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Article

Bonamia exitiosa in European Flat Oyster (*Ostrea edulis*) on the Croatian Adriatic Coast from 2016 to 2020

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Abstract: The annual production of European flat oysters (*Ostrea edulis*) in Croatia is about 50 to 65 tons, and it has a long tradition. All Croatian oyster farms are subjected to the national surveillance program aiming to detect the presence of *Bonamia ostreae* and *Marteilia refringens* according to the Council Directive 2006/88/EC. Within the surveillance program, the first findings of the parasite *Bonamia* spp. occurred in 2016 in two production areas in the north and south of the Eastern Adriatic coast. The repeated findings of the parasite were noted up to 2020 but also on two additional sites in the north. The parasite was detected by cytological analysis of stained heart smears, histological examination, and PCR. PCR positive samples were sequenced for SSU rDNA gene, and BLAST analysis confirmed infection with *Bonamia exitiosa*. Attempts to prove the Pacific oyster as a putative vector of the parasite failed. The infection prevalence from 2016 until 2020 ranged from 3.3 to 20% in different sites. No mortalities were reported from the infected sites, and it seemed that infection of flat oysters with *B. exitiosa* did not affect their health. The study has not shown the source and way of infection spread, which imposes the need for more comprehensive molecular and epidemiological studies.

Keywords: *Bonamia exitiosa*; bonamiosis; Croatian Adriatic coast; *Ostrea edulis*; prevalence; surveillance

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1. Introduction

The production of European flat oyster (*Ostrea edulis*) has a long tradition and economic significance in many countries of the Mediterranean basin and the East Atlantic coast. Along the Croatian coast, flat oysters have been cultivated for centuries, and in recent decades, the annual production has ranged from 50 to 65 t [1]. Production of *O. edulis* has stagnated since the 70s of the last century as a result of the introduction of pathogens into the susceptible population. Therefore, diseases have caused large losses and dramatically affected production [2–4]. One of these pathogens is the protozoan parasite *Bonamia ostreae*, belonging to the clade "microcell" within Haplosporidia [5]. Various methods have been developed to identify and confirm the presence of parasites of the genus *Bonamia*, but each of them has certain limitations. Microscopy of gill and heart-stained tissue impressions and histological slides are methods to identify and confirm *Bonamia* species. Identifying parasite species due to morphological similarity is time-consuming and requires experience and expertise. However, both these methods have low sensitivity and specificity [6–9], similar to TEM, which seems to be insufficient in the identification of species [10]. Diagnostic sensitivity has significantly improved with the implementation of molecular methods, such as PCR [11,12] or in situ hybridization.

Based on molecular studies of small ribosomal subunit rRNA genes, the genera *Bonamia* is phylogenetically positioned into a clade microcell within the genus *Haplosporidia*, and within it, includes four species: *B. ostreae*, *B. exitiosa*, *B. roughleyi*, and *B. perspora* [13–16].

All species of *Bonamia* cause infection of flat oyster hemocytes, leading to death [12]. In some cases, parasites are present in low prevalence and with little impact on the flat oyster population [17]. It has been reported that flat oysters exposed to parasites over a long period develop a certain degree of resistance [18]. *Bonamia exitiosa* was for the first time reported as the cause of the Chilean oyster's (*Ostrea chilensis*) high mortality in New Zealand when only 9% of affected flat oysters survived [19]. Catastrophic and rapid mortality was caused by the same parasite on triploids of the Suminoe oyster (*Crassostrea ariakensis*), introduced for experimental cultivation in 2003 in Bogue Sound, North Carolina [20], in an area where the crested oyster (*Ostreola equestris*) was an indigenous species [15].

This paper presents the findings, distribution, and prevalence of the *Bonamia exitiosa* in the samples of flat oyster analyzed within the national surveillance program for the infection with *Bonamia* parasites.

2. Materials and Methods

2.1. Sampling

The sampling points within production areas were determined through an intensive monitoring program in the period from 1998 to 2000. Each year, according to the national surveillance program, authorized veterinarians sampled 30 adult flat oysters on each of nine sampling points along the Croatian Adriatic coast (Figure 1, Table 1). The samples were packed in a transport refrigerator and delivered to the laboratory within 24–36 h.

Figure 1. Map showing European flat oyster (*O. edulis*) production areas with sampling points included in the national surveillance plan. A production area in the south with five sampling points and three production areas in the north Croatian Adriatic Sea tested positive (red numbers) for *B. exitiosa* in 2016–2020. The numbers refer to production areas/sampling sites listed in Table 2.

Table 1. Positive findings of *Bonamia exitiosa* within sampling area showing sampling dates, sampling points, and prevalence using different diagnostic techniques in Croatia during the period 2016–2020.

Species	Flat Oyster (*Ostrea edulis*)										Pacific Oyster (*Magallana gigas*)			
Production Area	**Mali Ston Bay**		**Medulin Bay**		**Lim Bay**		**Savudrija Bay**		**Marina Bay**		**Medulin Bay**		**Lim Bay**	
Date and number of sample animals	Date M/Y	Nº	Date M/Y	Nº	Date M/Y	Nº	Date M/Y	Nº	Date M/Y	Nº	Date M/Y	Nº	Date M/Y	Nº
	05/2016	150 *	05/2016	30 *	05/2016	30	05/2016	30	11/2016	30				
	11/2016	150	11/2016	30 *	09/2016	30	10/2016	30	06/2017	30				
	05/2017	150	07/2017	30 *	05/2017	30	05/2017	30	05/2018	30	07/2017	18		
	11/2017	150	11/2017	30 *	06/2018	30 *	06/2018	30 *	09/2018	30				
	11/2018	150	10/2018	30	09/2019	30	10/2019	30	08/2019	30			09/2018	30
	10/2020	150	10/2020	30 *	09/2020	30	09/2020	30 *	08/2020	30				
Totally		**900**		**180**		**180**		**180**		**180**		**18**		**30**

* Samples with positive finding of *B. exitiosa.*

Table 2. Total number of oysters analyzed for the presence of *Bonamia* spp. in the period 2016–2020.

Date of Sampling (M/Y)	Production Area/Name of Sampling Site	Number of Sampled Animals	Sampled Species	Results of Laboratory Analysis					
				Heart Imprints		PCR		Real Time PCR	
	MALOSTONSKI BAY AREA			+-ve	%	+-ve	%	+-ve	%
05/2016	6 * Mali Ston	30	*Ostrea edulis*	3	10	5	16.7	n/d	-
05/2016	2 * Brijesta	30	*Ostrea edulis*	3	10	6	2.,0	n/d	-
05/2016	5 * Bistrina	30	*Ostrea edulis*	2	6.7	2	6.7	n/d	-
05/2016	3 * Bjejevica	30	*Ostrea edulis*	2	6.7	3	10.0	n/d	-
05/2016	4 * Sutvid	30	*Ostrea edulis*	1	3.3	1	3.3	n/d	-
	MEDULINSKI BAY								
05/2016	7 * Medulinski Bay	30	*Ostrea edulis*	2	6.7	3	6.7	n/d	-
11/2016	7 * Medulinski Bay	30	*Ostrea edulis*	1	3.3	1	3.3	n/d	-
07/2017	7 * Medulinski Bay	30	*Ostrea edulis*	2	6.7	3	10.0	n/d	-
11/2017	7 * Medulinski Bay	30	*Ostrea edulis*	1	3.3	1	3.3	n/d	-
11/2017	7 * Medulinski Bay	18	*Magallana gigas*	0	0	0	0	n/d	-
10/2020	7 * Medulinski Bay	30	*Ostrea edulis*	4	13.3	5	16.7	5	16.7
	LIM BAY								
07/2018	1 * Lim Bay	30	*Ostrea edulis*	1	3.3	2	6.7	n/d	-
2018	1 * Lim Bay	30	*Magallana gigas*	0	0	0	0	n/d	-
	SAVUDRIJA BAY								
06/2018	12 * Savudrija Bay	30	*Ostrea edulis*	2	6.7	3	10.0	n/d	-
09/2020	12 * Savudrija Bay	30	*Ostrea edulis*	1	6.7	4	13.3	4	13.3

* labels the number of the sampling site corresponding to the map of sampling sites.

All samples submitted within the surveillance program in the period 2016 to 2020, from the *B. exitiosa* positive sites are listed in Table 2 and sites are shown in Figure 1. Namely, from the site of the Medulin Bay during the mentioned period (2016–2020), 180 flat oysters were submitted; from the Mali Ston Bay with five sampling sites, there were 900 individuals, while 180 individuals were submitted from the sites from Lim Bay, Savudrija Bay, and Marina Bay, respectively.

After the first finding of *B. exitiosa* positive flat oysters in the northern part of the Eastern Adriatic coast, epidemiological surveillance was carried out. It was found that there were some populations of Pacific oyster (*Magallana = Crassostrea gigas*) present in the Medulin Bay production area, and during 2017, a sample of 18 animals was collected to

examine them as a possible source of infection. In 2018, after the detection of *B. exitiosa* in Lim Bay, another batch of 30 Pacific oyster was tested for the presence of this parasite.

2.2. Tissue Processing, Cytological and Histological Examination

After dissection of each oyster submitted for diagnostics, including Pacific oysters, tissues were collected for cytology, histology, and molecular analysis.

For cytological diagnosis of *Bonamia* spp., a piece of the heart of each oyster was taken and briefly dried on absorbent paper. A series of heart imprints were made in two rows on a glass slide. Imprints of five specimens were made on one slide and stained with Hemacolor® staining kit according to manufacturer instructions (Merck, Darmstadt, Germany).

For histological examination, transversal sections of soft tissue, including gills and the digestive gland of each oyster, were placed in histo-cassettes and immersed in Davidson's formalin, alcohol, acetic acid (AFA) fixative. Davidson-fixed tissues were dehydrated through a graded series of ethanols, succeeded by xylene, and embedded in paraffin, sectioned at 3 µm, and mounted on Microme EC 350-2 slides (Thermo Scientific, Waltham, MA, USA). Mounted slides were heated to 60 °C, deparaffinized, and rehydrated in xylene, a graded series of alcohol, and finally, water, followed by staining with hematoxylin and eosin (H&E).

For detection of *Bonamia* spp. by PCR, gill samples were taken and preserved in ethanol 96% until DNA extraction.

Stained tissue impressions and histological sections were examined on a Zeiss Axiskop-2 binocular microscope (Carl Zeiss, Jena, Germany) at 400× and 1000× magnifications with immersion oil.

2.3. Molecular Detection and Characterization

2.3.1. DNA Extraction

DNA used in all molecular analyses was extracted from gill tissues from each animal. From the samples collected from 2016–2019, DNA was extracted using MagMAX CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific, USA) on KingFisher Flex System (Thermo Fisher Scientific, Waltham, MA, USA). From the samples collected in 2020, DNA was extracted using innuPREP AniPath DNA/RNA Kit—IPC16 (Analytik Jena, Germany) on InnuPure C16 touch (Analytik Jena, Jena, Germany). Both methods of extraction were done according to the manufacturer's instructions.

2.3.2. PCR and Sequencing

In the period from 2016 to 2018, a modified method developed by Cochennec et al. [11] for the detection of *Bonamia* sp was used. The reaction mix contained 10 µL of GoTaq G2 Hot Start Master Mix (Promega, Madison, WI, USA), 60 ng of DNA as measured on a DS-11 Series Spectrophotometer (DeNovix, Wilmington, NC, USA), 0.5 µM of each primer (BO 5′ 3′, BOAS 5′ 3′), and nuclease-free water to the final volume of 20 µL. The amplification was performed using ProFlex PCR System (Applied Biosystems, Waltham, MA, USA) with an initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, elongation at 72 °C for 1 min, and ending with the final elongation step at 72 °C for 7 min. The presence of PCR products was examined by electrophoresis on a QIAxcel system (Qiagen, Hilden, Germany) using the QIAxcel DNA Screening Kit. For species identification, direct Sanger sequencing of the PCR products was performed by Macrogen Europe. Sequence identity was confirmed by BLAST [21].

2.3.3. Real-Time PCR

For the samples from 2019 and 2020, for the simultaneous detection of *B. ostreae* and *B. exitiosa* DNA, a probe-based real-time qPCR assay developed by the European Union Reference Laboratory (EURL) for Mollusc Diseases (Ifremer, La Tremblade, France) was

performed [22]. The reaction mix contained 10 µL of GoTaq® Probe qPCR Master Mix, 2X (Promega, Madison, WI, USA), 0.3 µM of each primer (BO2_F 5′ AAATGGCCTCTTCCCAATCT 3′, BO2_R 5′ CCGATCAAACTAGGCTGGAA 3′, BEa_F 5′ GACTTTGACCATCGGAAACG 3′, BEa_R 5′ ATCGAGTCGTACGCGAGTCT 3′), 0.2 µM of each double-labelled probe (BO2_probe 5′ HEX—TGACGATCGGGAATGAACGC—BHQ-1 3′, BEa_probe 5′ FAM—GGCAGCGAATCGATGGGAAT—BHQ-1 3′), 25 ng of template DNA as measured on a DS-11 Series Spectrophotometer (DeNovix, Wilmington, USA), and nuclease-free water to the final volume of 20 µL. The amplification was performed on qTower^3 thermal cycler (Analytik Jena, Jena, Germany) with the following program: 95 °C for 2 min for polymerase activation, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 30 s. The fluorescence was recorded at the end of each cycle with HEX and FAM filters. To detect possible differences, all positive samples were amplified and sequenced using the same method described above.

2.3.4. Sequencing and Phylogeny

Obtained sequences were analyzed using Geneious Prime 2020.0.1 software. For the phylogenetic analysis of the obtained sequences, similar sequences available in GenBank [23] were used and identified using BLAST [21]. Sequences were aligned with MAFFT v7.388 [24] using default parameters. Phylogenetic analysis was performed in MEGA 10.0.5 [25]. The best model was selected based on the lowest BIC score. The maximum likelihood tree was calculated with the following settings: K2+G substitution model, five discrete gamma categories, used all sites, and SPR level 5 heuristic method with very strong branch swap filter. Phylogeny was tested by the bootstrap method with 1000 replications. The obtained tree was visualized in iTOL [26]. Four sequences belonging to *Minchinia* spp. were used as an outgroup.

3. Results

Until 2016, the results of cytological examination of heart imprints, histological slides of oysters' organs, and PCR tested negative for the presence of parasite *Bonamia* spp. There were no reported mortalities, and there were no symptoms of the disease on the oysters from the production areas included in the surveillance program. In the May of 2016, for the first time, there were positive findings of the parasite *Bonamia exitiosa* from two production areas (Figure 1, Table 2).

The first one was in the north of the Eastern Adriatic Coast in Medulin Bay where 3 out of 30 animals tested positive for *Bonamia* sp. using conventional PCR and heart imprints, and the second one was in the south of the Croatian Adriatic Sea in the Mali Ston Bay. In all five sampling points, there were *Bonamia* sp. positive samples; (in Mali Ston and Brijesta, 3 out of 30, respectively, by heart imprints and five and six using conventional PCR; in Bistrina and Bjejevica, 2 out of 30, respectively, in imprints and two and three by PCR; and Sutvid, 1 out of 30 by each technique. Following the first finding in Medulin Bay in May, 1 out of 30 animals sampled in November of the same year tested positive. In 2017, positive samples were detected again in Medulin Bay in May and November and also in October of 2020. In 2019, a conventional PCR was replaced with a new molecular method, real-time PCR developed by EURL for mollusc diseases, and validated through interlaboratory proficiency testing as a screening method in the surveillance program. All samples analyzed in 2019 and 2020 were tested using real-time PCR. Therefore, samples collected in Medulin Bay in October 2020 were screened using real-time PCR, and positive samples were tested using conventional PCR and submitted for sequencing. In Mali Ston Bay, there have been no positive findings since 2016. In June 2018, there were positive findings of *B. exitiosa* in 2 out of 30 flat oysters sampled in the Lim Bay detected by imprints and three by PCR and in the same number by each method (two by imprints and three by PCR out of 30 flat oysters) sampled in the Savudrija Bay, two sampling points in the Northeastern Adriatic coast. In Savudrija Bay, in 2020, 4 out of 30 flat oysters were found positive by real-time PCR, confirmed by conventional PCR and sequencing. However, all

samples from Marina Bay tested negative for the presence of *Bonamia* sp. during the whole studied period using heart imprints, conventional and real-time PCR.

All pacific oysters collected in the Medulin Bay (n = 18) and from the Limski Bay (n = 30) tested negative for the presence of *Bonamia* sp by both used techniques.

Microscopic examination of heart imprints revealed mononuclear cells (Figure 2a) and, in fewer numbers, binuclear cells of light blue-stained cytoplasm and red-stained nuclei that were located centrally or near the center in mononuclear stages (Figure 2b).

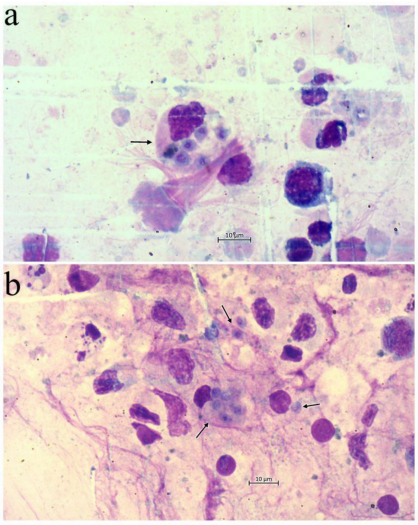

Figure 2. (**a**) Heart imprints of *Ostrea edulis* infected by cells identified as *Bonamia exitiosa* within hemocyte; (**b**) Heart imprints of *Ostrea edulis* infected by mononuclear and binucleated cells identified as *Bonamia exitiosa.* Hemacolor staining, 1000× magnification.

Microscopic examination of histological slides showed weak to moderate infiltration of hemocytes in the connective tissue of gills, digestive glands, and gonads. Weak to medium necrosis with low to medium presence of *Bonamia*-like parasites (Mean size: 1.95 µm) were found in the same tissues. In a small number of infected animals, the connective tissue of the gonads had marked necrotic changes and *Bonamia*-like cells were found (Figure 3a,b).

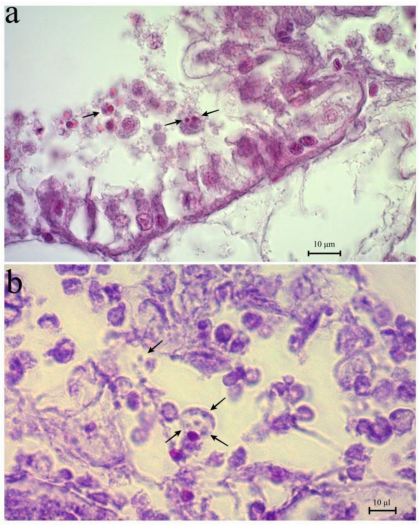

Figure 3. (**a**) Histological section of *Ostrea edulis*; *Bonamia*-like parasite in necrotic tissue of digestive gland (**b**) Gonadal connective tissue cells infected with *Bonamia*-like parasite. H&E staining, 1000× magnification.

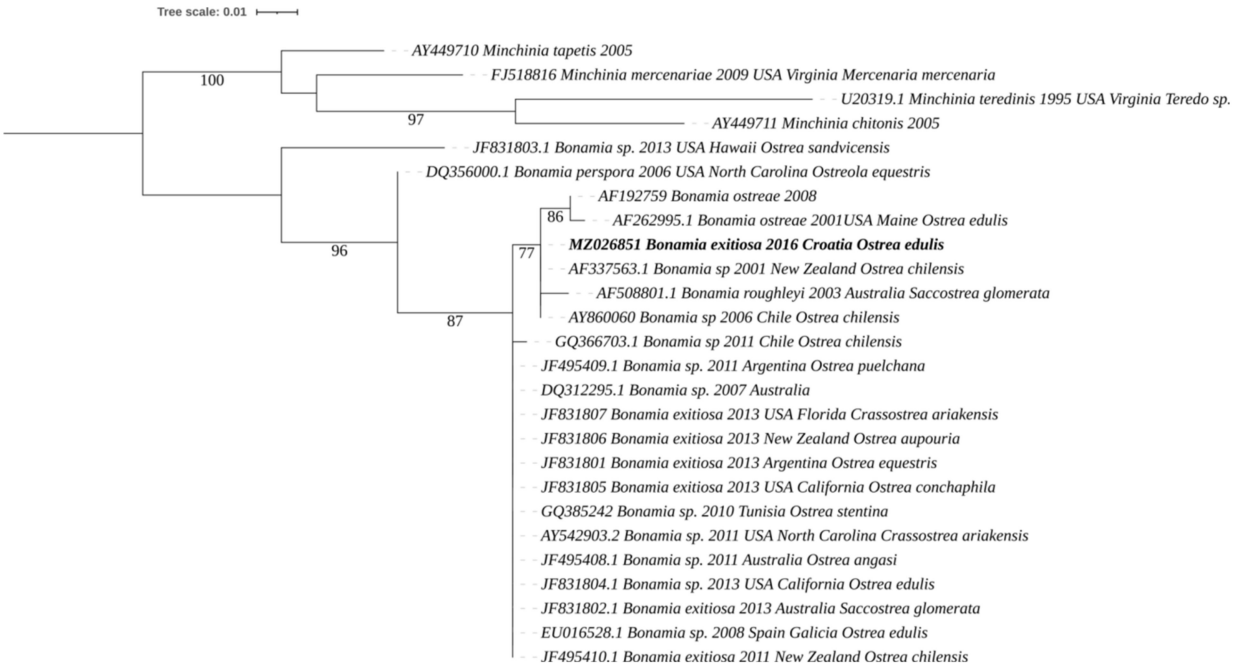

Figure 4. *Bonamia* spp. maximum likelihood phylogenetic tree. Bootstrap values higher than 75 are displayed.

4. Discussion

Until 2010, the diagnostic procedure for detection of *Bonamia* spp. was based on the results of heart imprints and histopathological evaluation of each oyster. Subsequently, since 2010, examinations of stained imprints and PCR screening methods for the same sample have been combined according to a dual detection strategy described by Carnegie and EU legislative [27,28]. In the period from 2010 to the first finding of *Bonamia* spp. positive oysters, 1870 individuals were tested in total.

In the spring of 2016, *Bonamia* parasite was detected for the first time by conventional PCR in the samples collected from the production area in the northern part of the Eastern Adriatic Sea, sampling point 7 (Figure 1). The sample consisted of 30 adult individuals (more than 2-y old), which were reported to be more susceptible to infection with *Bonamia* spp. compared to younger ages [29,30]. The microcellular finding was confirmed in stained heart imprints. The suspicion for the presence of *Bonamia ostreae* was set up, but sequencing revealed the presence of *Bonamia exitiosa*. Shortly following, the samples collected from the production area in the southern part of the Croatian Adriatic coast, the Mali Ston Bay, were submitted for diagnostics. In each of five samples (n = 150) from different sampling points, the same diagnostic procedure revealed that 17 flat oysters were PCR positive for *Bonamia* spp. Again, an affiliation of *B. exitiosa* was determined by sequencing of the PCR positive product. It was found that the prevalence of *B. exitiosa* in the different sampling points within Mali Ston Bay notably varied from 3.3% to 20.0% per sampling point (Table 2) when tested by PCR compared to prevalence of 3.3% to 13.3% after evaluation of stained heart imprints. In stained heart imprints, single cells with a central nucleus were observed by microscopic examination. However, outside hemocytes, there was a lower abundance of cells with two nuclei. This corresponds to the previously described imprints findings of *Bonamia exitiosa* in flat oysters [31–34]. Histopathological examination of positive PCR samples of *B. exitiosa* confirmed *Bonamia*-like cells in one-third of samples. In this study, the histological examination was proven as a less sensitive method in line with the finding of Lynch et al., who previously related the low prevalence of parasite to the low sensitivity [35]. It can also mean that environmental conditions were not favorable for the spread and development of infection or that our PCR positive findings were related to the detection of non-infective parasite stages [36].

Interestingly, *B. exitiosa* was detected in all five sampling points in the Mali Ston Bay only in spring of 2016 and never again until 2020, although surveillance was carried out regularly each year, and in total, 750 oysters were examined during this period and tested negative for the presence of *B. exitiosa*. At the same time, in the north of the Adriatic coast, *B. exitiosa* was confirmed for the first time by sequencing and heart imprints in spring 2016, and in following years, it was detected in another two production areas. In Medulin Bay, the findings of *B. exitiosa* continuously occurred from the spring of 2016 to autumn of 2020, except in 2019. During the seasons with positive findings of the parasite, the prevalence of the PCR positive samples varied from 3.3% to 16.7%. Unexpectedly, in 2019, there were no *B. exitiosa* positive/infected oysters, which could be explained with a low number of analyzed samples. There were only 90 individuals submitted for diagnostics of *Bonamia* spp.

It has been experimentally proven that *B. ostreae* favors lower sea temperature for survival [37] and higher prevalence of bonamiosis in oysters, and increased mortality in the colder period with lower sea temperatures has been described. On the contrary, in the case of *Bonamia* spp. in *C. ariakensis*, it has already been observed that the disease occurred in the warmer period when temperatures exceeded 20 °C [38]. Epidemiological circumstances of our *B. exitiosa* positive cases are unlike each of the reported cases, as no increase in mortalities or disease prevalence has been observed over the last five years.

For the reason mentioned above, the real impact of the infection is hard to evaluate. The prevalence of the *B. exitiosa* along the Croatian Adriatic Coast varied from 3.35% to a relatively high prevalence of 20% in the sampling point Brijesta in the Mali Ston Bay, and the recent recorded prevalence was 16.7% in the Medulin Bay, the site of the first finding (Table 2). These results show a higher prevalence of *B. exitiosa* compared to the findings in the study from the Manfredonia Bay in the southern part of the Italian Adriatic coast [32] in 2007, in which only two oysters out of a total of 750 were positive for *B. exitiosa*. There were no constancy in the prevalence on the sampling site over the studied period. It was shown that sequences of *B. exitiosa* detected in *O. edulis* from the Mediterranean area, including the one from Manfredonia Bay, Spain and also *O. stentina* in Tunisia [31,32,39], are 99.3% similar to those in Croatia, which supports the fact that the same strain is circulating throughout the Mediterranean basin.

Another concern is the answer to the question of how the flat oysters on Croatian production sites became infected with *B. exitiosa* It is reported that Pacific oysters are a vector or reservoirs of *Bonamia* spp. [40,41]. Although there is no farming of this species in the Croatian part of the Adriatic Sea, it has been present in the Lim Bay since 1963 [42]. This invasive species was also confirmed in the central Adriatic, but it was not found in the locality of Mali Ston, a southern production area with positive findings of *B. exitiosa* in the spring of 2016 [43]. The first positive finding of *B. exitiosa* in the Medulin Bay followed by a positive finding in Limski Bay in 2018 aroused suspicion of Pacific oysters as a source of infection for flat oysters. Unfortunately, the suspicion had to be discarded as both samples, one consisting of 18 Pacific oysters from the Medulin Bay in 2017 and another consisting of 30 Pacific oysters from the Lim Bay, tested negative for the presence of *Bonamia* spp. Furthermore, until now, there have been no records of mortality or introductions of new batches of either Pacific or flat oysters or any other mollusc species into the infected production areas. The possibility of some vector that might transfer the parasite across the Adriatic Sea should not be fully discarded, but it is hard to prove it.

Since the source of infection with *B. exitiosa* in flat oysters in all recorded production areas in Croatia remains unknown, another susceptible species may be considered a source of infection for the flat oyster. It is known that another oyster species, dwarf oysters (*O. stentina*), also exist in the Adriatic Sea [44,45], and phylogeographic research has confirmed that *B. exitiosa* has always been found in *O. stentina* [39]. The hypothesis could be made that *O. stentina*, an inhabitant in the Adriatic Sea, could be a source of *B. exitiosa*, as *O. stentina* in Tunisia were found to be positive for this parasite [39,46].

Moreover, it was not possible to get any additional information on the source of *B. exitiosa*'s origin from phylogenetic analysis. As it is visible from the phylogenetic tree, the similarity of the Croatian isolate of *B. exitiosa* to different previously sequenced isolates from Chile or Australia was 100%. The similarity of different *B. exitiosa* isolates found in different oyster species around the world emphasize the fact that the SSU rDNA gene is highly conserved. Therefore, the sequencing of additional genes or the whole genome should be carried out to provide us with more details on the phylogeny of the Croatian isolates.

More comprehensive molecular studies of the *B. exitiosa*, together with investigation of the natural population of *O. stentina* from production areas and natural beds along the Eastern Adriatic coast to confirm the natural-historical origin of the parasite *B. exitiosa*, will enable a better understanding of the pathogen's presence in the Croatian flat oyster production area.

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