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Occurrence of ascaridoid nematodes in *Illex coindetii*, a commercially relevant cephalopod species from the Ligurian Sea (Northwest Mediterranean Sea)

Vasco Menconi[1], Paolo Pastorino[1,2,*], Erika Astrid Virginie Burioli[1,3], Marzia Righetti[1], Tommaso Scanzio[1], Livio Favaro[1,4], Maria Cristina Bona[1], Elena Pavoletti[5], Alessandro Dondo[1], Marino Prearo[1]

[1] Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, via Bologna 148, 10154 Torino, Italy

[2] Dipartimento di Scienze della Vita, Università degli Studi di Trieste, via Giorgieri 10, 34127 Trieste, Italy

[3] LABÉO Frank Duncombe, 1 route de Rosel – Saint Contest – 14053 Caen, France;

[4] Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università degli Studi di Torino, via Accademia Albertina 13, 10123 Torino, Italy

[5] Servizio Veterinario ASL Vercelli, Area Igiene della produzione, trasformazione, commercializzazione, conservazione e trasporto degli alimenti di origine animale e loro derivati, Largo Giusti 13, 13100 Vercelli, Italy.

*corresponding author: Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, via Bologna 148, 10154 Torino, Italy. E-mail: paolo.pastorino@izsto.it (P.P.); telephone: +39 0112686295

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Abstract

In this study we investigated the occurrence of larval ascaridoid nematodes in *Illex coindetii*, a commercially relevant cephalopod, via a cross-sectional prevalence study (January 2015 to February 2018) in two fishing areas (Savona and Piombino) in the Ligurian Sea (northwest Mediterranean Sea). A total of 745 specimens of *I. coindetii* were caught by local fisherman through bottom trawling. Parasitological examination revealed ascaridoid nematodes (L3) larvae (n=9): *Hysterothylacium* sp. (n=6) and *Anisakis* spp. (n=3). All larvae morphologically referred to the genus *Anisakis* were identified by PCR-RFLP as *A. pegreffii.* The prevalence of *Hysterothylacium* sp. infestation was 0.88% for the Savona and 0.56% for the Piombino sampling site; the prevalence of *A. pegreffii* was 0.18% for Savona and 1.11% for Piombino and did not differ

between the two sampling sites (chi-square test; p=0.0848). Better knowledge of the distribution of ascaridoid nematodes, especially of *Anisakis* species is essential for defining their epidemiological role and the potential risk for consumer health.

1. Introduction

Anisakid nematodes have a complex life cycle that progresses through four larval stages (L1–L4) and involves a broad spectrum of hosts (crustaceans, cephalopods, fish, marine mammals, and sea birds). The heteroxenous life cycle involves marine mammals as definitive hosts, crustaceans as first intermediate hosts, and fish, squid and other invertebrates as intermediate or paratenic hosts (Køie 1993; Mattiucci et al., 2018). Infection is transmitted through the marine food web (Klimpel and Palm 2011).

Humans become accidental hosts after consuming raw or semi-cooked fish or cephalopods infected with third-stage larvae (Audícana and Kennedy, 2008). The consumption of improperly processed or uncooked seafood (i.e., sushi and sashimi, salted or smoked herring, Nordic gravlax, Lomi Lomi, salmon, American cebiche and marinated anchovies) is a key factor in human infection (Audicana et al., 2002). The most common symptoms of human anisakiasis are abdominal pain, nausea, emesis, and mild fever (Arizono et al., 2012). In most clinical cases, the larvae have penetrated the stomach mucosa; larvae have rarely been found in the small intestine and even more rarely in the colonic mucosa (Arizono et al., 2012). Several episodes of allergic reactions have been described as a possible consequence of *Anisakis* spp. infection, even with the ingestion of well-cooked fishery products (Daschner et al., 2000; Mattiucci et al., 2013).

Public health authorities and the seafood industry are aware that fish-borne zoonotic nematode infection can affect both consumer health and the economy of fish production. In response to these issues, the European Food Safety Authority (EFSA) published a Scientific Opinion on risk assessment of parasites in fishery products (EFSA, 2010). Nonetheless, the zoonotic implications and the distribution and epidemiology of anisakid nematodes in the Mediterranean Sea remain poorly understood. Furthermore, the bulk of published data refer to the occurrence of *Anisakis* spp. larvae in teleost species. Few studies have described the occurrence of ascaridoid larvae in cephalopods from the Mediterranean Sea (Gestal et al., 1999; Farjallah et al., 2008; Angelucci et al., 2011; Petrić et al., 2011; Serracca et al., 2013; Picó-Durán et al., 2016; Goffredo et al., 2019).

Cephalopods are important predators in the marine trophic chain (Boyle and Pierce 1994; Clarke, 1996). Furthermore, the global landings of cephalopods have increased with growing consumer appreciation. Broadtail shortfin (*Illex coindetii*) is a benthic ommastrephid squid commonly fished in the Mediterranean Sea by bottom trawl (Lefkaditou et al., 2008; Picó-Durán et al., 2016). It is widely distributed throughout the Mediterranean, the eastern Atlantic (from south of Great Britain to Namibia), and the western Atlantic (from the Caribbean to the Gulf of Mexico and the Straits of Florida) (Sanchez et al., 1998). *I. coindetii* has been recorded up to 1100 m depth, but commercial catches are normally obtained at an average depth of 60-400 m (Guerra 1992; Jereb and Roper, 2010). Records of cephalopods caught at depths greater than 500 m are few and come mainly from the western Mediterranean (Quetglas et al., 2000). With this study we wanted to investigate the prevalence and the anatomical location of ascaridoid nematodes infestation, as well as the geographical distribution of ascaridoid nematodes in *I. coindetii* from the Ligurian Sea. Our findings may inform the creation of risk maps of cephalopod consumption.

2. Material and Methods

2.1 Study area and squid sampling

Between January 2015 and February 2018 (3-year monitoring period), a cross-sectional prevalence study was carried out on ascaridoid nematodes recovered from broadtail shortfin (*Illex coindetii*). A total of 745 specimens of *I. coindetii* were caught by local fisherman through bottom trawling in two fishing areas: Savona (n=565) and Piombino (n=180) in the Ligurian Sea (Fig. 1).

Squid samples were put in cold boxes at 4°C and transferred to the Fish Diseases Laboratory of the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta for analysis. For each specimen, species identification and measurement of mantle length (cm) were performed. Anatomopathological and parasitological examination focused on the occurrence of larval ascaridoid nematodes. The internal organs were placed in Petri dishes with physiological saline solution and left for 30 min under a heat source to allow mobilization of the anisakid larvae (Serracca et al., 2013). The visceral surfaces were visually inspected and observed under a stereomicroscope (Zeiss Stemis V8, Zeiss, Oberkochen, Germany) to detect the presence of larval ascaridoid nematodes. The muscle tissue was sectioned in thick slices (< 5 mm) and analysed by transillumination (UVP White Light Transilluminators, TW-43, Analytik Jena AG, Jena, Germany). The muscle tissue and visceral organs were digested separately using an enzymatic digestion method based on the Codex Alimentarius Commission (Codex Alimentarius Commission, 2004) and EU Regulation (EC) No. 2075/2005 (European Commission, 2005). The larvae were rinsed in deionized water and counted. Morphological identification was performed by light microscopy (Olympus BX40, Olympus, Tokyo, Japan). The anterior and posterior parts from each nematode were cleared using Amman's lactophenol to allow morphological identification. Morphological classification was performed as described in Berland (1961). A small piece of the mid-part of the larvae morphologically identified as *Anisakis* spp. underwent molecular analysis by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to the protocol reported by Serracca et al. (2013).

2.2 Statistical analysis

Normality of data was checked using the Shapiro-Wilk test. The non-parametric Mann-Whitney U test was used to check for differences in mantel length (the biometric parameter used to measure squid size) of *I. coindetii* samples from the two sampling sites (Arkhipkin, 1996). The prevalence of nematode infestation was calculated for the two sampling sites (Bush et al., 1997) and the differences between them were tested using the chi-square test. The 95% confidence intervals (95% CI) were also presented. The criterion for significance was set at $p < 0.05$. Statistical analyses were performed using GraphPad Prism version 8.0.2.

3. Results

The range in mantle length was 3-15 cm (8.65 ±2.11 cm mean ± standard deviation [±SD]) for the squid from the Savona sampling site and 7-20 cm (10.67 ±2.69 cm) for the squid from the Piombino site. A significant difference in mantel length between the two sites was noted (Mann-Whitney U test; p=0.0001).

Parasitological examination revealed ascaridoid nematodes larvae (n=9) (Table 1): *Hysterothylacium* sp. (n=6, 5 of which from the Savona and 1 from the Piombino site) and *Anisakis* spp. (n=3, 1 of which from the Savona and 2 from the Piombino site). We found third larval stage (L3) of *Anisakis* larvae type I (*sensu* Berland, 1961) and third larval stage (L3) of *Hysterothylacium* sp. All larvae morphologically referred to the genus *Anisakis* were identified as *A. pegreffii* by PCR-RFLP.

None of the ascaridoid nematodes had penetrated the musculature but were found encapsulated on the surface of the peritoneal membrane lining the mantle cavity. The prevalence of *A. pegreffii* infestation was 0.18% (95% confidence interval [CI] 0 - 0.52) for the Savona site and 1.11% (95% CI 0 - 2.64) for the Piombino site; the prevalence of *Hysterothylacium* sp. was 0.88% (95% CI 0 - 2.64) for the Savona site and 0.56% (95% CI 0.01 - 3.06) for the Piombino site. There was no difference in the prevalence of *A. pegreffii* between the two sites (χ^2=2.97; p=0.0848).

4. Discussion

With this study we investigated the occurrence of *Anisakis pegreffii* and *Hysterothylacium* spp. in *I. coindetii* from the Ligurian Sea. Few studies to date have described the occurrence of ascaridoid nematodes in *I. coindetii* from the Mediterranean Sea. This knowledge gap poses a potential health risk for squid consumption.

Published data show a higher prevalence of ascaridoid nematodes infestation in *I. coindetii* than in our samples: Gestal et al. (1999) found a prevalence of 4.8 % for *A. simplex* and of 28 % for *Hysterothylacium* spp. (n=42; Tyrrhenian Sea); Angelucci et al. (2011) reported a prevalence of 50 % for *Anisakis* spp. but only in 4 samples from the Sardinian Sea; Picó-Durán et al. (2016) reported a prevalence of 11.4 % for *A. pegreffii*, 16.6 % for *A. physeteris*, 0.8 % for *A. simplex* x *A. pegreffii* hybrid, and 4.1 % for *Hysterothylacium* spp. in samples from Spanish Mediterranean areas (n=123). All specimens of *I. coindetii* (n=137) from Apulia (Italy) analysed by Goffredo et al. (2019) tested negative for anisakid nematode larvae. Finally, Serracca et al. (2013) found only one larva of *Hysterothylacium* spp. (prevalence 1.6 %; n=60) in samples from the Ligurian Sea.

Cipriani et al. (2019) also investigated the occurrence of ascaridoids nematodes in squids but belonged to the species *Illex argentinus* (n=70) caught off the Falkland Islands (South Atlantic Ocean). Prevalence values of *Anisakis pegreffi* (15.7 %) and *Hysterothylacium aduncum* (2.9 %) were higher in comparison to our results, probably due to the different environmental conditions of ocean waters that affect the biological cycle of ascaridoids nematodes.

In our sample, the average mantle length (8.65 cm for the Savona and 10.67 cm for the Piombino sampling site) was smaller than in the study samples mentioned above, in which the mean mantle length reached up to 36.5 cm (Gestal et al., 1999; Petrić et al., 2011; Serracca et al., 2013; Picó-Durán et al., 2016). The higher prevalence the previous studies reported may stem from the fact that older *I. coindetii* host more nematodes than young ones since squids change their food habits as they grow. *I. coindetii* change diet during the life cycle: juveniles generally prey on crustaceans (Euphausiacea, Penaeidae and Phrosinidae) before switching to fish and cephalopods as adults (Boyle and Rodhouse, 2005; Rosas-Luis et al., 2014). Broadtail shortfin most likely acquires anisakid larvae through the predation of infected euphausiids or from small teleosts (Pascual et al., 1996; Abollo et al., 2001). Based on the results of this study and previous findings, it may be assumed that the consumption of smaller *I. coindetii* carries a lower health risk for nematode zoonoses.

This study is the first to reveal *A. pegreffii* identified at the species level by PCR-RFLP in *I. coindetii* from the Ligurian Sea. This finding is consistent with published data documenting it as the most widespread species in the Mediterranean Sea (Mattiucci and Nascetti, 2006). Furthermore, our observation of visceral infestation of ascaridoid nematodes larvae is shared by several previous studies (Smith, 1984; Gestal et al., 1999; Petrić et al., 2011) but not by Picó-Durán et al. (2016) who found 10 larvae of *Hysterothylacium* sp. in the muscle tissue.

The role of *I. coindetii* in the *Anisakis* spp. life cycle may be limited by their shorter lifespan compared to fish (Packard, 1972). Nevertheless, they make up an important part of the diet of odontocete cetaceans (definitive hosts) (Smith, 1984). The Ligurian Sea, also designated as the Pelagos Sanctuary for Cetaceans, is a protected area where cetaceans are more abundant than in the rest of the western Mediterranean Sea (Di Sciara et al., 1993; Azzellino et al., 2008; Serracca et al., 2013). Anisakidae transmission pathways are strictly habitat-dependent and involve a wide spectrum of invertebrates and intermediate or paratenic fish hosts (Klimpel and Rückert 2005; McClelland 2005; Palm and Klimpel, 2007). Despite predisposing factors present in both sampling sites (Pelagos Sanctuary for Cetaceans), the survey data show a very low prevalence of *Anisakis* and *Histerothylacium* species.

Further investigations on cephalopods, as well as parasite hosts, may improve our knowledge of the food web structure and better define their role in the ascaridoid nematodes life cycle.

5. Conclusions

Cephalopods are intermediate or paratenic hosts in the biological cycles and transmission of several parasites (Petrić et al., 2011). This study provides new information on the distribution of ascaridoid nematodes larvae in broadtail shortfin from the Ligurian Sea. Improving knowledge of zoonotic food-borne parasites is a milestone in health risk assessment of fish consumption (Menconi et al., 2020). The survey data suggest that the risk of fish-borne zoonotic nematode infection after the consumption of broadtail shortfin from the Ligurian Sea is very low. Prevalence rates, low consumption of raw and/or undercooked cephalopods, and the tendency of the larvae to encapsulate in the visceral compartment rather than in the muscle tissue of the host bear this out. Further studies are needed for detailed risk assessment of ascaridoid nematodes larvae in other commercially relevant cephalopod species to define consumer health risk.

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Declaration of competing interest

None.

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Table 1. Mantel length (cm) and number (N) of *Illex coindetii* caught from the two sites with the number and mean intensity of infestation by *Hysterotylacium* sp. and *Anisakis pegreffii*.

Sampling site	*Illex coindetii*	Mantel length	*Hysterotylacium* sp.		*Anisakis pegreffii*	
	N	cm (mean ± SD)	N	mean intensity	N	mean intensity
Savona	565	8.65 ± 2.11	5	1	1	1
Piombino	180	10.67 ± 2.69	1	1	2	1

Figure 1. Western Mediterranean Sea and location of sampling sites (in red). Site 1 (Savona): 44°17'25.58"N 8°39'6.23"E; site 2 (Piombino): 42°56'34.164"N 10°25'37.713"E.

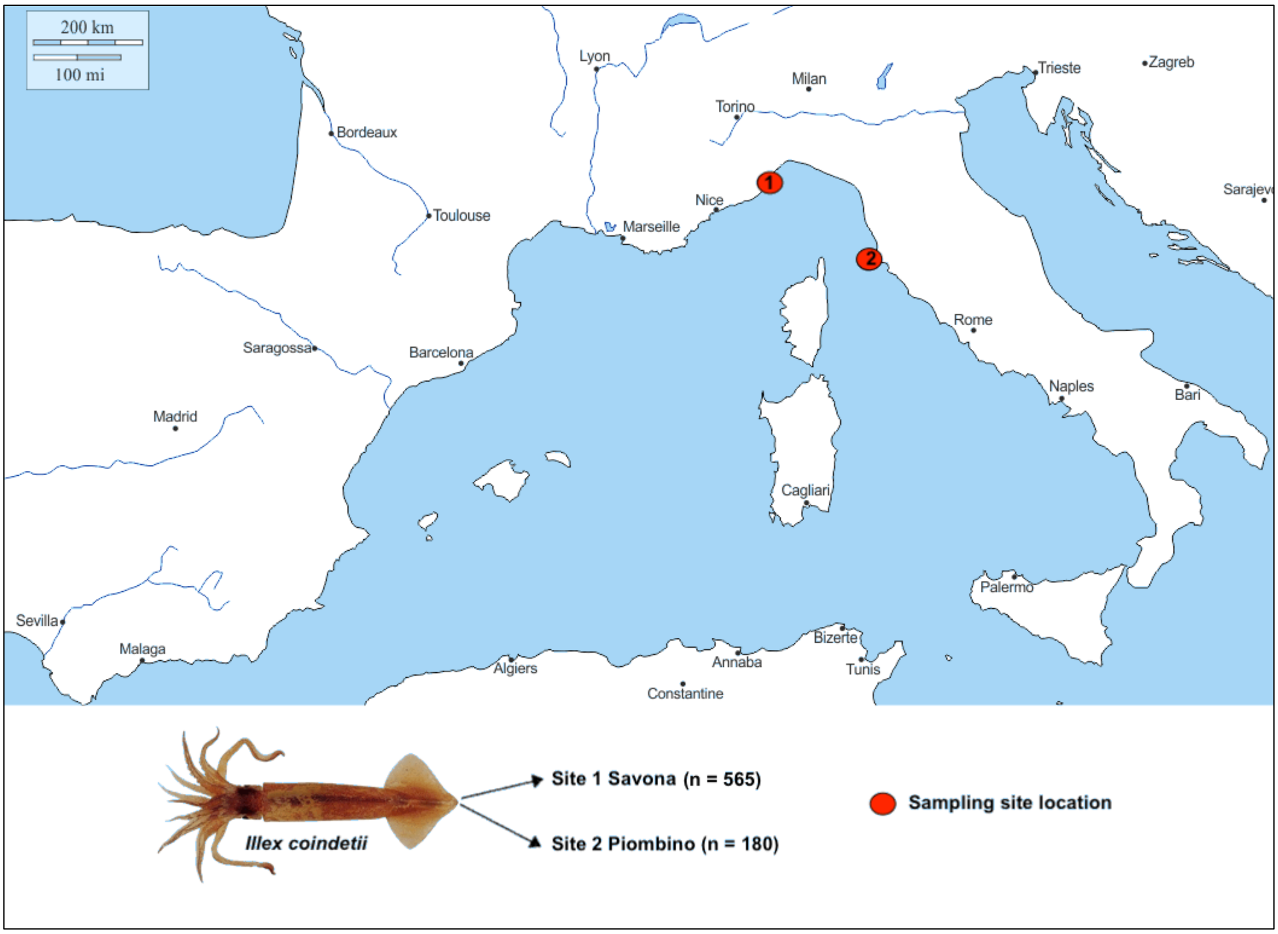
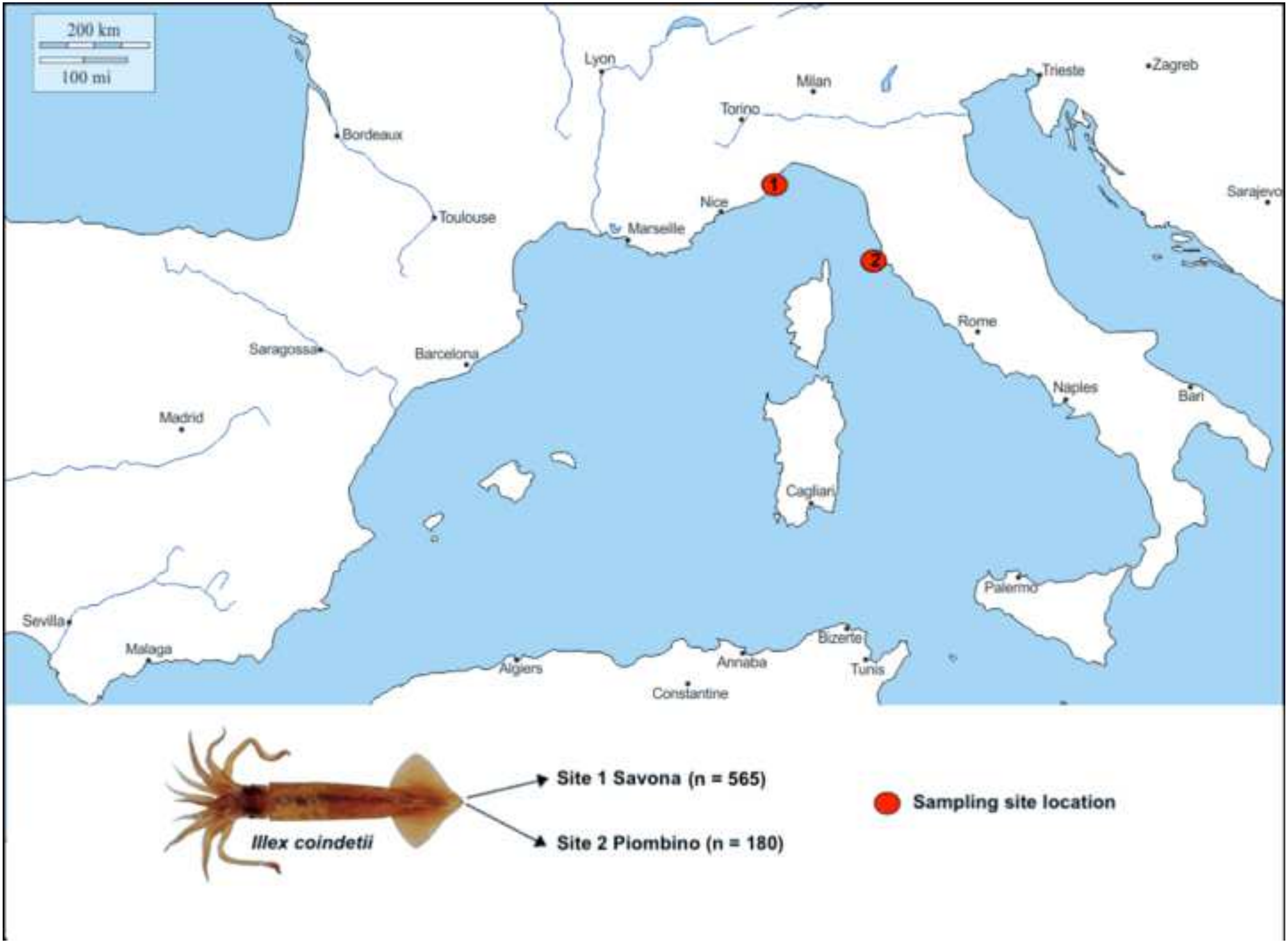


Figure 1



200 km
100 mi
Lyon
Milan
Trieste
Zagreb
Torino
Bordeaux
Sarajevo
Nice
Toulouse
Marseille
Rome
Saragossa
Barcelona
Naples
Bari
Madrid
Cagliari
Palermo
Sevilla
Malaga
Bizerte
Algiers
Annaba
Tunis
Constantine
Illex coindetii
Site 1 Savona (n = 565)
Site 2 Piombino (n = 180)
Sampling site location

***Credit Author Statement**

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Conceptualization: **Vasco Menconi**, **Paolo Pastorino**, **Maria Cristina Bona**, and **Marino Prearo**; Data curation: **Vasco Menconi**, **Paolo Pastorino** and **Maria Cristina Bona**; Investigation: **Vasco Menconi**, **Paolo Pastorino**, **Erika Astrid Virginie Burioli**, **Marzia Righetti**, **Tommaso Scanzio**, **Livio Favaro**, **Maria Cristina Bona**, **Elena Pavoletti**, **Alessandro Dondo** and **Marino Prearo**; Methodology: **Vasco Menconi**, **Paolo Pastorino** and **Marino Prearo**; Supervision: **Marino Prearo**; Writing – original draft: **Vasco Menconi** and **Paolo Pastorino**; Writing – review & editing: **Vasco Menconi**, **Paolo Pastorino** and **Marino Prearo**. All authors have read and agreed to the published version of the manuscript.

***Conflict of Interest Form**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: