firstly, dolphins of different ages change their preferences for prey species or feeding sites (Cockcroft and Ross, 1990; Wells and Scott, 2002; Meissner et al., 2012), which results in increased or decreased *Anisakis* intake through the fish prey. Another possible explanation is the effect of the sample size, where an underestimation of real prevalence in age group IV due to a smaller sample size can affect the shape of the convex curve. A positive correlation was also observed in the relationship between the abundance of adults and L4 *Anisakis* and bottlenose dolphin body length, which mirrors the relationship between age and body length. The lack of statistically significant correlation for larval (L3) abundance might be due to the short time necessary for the larvae to moult in pre- and adult stages, as well as to a higher possibility of error when determining larval numbers, given their small size and inconspicuous appearance, especially in stomachs with contents. The strongest correlation for anisakid abundance was observed in striped dolphins with respect to the individual host's body mass, which decreased with greater nematode abundance, although we cannot confirm that the increased accumulation of nematodes is exclusively a consequence of poor host health condition or vice versa. Similarly, we observed a decrease in bottlenose dolphin abdominal blubber depth with an increase in *Anisakis* larval abundance. According to Struntz et al. (2004), this morphometric trait changes throughout life for different reasons (ontogenic development, different reproductive status, different geographical area) and therefore the observed relationship cannot be unequivocally explained only by host health aspects. The relationship between *Anisakis* infection and host sex was insignificant, although Poulin (1996) demonstrated, in numerous studies, a higher prevalence and intensity of nematodes in mammalian male hosts, similar to what was observed in our samples.

Inconsistencies in correlation between traits of different host species and *Anisakis* parameters only underline the multidimensional nature of mechanisms that shape this relationship and that should be monitored at the larger time/space scale.

Anisakis pegreffii populations infecting different toothed whale species in the Adriatic Sea are unstructured and heterogeneous. The genetic diversity indices in *Tursiops* and *Stenella* populations are similar, in contrast to very low values in the *Grampus* population. Such differences largely reflect differences in niche ranges and feeding habits between those host species, although it might be attributable, to some degree, to sampling effort. Risso's dolphins feed almost entirely on neritic and oceanic squid (Baird, 2002), while the bottlenose dolphin diet includes a large variety of benthic and/or pelagic fish and/or squid (Wells and Scott, 2002). Similarly, striped dolphins feed on a variety of pelagic and benthopelagic fish and squid (Archer, 2002), although their behavioural plasticity also results in the use of coastal habitats (Spitz et al., 2006). It seems that such differences in cetaceans' foraging grounds do not represent a barrier to *Anisakis* gene flow, given the parasite's failure to form distinct populations within a host with separate foraging grounds. Congruently, Mladineo and Poljak (2014) observed that, in the Adriatic Sea, *Anisakis* populations also remained panmictic among paratenic fish hosts inhabiting demersal, pelagic or oceanic zones.

The haplotype diversity average value (0.64) in the Adriatic Sea was similar to the Mediterranean Sea for *A. pegreffii* (0.67; Mattiucci et al., 2009), while the overall nucleotide diversity (0.0045) was lower than in the Mediterranean Sea (0.009), or very low in comparison to the sub-Antarctic region (0.020) (Mattiucci et al., 2009). Such high haplotype and low nucleotide diversity of *A. pegreffii* in the Adriatic Sea indicates small differences between haplotypes, mainly resulting from a single nucleotide base difference. We also observed this through a phylogenetic haplotype network that presented one as the most common number of mutations and three as the largest number of mutations per site. Furthermore, a combination of high haplotype and low nucleotide diversity can suggest rapid demographical expansion of *A. pegreffii* from a small effective population size. Observed sequence divergence (Tamura's and Nei's distances, average 0.7%) among the Adriatic Sea sequences is higher than the reported 0.1% in Mattiucci et al. (2013), most probably as a consequence of the sampling effort.

Genetic differentiation tests (AMOVA, F_{ST} and non-differentiation exact test) showed no genetic structure between *A. pegreffii*

populations infecting different toothed whale species, suggesting the existence of a single population in the Adriatic Sea. The genetic structuring of parasite populations is usually attributed to ecological characteristics of a parasite: wide distributional range, fragmented nature of the habitat and/or low expected rate of long distance dispersal (Mes, 2003). In contrast, high gene flow is enabled by a wide distribution of paratenic hosts and the migratory behaviour of the final hosts, which determine generally accepted absence of genetic structure in *A. pegreffii*. In the Adriatic Sea *Anisakis* samples, the majority of genetic diversity lies within populations rather than among them, congruent with previous allozyme analysis (Mattiucci et al., 1997).

Testing of the demographic history of *A. pegreffii* from the Adriatic Sea resulted in varying results, possibly influenced by the statistical strength of each of the demographic tests used (Fu, 1997; Ramos-Onsins and Rozas, 2002). Accordingly, Fu's *Fs* test is the most reliable and powerful in detecting selective neutrality of mutation and population growth followed by Tajima *D*, while goodness of fit and mismatch distribution tests have lower statistical power. One of the reasons is the stepwise growth model presumed by the test, which can be unsuitable for some populations, resulting in bias and incomplete assessment of demographic history. The second reason might lie in mismatched distribution criteria for pairs of genes (or sequence differences) being independent and randomly chosen in the population, which does not have to be the case with a population that has arisen from a recent and sudden expansion and inherits the majority of linked genes (Schneider and Excoffier, 1999). Consequently, it is most probable that *A. pegreffii* in *Tursiops* and *Stenella* populations have gone through a demographic expansion but not stepwise expansion. Small differences in the distribution shape of pair-wise sequence differences can also affect the assessed values of mismatch distribution parameters (τ, θ_0, θ_1) (Schneider and Excoffier, 1999) and therefore cause a null expansion time (τ) despite observed differences in initial (θ_0) and post-expansion population sizes (θ_1). Fu's *Fs* negative but non-significant value for *A. pegreffii* in the *Grampus* population ($n = 25$) can be attributed to the small sample size due to the test's sensitivity to small samples (Ramos-Onsins and Rozas, 2002; Pilkington, M.M., 2008. An apportionment of African genetic diversity based on mitochondrial, Y chromosomal, and X chromosomal data (PhD thesis). The University of Arizona, Tuscon, Arizona, USA). The observed phylogenetic relationships between haplotypes of *A. pegreffii* confirm the demographic expansion of the species with a characteristically shaped radial net of haplotypes distributed around the most common founder haplotype.

The explanation of recent demographic expansion for *A. pegreffii* corresponds to a widely observed pattern of different taxa population expansions after the last glacial period, dating back 12,500 years, although this was not thoroughly investigated for other areas. Neutral selection and constant population size (Tajima *D*) for *A. pegreffii* was analysed in Pacific sardines in the Pacific Ocean region, along the western coast of North America (Baldwin, R.E.B., 2010. Using parasite community data and population genetics for assessing pacific sardine (*Sardinops sagax*) population structure along the west coast of North America (PhD thesis). Oregon State University, Corvallis, Oregon, USA). The Tajima *D* value, based on an adequate sample size ($n = 76$), suggested no population growth, but it remains inconclusive whether there is an actual difference between that and the Adriatic Sea results because only one test (Tajima *D*) was used.

Anisakis pegreffii sequences isolated from a Cuvier's beaked whale (*Ziphius* population) cannot be interpreted adequately due to a negligible number of nematodes ($n = 6$) collected from a single host. Interestingly, the Cuvier's beaked whale carried two individuals of *A. physeteris*, a species not previously recorded in the Adriatic Sea. This represents a very important finding for several reasons: it is the first known record of adult *A. physeteris* in Cuvier's beaked whales, where only larval stages had been isolated to date, and this anisakid species is the most common in sperm whales (*Physeter macrocephalus*). Additionally, *A. physeteris* has never been reported in the Adriatic Sea, indicating that it was probably transferred from the Tyrrhenian Sea where it is common in the Atlantic horse mackerel, *Trachurus trachurus*, in mixed infections with *A. pegreffii* (Mattiucci et al., 2008). The migration route for this particular Cuvier's beaked whale specimen can be confirmed by the prey analysed from its stomach contents (Kovačić et al., 2010). Authors determined different cephalopod species, of which two were deep sea cephalopods (*Octopoteuthis sicula* and *Galiteuthis armata*) found only in the Mediterranean and not in the Adriatic Sea (Coll et al., 2010).

Although the Gibraltar Strait area is generally considered a border for *A. simplex* s. s. distribution between the Atlantic Ocean and the Mediterranean Sea, presence of this anisakid also occurs in mixed infections with *A. pegreffii* in pelagic fish from the Alboran Sea (Mattiucci et al., 2004, 2007; Mattiucci and Nascetti, 2006) and off the Tunisian coast (Farjallah et al., 2008). In the Adriatic Sea, *A. simplex* s. s. has only recently been recorded in very low numbers in both final (Blažeković, K., Lepen Pleić, I., Đuras Gomerčić, M., Gomerčić, T., Mladineo, I., 2012. Molecular identification of *Anisakis* spp. complex from gastrointestinal tract of stranded cetaceans in Adriatic Sea. In: McGovern, B., Berrow, S., McKeogh, E., O'Connor, I. (Eds.), 26th European Cetacean Society Conference. European Cetacean Society, Galway, March 26–28, p. 89) and paratenic hosts (Vardić Smrzlić et al., 2012; Mladineo and Poljak, 2014) in sympatry with *A. pegreffii*. The high mobility of its final (Wells and Scott, 2002) and paratenic hosts contribute to such an "influx" of an Atlantic anisakid species in the Adriatic Sea. It remains to be observed over a longer time span whether findings of *A. simplex* s. s. in the Adriatic Sea, both in the final and paratenic hosts, suggest its successful propagation in the area, or whether there are more occasional inflows with final host migrations. We believe that in the case of the final hosts, *A. simplex* s. s. has been carried by toothed whale migrations because all infected individuals (two bottlenose and one striped dolphin) were adult males (19, 22 and 21 years old). Adult male bottlenose dolphins at that age are known to live solitarily or in male partnerships (Wells and Scott, 2002), tending to migrate (travel) over a larger range (Wells, 1991). Furthermore, all three carcasses were recovered near the central Adriatic Sea area, and bottlenose dolphins from the Adriatic Sea do not differ genetically from those from the Mediterranean Sea (Galov, A., 2007. Genetička raznolikost populacije dobrog dupina (*Tursiops truncatus*) s osvrtom na druge vrste kitova (Cetacea) Jadranskog mora (PhD thesis). Faculty of Science University of Zagreb, Zagreb, Croatia (in Croatian); Galov et al., 2011), consequently implying the inflow of *A. simplex* s. s. via dolphin migration.

To undertake the global assessment of *A. pegreffii* genetic structure inferred through a mitochondrial DNA *cox2* fragment, we separated *A. pegreffii* into four populations belonging to four geographical areas: Adriatic Sea, Mediterranean Sea, western Pacific Ocean and eastern Pacific Ocean. The Adriatic Sea population ($n = 233$) exhibited the lowest value of haplotype diversity (0.76), while the eastern Pacific Ocean population ($n = 33$) had a value higher (0.98) than previously reported for the sub-Antarctic region (0.80) (Mattiucci and Nascetti, 2007). Nucleotide diversity showed a similar pattern, being low in the Mediterranean/Adriatic Sea populations (0.005; 0.006) and increasing in the western/eastern Pacific Ocean regions (0.011; 0.017), compared with the highest in the sub-Antarctic area (0.020). The high haplotype and low nucleotide diversity of *A. pegreffii* suggests rapid historical demographic expansion of this species worldwide, which was also confirmed by Tajima's *D* and Fu's *Fs* statistics. However, areas that depict lower genetic diversity compared with others also demonstrate

the possibility of genetic erosion in *Anisakis* populations, reflected by the habitat and food web degradation of those regions (Van Straalen and Timmermans, 2002; Mattiucci and Nascetti, 2008). Interestingly, the divergence time indicated different historical periods in which population expansion occurred in the Mediterranean Sea region (Adriatic and Mediterranean Sea populations) compared with the Pacific Ocean regions (western and eastern Pacific Ocean populations). This is congruent with the structuring of *A. pegreffii* populations between these distant geographical locations, inferred by AMOVA and F_{ST} results. No significant structure, however, was observed between the Adriatic and the Mediterranean Sea *Anisakis* samples. Although *A. pegreffii* has been shown to have a gene flow that secures panmixia over broad areas (Mattiucci et al., 1997), it has a spatial limitation when observed at the global level, resulting from cetacean migrations patterns. The exact test of population non-differentiation revealed overall differentiated populations, except between the Mediterranean Sea and the Pacific Ocean nematode populations, which was attributed to the small sample size of the former ($n = 6$). Previously, Quiazon et al. (2011) failed to prove structure in an *A. pegreffii* global population assessment using an internal transcribed spacer (ITS) region and a negligible number of mitochondrial DNA sequences (six sequences). This further supports the importance of analysing large data sets when possible.

A global genetic structure of four global *Anisakis* populations (genetic differentiation) exists in 41% of data, indicating a 59% level of gene flow. Between the Mediterranean Sea and Indian Ocean anisakid populations, gene flow can be attributed to lessepsian migration of paratenic hosts through the Suez Channel, previously observed for *A. typica* (Mattiucci et al., 2007). Gene flow between the western (China and Japan) and the eastern Pacific Ocean (California, USA) is restricted by limited final host movements over a considerably large area (Hooker, 2002; Whitehead et al., 2008; Weller et al., 2012). Although these two areas share the largest number of common haplotypes (six), and the lowest with the Mediterranean Sea (two), genetic structuring exists between western and eastern Pacific Ocean samples. A limitation in nematode gene flow is also caused by their final host's inability to range over such large distances in the time frame (2–3 months) required for *Anisakis* (one generation) residing in their gastrointestinal tract to stay alive and reproduce. For instance, sperm whales, although the largest toothed whales species, travel at 4 km/h for a year to cover a distance of 1,000 km (Whitehead et al., 2008).

Although the Adriatic Sea population exhibits less common haplotypes (three) with the Mediterranean Sea than with the western (four) or the eastern Pacific Ocean (five) populations, this bias is introduced by the small sample size of the Mediterranean population. Haplotype 1 (H1 – TT168A) had the highest total frequency (38%), and it was the only one shared among all four populations. The most distinctive and newest haplotypes isolated from the Adriatic Sea (TT212B, TT212C, TT142B) infected only adult males of bottlenose and striped dolphins and were shared by L3s parasitizing the whiting *M. merlangus* (P-1) and the Atlantic bluefin tuna *T. thynnus* (ANI-2) in the Adriatic Sea. The bathipelagic and pelagic life styles of these paratenic hosts (Jardas, 1996) suggests highly migratory behaviour far from a coastal area, typical of adult dolphins. Moreover, these haplotypes are closely related to haplotypes (412EPA, 47EPA, 48EPA and 49EPA) infecting the eastern Pacific Ocean sardine, *S. sagax*, evidencing some degree of gene flow at the global level. Interestingly, haplotype 111ADR, which is the most abundant in the western Pacific Ocean samples, also occurs in the Adriatic Sea samples although at a much lower rate. It was isolated from the Atlantic horse mackerel, *T. trachurus* (Wells, 1991), an oceanodromous paratenic fish, further supporting global gene flow, although still not significant enough to confirm *A. pegreffii* panmixia worldwide.

Divergence (Tamura's and Nei's distances) between *A. pegreffii* sequences at a global level (average 1.9%) is between those already reported (5.5%, Valentini et al., 2006; 1.8%, Baldwin, R.E.B., 2010. Using parasite community data and population genetics for assessing pacific sardine (*Sardinops sagax*) population structure along the west coast of North America (PhD thesis). Oregon State University, Corvallis, Oregon, USA; 0.1%, Mattiucci et al., 2013) for different geographical areas. The observed estimates of evolutionary divergence are clearly linked to the geographical distance between them, as the divergence value increases with distance (Adriatic–Mediterranean = 0.8%; Mediterranean–western Pacific = 3.1%). This is in accordance with Nei (1972), who reported that in some migration models, genetic distance is linearly connected to geographical distance or area, suggesting the existence of isolation of *A. pegreffii* populations by distance and barriers in the cetacean global migration. Interestingly, a similar pattern was observed in *A. simplex* s. s. (allozyme data) (Mattiucci and Nascetti, 2008), while the same genetic markers failed to prove structuring in *A. pegreffii* species worldwide.

The global genetic structuring of *A. pegreffii* populations is a reflection of dolphins' limitations in travelling over a range of extreme distances, such as those between the eastern and western Pacific Oceans. Those limitations do not exist over a smaller geographical range, and observed panmixia of an *A. pegreffii* population among the Adriatic and Mediterranean Seas results from dolphin migrations between these areas.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2014.07.012.

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