Ecological Life Sciences
ISSN: 1308 7258 (NWSAELS)
ID: 2020.15.2.5A0133

Status : Research Article
Received: 06.12.2019
Accepted: 23.04.2020

Harun Yılmaz
Ercüment Genç
Esra Ünal Mine
Ankara University, Ankara-Turkey
H_yilmaz@hotmail.com; egenc@ankara.edu.tr; unalesramine@gmail.com
Şükrü Yıldırım
Ege University, sukru.yildirim@ege.edu.tr, İzmir-Turkey
Emre Keskin
Ankara University, keskin@ankara.edu.tr, Ankara-Turkey

DOI	http://dx.doi.org/10.12739/NWSA.2020.15.2.5A0133		
ORCID ID	0000-0002-1143-4862	0000-0001-7474-2208	0000-0003-3324-4493
	0000-0001-6411-8460	0000-0002-7279-313X	
CORRESPONDING AUTHOR	Ercüment Genç		

MOLECULAR IDENTIFICATION OF PARASITES ISOLATED FROM MEDITERRANEAN MUSSEL (*Mytilus galloprovincialis* Lamarck 1819) SPECIMENS

ABSTRACT

In this study, Mediterranean mussels (*Mytilus galloprovincialis* Lamarck, 1819) were collected from the most important mussel distribution/fishing areas in Turkish coastal waters (Çanakkale, Balıkesir, İzmir). The presence of parasite was investigated based on molecular methods. Molecular identification of parasite species was established by designing parasite-specific primers for PCR amplification and resulting nucleotide sequences were analysed. As a result of this study, four parasite species were identified as *Mytilicola intestinalis* Steuer 1902, *Mytilicola orientalis* Mori 1935, *Urastoma cyprinae* Graff 1882, *Parvatrema duboisi* Dollfus 1923 respectively. All identified species were found in İzmir (Agean Sea) specimens while *Urastoma cyprinae* and *Parvatrema duboisi* were found only in Çanakkale (Dardanelle) and Balikesir (Marmara Sea) specimens. Nucleotide composition, pairwise genetic distance and phylogenetic trees of the detected species were given. This research takes place to be the first study on identifying mussel parasites by using molecular techniques. Also, *Mytilicola orientalis* is the first record from Turkish coastal waters.

Keywords: Mollusca, Mediterranean, *Mytilus*, Parasite, Molecular Identification

1. INTRODUCTION

The mussel is commercially valuable molluscs that have been preferred for bivalve aquaculture for many years. Mediterranean mussel (*Mytilus galloprovincialis*) is naturally present from Northwest Spain in Europe to the coastal area of the Black Sea [1]. Due to high market value and demand, researchers have been investigating for growth, disease control and management of Mediterranean mussel culture. Especially parasitic agents are noteworthy among these. Many pathogens such as *Mytilicola intestinalis*, *Mytilicola orientalis*, *Parvatrema duboisi*, *Urastoma cyprinae* parasites from different parts of the Mediterranean Sea had been reported from Mediterranean mussel [2, 3, 4 and 5]. Additionally, *Marteilia refringens* is a "notifiable disease" in the World Animal Health Organization (OIE) list [6 and 4]. Those parasites caused outbreaks and destructive damages in mussel culture.

How to Cite:
Yılmaz, H., Genç, E., Ünal Mine E., Yıldırım, Ş., and Keskin, E., (2020). Molecular Identification of Parasites Isolated from Mediterranean Mussel (*Mytilus galloprovincialis* Lamarck 1819) Specimens, Ecological Life Sciences (NWSAELS), 15(2):61-71, DOI: 10.12739/NWSA.2020.15.2.5A0133.

Turbellarian parasite, *Urastoma cyprinae* is located in the mouth and gill filaments of mussels which found in many parts of the world [7]. This parasite causes reduced feeding capacity and tissue damages in infected mussels [8 and 9]. Murina and Solonchenko [10] reported that the infection of *Urastoma cyprinae* was intense during winter period in Caucassus coast (Black Sea). The presence of this parasite was noted from Black Sea, Sinop coast, Turkey [11, 12 and 5], and from Aveiro Lagoon, Portugal [3] in Mediterranean mussels. The trematode parasite *Parvatrema duboisi* was also reported from the gill filaments of the Mediterranean mussel in Black Sea, Sinop, Turkey [5]. Karagiannis et al. [4] conducted a study in order to understand the presence of some parasitic disease and mortality problems in aquaculture system. These researchers have reported that turbelarian *Urastoma cyprinae* and paramyxean parasite *Marteilia* sp. species were common, while copepod parasite *Mytilicola intestinalis* was low in Thermaikos Bay (Greece). Copepodids from digestive gland of Mediterranean mussel were found as *Mytilicola intestinalis* and *Mytilicola orientalis* in Aveiro Lagoon, Portugal [3]. They argued that the period when these parasites were most intense was summer and autumn in Portugal. According to Gresty [13], *Mytilicola intestinalis* is a common parasite of *Mytilus galloprovincialis*. This parasite occurs a red colour in the host. Adults may have worm-like appearances and extensions may be described as fairly short wolves [14]. This parasite had reduced growth rate in the mussels [15]. It was also noted that *Mytilicola intestinalis* caused sudden deaths of mussels in Netherlands [16] and filtration-feeding problems were recorded by Korringa [17]. It has been suggested that biochemical changes occur in the tissues of parasitized mussels [18]. Another Mytilicolid, *Mytilicola orientalis* is a red copepod like *M. intestinalis* can be seen in oysters such as *Ostrea lurida* and *Ostrea gigas* and also in blue mussel (*Mytilus edulis*). Previous researchers claim that *Mytilicola orientalis* were accidentally contaminated from Asia to Europe and North America [19, 16, 20 and 21]. It has been demonstrated that blue mussels are sensitive to this parasite [22]. On a global scale, marine bivalves are transported from one place to another for consumption and aquaculture purposes can cause the spread of parasites [23]. Mialhe et al. [24 and 25] stated that since the conventional parasite identification techniques are limited with microscopy, histology and rarely ultrastructural ways therefore using molecular methods are important in terms of time and reliability. Conventional techniques may be problematic in defining many pathogens by these methods [24, 26, 27, 28, 25, 29, 30, 31, 32, 6 and 33]. With this approach, many researchers have been trying to develop DNA-based diagnostic techniques for mollusc pathogens [29] for decades.

These techniques are defined as routinely used to determine the pre-disease presence of pathogens and to be used in international status in disease monitoring programs. It is also emphasized that the diagnosis of mussel diseases should be made fast, reliable and sensitive. In this context, Pernas et al. [31] stated that the identification by DNA based method for *Marteilia refringens* which is a bivalve notifiable disease agent is sensitive and applicable in comparison with the conventional method. DNA based molecular studies are limited on mussel parasites up to date. With PCR based methods, extraction, amplification and sequencing of DNA can be done quickly with a small tissue sample [34]. Therefore, this study aimed to investigate the presence of parasites in *Mytilus galloprovincialis* from three different fishing areas/local fish markets in Mediterranean region (Agean Sea: İzmir, Dardanelle: Çanakkale and the Marmara Sea:

Balıkesir) by using the species-level molecular identification approach.

2. RESEARCH SIGNIFICANCE

It is complicated to identify parasites from tissue sections by the conventional method. Only two species of parasites could be identified from histological sections. Molecular diagnostic techniques revealed four different species of parasites. Nucleotide composition, pairwise genetic distance and the phylogenetic trees of the examined parasites of mussels were given. In this study, mussel parasites have been identified using molecular techniques. And, *Mytilicola orientalis* was reported for the first time in Turkish coastal waters.

3. MATERIALS AND METHODS

3.1. Sample Collections

Mediterranean mussels were collected from the essential natural mussel distribution areas of Turkish coastal waters. One hundred sixty-five alive mussel samples were taken from each sampling locations in Çanakkale (N=55, 8.27±2.03cm), Erdek-Balıkesir (N=55, 9.14±2.30cm) and Buca-İzmir (N=55, 8.56±2.16cm) local fish markets.

3.2. DNA Extraction and PCR

The mussels were wrapped in parchment paper during transportation, put in plastic nets with a volume of 10 L and placed in cold conditions (5-7°C) were transported to the Evolutionary Genetic Laboratory in Ankara University, Faculty of Agriculture, Fisheries and Aquaculture Research and Application Unit within 24 hours. A total of 165 mussels were pooled in 3 location groups. Each location groups were divided into ten pools. The samples from each location were fixed in 70% ethanol and were frozen with liquid nitrogen and homogenized by mechanically smashed with a mortar and pestle. Each pool was tested with seven different primers of target species. The samples were isolated using a commercial DNA purification kit (K0722, Thermo Fisher Scientific, Lithuania) according to manufacturer protocol. The extracted DNA samples were run on 1% agarose gel and samples DNA's quality and concentration were measured using Colibri Microvolume Spectrometer (Titertek, Germany). After that DNA concentration was stored diluted to be 50 ng/µL. The primers were designed in accordance with the previous studies results which reported of the common pathogens for the mussels [1 and 25]. The samples were amplified by the PCR method using a new designed taxa specific primers (Table 1).

Table 1. Species-specific designed primers

Species	Forward	Reverse
Mytilicola intestinalis	5'-TGTACTGGATGGTGACA-3'	5'-GTTAGAACTAGGGCGGTA-3'
Mytilicola orientalis	5'-ACAGCCCAAGTAAGGTGA-3'	5'-CCGCAGAGCCTAGAAGAA-3'
Urastoma cyprinae	5'-AGGGAGGTAGTGACGAAA-3'	5'-TGATAGGCAGAGTCGGTA-3'
Parvatrema duboisi	5'-TGCTGTCGAGCTGCGAA-3'	5'-GAGCGGCCGAAACCACTA-3'
Nematopsis legeri	5'-TTGGACTACCGTGGCTTTTACA-3'	5'-CTCAGGCGTTCTCTCCGAAA-3'
Marteilia refringens	5'-GTTCGGTCGCCACTACGA-3'	5'-GCGGAAAAGCGTGTGATCA-3'
Peniculistoma mytili	5'GGGAGGTAGTGACAAGAAATAGCA-3'	5'-TCGAAAGCTGATAGGGCAGAAA-3'

PCR reactions were conducted with 4.0µl from 5xFIREPol Master Mix, 0.5µl from each primer (10pmol/µl), 1.0µl from DNA (50ng/µl) and 14µl from ddH_2O. PCR thermal profile was set to 5 min at 95°C, 30 cycles of 15 sec at 95°C, 45 sec at 54-66°C, 2 min at 72°C, completed with a final extension for 10 min at 72°C. The PCR products were analysed 2% agarose gel electrophoresis and the amplicon size (bp) was confirmed.

3.3. DNA Sequencing and Phylogenetic Analysis

The PCR products were purified with a commercial clean-up kit (Wizard® SV Gel and PCR Clean-Up System, Promega). The amplicons were sequenced (BMLabosis-Macrogen, Ankara) by using the same oligonucleotides. The DNA sequences were performed in the form of a dideoxynucleotide chain termination reaction using the Big Dye Cycle Sequencing Kit V.3.1 on the ABI 3130XL Genetic Analyzer platform as service acquisition. The obtained nucleotide sequences were aligned and compared to registered reference sequences in the Gene Bank and BOLD database and standardized for phylogenetic analysis. Afterwards nucleotide compositions, nucleotide pair frequency and the genetic distance matrix have been generated and relationship among those presented by neighbor joining tree using MEGA 5 software (Tamura et al., 2011). Afterwards, the nucleotide composition, nucleotide pair frequency and transcription/transversion ratio were calculated, and at last, genetic distance matrices were generated according to the Kimura 2-parameter model and the relationship among these genes and neighbor joining trees were determined. The DNA sequences for all the samples were alingned by clustalW and Sequencher 5.0 software. MEGA 5 (SEQ ID NO: 5) with the help of reference sequences obtained from the GenBank database (HM775188 for *Mytilicola intestinalis* 18S, HM775189 for *Mytilicola orientalis* 18S, AF167422 for *Urastoma cyprinae* 18S and AB478509 for *Parvatrema dubosi* 5.8S) [35]. Nucleotide compositions and average A-T/G-C contents of the aligned DNA sequences were analyzed as percentages. MEGA 5 and Sequencher 5.0 software were used in the analysis. The codons generated by the DNA sequences were analysed and the codon frequencies and relative synonymous codon usage (RSCU) rates were determined. The RSCU ratios were calculated as the ratio of the observed codon frequencies to the expected codon frequencies (in the case of equalization of all the codons used for the same amino acid) [36]. Analyses were performed using MEGA 5 software. Analysis of genetic distance between species was carried out using MEGA 5 and Arlequin 3.5 software using Kimura 2-parameter model. 1000 repetitive bootstrap analyses were used for standard error calculations of distance values. MEGA 5, PAUP 4.0 (Swofford 2002) and PHYLIP [37] software was used in the analysis of evolutionary relationships. The neighbor joining (NJ) method [38] was used to evaluate the effect of the method used to calculate the evolutionary relationship between the samples. One thousand repeat bootstrap tests were used to control the reliability of the trees. The relationship between parasite types has also been evaluated via the median joining network which will be established with Network 4.6.1.0 [39].

4. FINDINGS AND DISCUSSIONS

The positive PCRs were obtained from only 4 primer sets. *Urastoma cyprinae*, *Parvatrema duboisi* have been identified from all tested locations. *Mytilicola intestinalis* and *Mytilicola orientalis* were only detected from Buca-İzmir (Table 2). The nucleotide composition of the four replicated regions, the phylogenetic tree was also evaluated (Figure 1).

Table 2. Identified parasite species and sampling locations

Parasites/Locations	Buca-İzmir	Erdek-Balıkesir	Çanakkale
Mytilicola intestinalis	+	-	-
Mytilicola orientalis	+	-	-
Urastoma cyprinae	+	+	+
Parvatrema duboisi	+	+	+

Table 3. Genetic distance matrix

		1	2	3
1	*Mytilicola intestinalis*	-	0.003	0.013
2	*Mytilicola orientalis*	0.012	-	0.014
3	*Urastoma cyprinae*	0.246	0.240	-

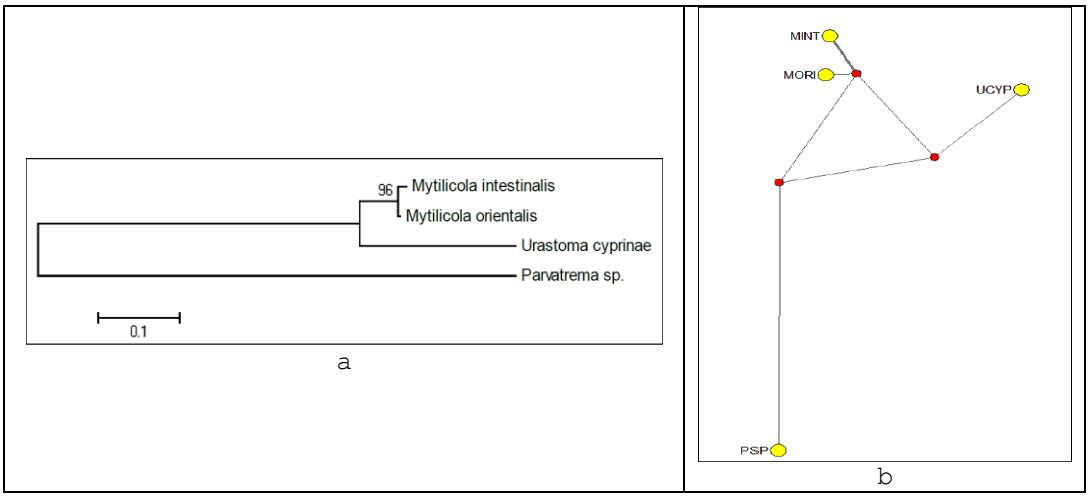

Figure 1. a: Neighbor joining phylogenetic tree, b: Median joining network (MINT: *M. intestinalis*, MORI: *M. orientalis*, UCYP: *U. cyprinae*, PSP: *Parvatrema* sp., Mutational vectors are shown in red)

Molecular analyses were performed from 18S (*Mytilicola intestinalis*, *Mytilicola orientalis* and *Urastoma cyprinae*) and 5.8S *(Parvatrema duboisi)* regions. Therefore, the *Parvatrema dubosi* data set was excluded from the genetic distance matrix (Table 3).

The nucleotide sequences obtained were paired with BLAST search engine aid and clustalW, MEGA 5, and Sequencher 5.0 (SEQ ID NO:2) were generated using the matched reference sequences (HM775188 for *Mytilicola intestinalis* 18S, HM775189 for *Mytilicola orientalis* 18S, AF167422 for the *Urastoma cyprinae* 18S and AB478509 for *Parvatrema dubosi* 5.8S) aligned with software. When the nucleotide sequences were examined, it was determined that there was no intra-species variation. All four identified species were represented by a single haplotype. The nucleotide compositions and average A-T/G-C contents of the aligned DNA sequences were analysed in percentage and the average values are as follows: 26.2% for T, 21.7% for C, 25.0% for A and 27.0% for G. The highest value of G-C was *Parvatrema duboisi* and it was 52.6% while the lowest value was 44.1% and it belongs to *Urastoma cyprinae*.

Since the results of the universal primers and the protein-coding gene, cytochrome C oxidase I, were not obtained, the codon usage was not calculated. Analysis of genetic distance between species was carried out using MEGA 5 and Arlequin 3.5 software using Kimura 2-parameter model. 1000 repetitive bootstrap analysis were used for standard error calculations of distance values. The genetic distance between two species of *Mytilicola* genus is low as expected (1.2%) and the genetic distance between *Mytilicola* species and *Urastoma cyprinae* is high (24.0-24.6%). The low in-species genetic distance is related to the fact that the gene being analysed is not a gene encoding protein and thus has a low rate of evolution. For forming the phylogenetic tree for evolutionary relationship analysis, neighbor joining (NJ) method was applied with 1000 repetitive bootstrap tests drawn according to Kimura 2 parameter model. The generated tree is shown in Figure 1.

Since at least 4 taxa were required to be able to draw neighbour joining trees, the closest reference sequence (KM246856.1) for *Parvatrema dubosi,* the fourth species from GenBank, was downloaded and used as an outgroup in the analysis. Median joining network analysis has also been used to reveal the relationship between parasite species. As in the analysis of evolutionary relationships, the fourth species, *Parvatrema duboisi*, which is defined as working as an external group, has benefited from the reference nucleotide sequence of the nearest species (Figure 1b). *Mytilicola* species have a single mutational vector between them; 2 mutational vectors from *U. cyprinae*; Parvatrema sp. is separated by 3 mutational vectors. When evaluated together with phylogenetic tree and genetic distance matrix, it is seen that the results are consistent and all phylogenetic analyses have been concluded with the systematic distances. Systematic classifications of the parasites obtained using molecular methods were arranged as a chart according to Boxshall [40, 41, 42 and 43].

165 Mediterranean mussels (*M. galloprovincialis*) (Çanakkale, BalIkesir and İzmir) were obtained alive from local fish markets of Turkey. Different parasites have been reported by different investigators before the Mediterranean mussel, which is the subject of conventional parasitological studies Table 5. In this study, as a result of the molecular analysis; the parasite species were identified as *Mytilicola intestinalis, Mytilicola orientalis*, *Urastoma cyprinae* and *Parvatrema dubosi*.

Table 5. Reported parasites from *Mytilus galloprovincialis* in literature and in the present study

Host	Identified parasites in the present study	Previous studies
Mytilus galloprovincialis	*Mytilicola intestinalis*	[2, 3, 4, 8, 9, 15, 17, 18, 44, 45, 46, 47]
	Mytilicola orientalis	[3]
	Urastoma cyprinaei	[2, 3, 9, 10, 47, 48, 49, 50, 51, 52]
	Parvatrema duboisi	[5, 53, 54, 55, 56, 57]

Mytilicola orientalis was first reported in Mediterranean Sea by Francisco et al. [3] from the Aveiro Lagoon of Portugal. The present study also reports *Mytilicola orientalis* for the first time in Turkish waters by molecular analysis. When we evaluate molecular results, our results are consistent with the Mialhe et al. [24], Le Roux et al. [30] results. Our results are also parallel with Pernas et al [31] who have reported *Marteilia refringens* by conventional and PCR methods. Additionally, the present stduy results are in correlation with Zrncic et al. [58] who have reported this parasite from *Mytilus galloprovincialis* by *in situ* hybridization using the *Marteilia refringens* probe, Lopez-Flores et al. [6] which states that *Marteilia refringens* and *Marteilia maurini* are identified by the molecular method [59]. *Mytilicola intestinalis, Mytilicola orientalis, Parvatrema duboisi* and *Urastoma cyprinae* were reported from *Mytilus galloprovincialis* for the first record by using molecular methods in the waters of Turkey.

5. CONCLUSIONS AND RECOMMENDATIONS

In this study, molecular examination results showed that there were four different parasites. Two of these parasites belong to the Rhabditophora and Trematoda classes of the Platyhelminthes phylum and the others belong to the Copepoda subclass of the Arthropoda phylum. While four parasites were detected by the molecular method in Izmir specimens, only two species isolated from Balikesir and Canakkale specimens. With this research, *Mytilicola orientalis* takes place to be the first record for the waters of Turkey in Mediterranean mussel. It

can be considered that using molecular methods on bio-ecology and pathogenesis of mussel parasites will help to understand host-para site interactions and disease control management for the future studies.

ACKNOWLEDGEMENT

The authors thank the staffs of the Evolutionary Genetics Laboratory (EGL), at Ankara University for the laboratory work. The authors gratefully acknowledge Dr. Gökben Özbakiş Beceriklisoy, who is the responsible Veterinarian of Fisheries Research and Application Unit, Faculty of Agriculture, Ankara University. *Funding information*: This study (M.Sc. Thesis of Harun YILMAZ) was supported by the Ankara University Scientific Research Projects Coordination Unit (Project grant number 14H0447002). In addition, this research was supported by Akuamaks Ltd. (Rabat Sokak 22/6 G.O.P, Cankaya, Ankara, Turkey) for the contribution in the aquaculture sector. *Compliance with ethical standards*: Conflict of interest, the authors declare that they have no conflict of interest. *Ethical approval*, all applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

REFERENCES

[1] Figueras, A.J., (1989). Mussel culture in Spain and France. World Aquaculture, 20(4):8-17.

[2] Rayyan, A., Photis, G., and Chintiroglou, C.C., (2004). Metazoan parasite species in cultured mussel *Mytilus galloprovincialis* in the Thermaikos Gulf (North Aegean Sea, Greece). Diseases of Aquatic Organisms, 58(1): 55-62. http://dx.doi.org/10.3354/dao058055

[3] Francisco, C.J., Hermida, M.A., and Santos, M.J., (2010). Parasites and symbionts from *Mytilus galloprovincialis* (Lamark, 1819) (Bivalves: Mytilidae) of the Aveiro estuary Portugal. Journal of Parasitology, 96(1):200-205. http://dx.doi.org/10.1645/GE-2064.1

[4] Karagiannis, D., Vatsos, I.N., Theodoridis, A., and Angelidis, P., (2013). Effect of culture system on the prevalence of parasites of the Mediterranean mussel *Mytilus galloprovincialis* (Lamark, 1819). Journal of the Hellenic Veterinary Medical Society, 64(2):113-122. http://dx.doi.org/10.12681/jhvms.15484

[5] Özer, A. and Güneydağ, S., (2014). First report of some parasites from Mediterranean mussel, *Mytilus galloprovincialis* Lamarck, 1819, collected from the Black Sea coast at Sinop. Turkish Journal of Zoology, 38(4):486-490. http://dx.doi.org/10.3906/zoo-1401-2.

[6] López-Flores, I., De La Herran, R., Garrido-Ramos, M.A., Navas, J.I., Ruiz-Rejon, C., and Ruiz-Rejon, M., (2004). The molecular diagnosis of *Marteilia refringens* and differentiation between *Marteilia* strains infecting oysters and mussels based on the rDNA IGS sequence. Parasitology, 129(4):411-419. https://doi.org/10.1017/S0031182004005827.

[7] González, C.C., Alvarez, R.R., Domínguez, H.R., Bua, M.S., Iglesias, R., Fernández, C.A., and Estévez, J.G., (2005). In vitro reproduction of the turbellarian *Urastoma cyprinae* isolated from *Mytilus galloprovincialis*. Marine Biology, 147(3):755-760.

[8] Robledo, J.A.F., Santarém, M.M., and Figueras, A., (1994). Parasite loads of rafted blue mussels (*Mytilus galloprovincialis*) in Spain with special reference to the

copepod, *Mytilicola intestinalis*. Aquaculture, 127(4):287-302. https://doi.org/10.1007/s00227-005-1622-9

[9] Villalba, A., Mourelle, S.G., Carballal, M.J., and Lopez, C., (1997). Symbionts and diseases of farmed mussels *Mytilus galloprovincialis* throughout the culture process in the Rias of Galicia (NW Spain). Diseases of Aquatic Organisms, 31(2):127-139.https://doi.org/10.3354/dao031127

[10] Murina, G.V. and Solonchenko, A.I., (1991). Commensals of *Mytilus galloprovincialis* in the Black sea: *Urastoma cyprinae* (Turbellaria) and *Polydora ciliata* (Polychaeta). In Turbellarian Biology (pp:385-387). Springer, Dordrecht.

[11] Harunoglu, E.M., (2005). Parasites of Mediterranean mussel (*Mytilus galloprovincialis* Lmk., 1819) from Izmir bay (Aegean Sea, Turkey). Ege University, Graduate School of Natural and Applied Science, Department of Fisheries Basic Science, MSc Thesis (in Turkish), 120 p.

[12] Guneydag, S., (2013). Parasite fauna of *Mytilus galloprovincialis* living in Sinop area of Black Sea. Sinop University, Graduate School of Natural and Applied Science, Department of Aquaculture, MSc Thesis (in Turkish), 123 p.

[13] Gresty, K.A., (1992). Ultrastructure of the Midgut of the Copepod *Mytilicola intestinalis* Steuer, an Endoparasite of the Mussel *Mytilus edulis* L. Journal of Crustacean Biology, 12(2):169-177.

[14] Hockley, A.R., (1951). On the biology of *Mytilicola intestinalis* (Steuer). Journal of the Marine Biological Association of the United Kingdom, 30(2):223-232.

[15] Cole, H.A., and Savage, R.E., (1951). The effect of the parasitic copepod, *Mytilicola intestinalis* (Steuer) upon the condition of mussels. Parasitology, 41(3-4):156-161.

[16] Stock, J.H., (1993). Copepoda (Crustacea) associated with commercial and non-commercial Bivalvia in the East Scheldt, The Netherlands. Bijdragen tot de Dierkunde, 63(1):61-64.

[17] Korringa, P., (1952). Epidemiological observations on the mussel parasite *Mytilicola intestinalis* Steuer, carried out in the Netherlands 1952. Ann Biol, 9:219-224.

[18] Williams, C.S., (1969). The effect of *Mytilicola intestinalis* on the biochemical composition of mussels. Journal of the Marine Biological Association of the United Kingdom, 49(1):161-173.

[19] His, E., (1977). Observations préliminaires sur la présence de *Mytilicola orientalis* Mori (1935) chez *Crassostrea gigas* Thunberg dans le basin d'Arcachon. Bull Soc Géol amis Mus Havre, 64:7-8.

[20] Holmes, J.M.C., and Minchin, D., (1995). Two exotic copepods imported into Ireland with the Pacific oyster *Crassostrea gigas* (Thunberg). The Irish Naturalists' Journal, 25(1):17-20.

[21] Minchin, D., (1996). Management of the introduction and transfer of marine molluscs. Aquatic Conservation: Marine and Freshwater Ecosystems, 6(4):229-244.

[22] Grizel, H., (1985). Mytilicola orientalis MORI, parasitism parasitose a Mytilicola orientalis. MORI. Fiches d'identification des maladies et parasites des poissons, crustaces et mollusques. Conseil international pour l'exploration de la mer, Copenhagen, 1-4.

[23] Torchin, M. E., and Kuris, A.M., (2005). Introduced marine parasites. In: *Marine parasitology,* Rohde, K. (Ed.), Csiro Publishing. 358-366.

[24] Mialhe, E., Bachere, E., Le Bec, C., and Grizel, H., (1985). Isolement et purification de *Marteilia* (Protozoa: Ascetospora)

parasites de bivalves marins. *Comptes rendus de l'Académie des sciences. Série 3, Sciences de la vie*, *301*(4):137-142.
[25] Mialhe, E., Bachere, E., Boulo, V., and Cadoret, J.P., (1995). Future of biotechnology-based control of disease in marine. Molecular marine biology and biotechnology, 4(4):275-283.
[26] Sogin, M.L., and Gunderson, J.H., (1987). Structural diversity of eukaryotic small subunit ribosomal RNAs a: Evolutionary implications. Annals of the New York Academy of Sciences, 503(1):125-139.
[27] Figueras, A.J., (1988). Aber disease of edible oysters caused by *Marteilia refringens*. *Amer. Fish. Soc. Spec. Pub.*, 18:38-46.
[28] Gosling, E.G., (1992). The Mussel *Mytilus*: Ecology, Physiology. Genetics and Culture. Amsterdam: Elsevier.
[29] Berthe, F.C.J., Burreson, E.M., and Hine, M., (1999). Use of molecular tools for mollusc disease diagnosis. Bulletin of the European Association of Fish Pathologists, 19(6):277.
[30] Le Roux, F., Audemard, C., Barnaud, A., and Berthe, F., (1999). DNA probes as potential tools for the detection of Marteilia refringens. Marine Biotechnology, 1(6):588-597.
[31] Pernas, M., Novoa, B., Berthe, F., Tafalla, C., and Figueras, A., (2001). Molecular methods for the diagnosis of *Marteilia refringens*. Bulletin of the European Association of Fish Pathologists. 21:200-208.
[32] Fuentes, J., López, J.L., Mosquera, E., Vázquez, J., Villalba, A., and Álvarez, G., (2002). Growth, mortality, pathological conditions and protein expression of *Mytilus edulis* and *M. galloprovincialis* crosses cultured in the Rıá de Arousa (NW of Spain). *Aquaculture*, *213*(1-4):233-251. https://doi.org/10.1016/S0044-8486(02)00046-7
[33] Kalkan, E., Kurtuluş, A., Maraci, Ö., and Bilgin, R., (2011). Is the Bosphorus Strait a barrier to gene flow for the Mediterranean mussel, Mytilus galloprovincialis (Lamarck, 1819)?. *Marine Biology Research*, 7(7):690-700. https://doi.org/10.1080/17451000.2011.558098
[34] McManus, D.P., and Bowles, J., (1996). Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systematics. International Journal for Parasitology, 26(7):687-704.
[35] Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S., (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution, 28(10):2731-2739. https://doi.org/10.1093/molbev/msr121
[36] Sharp, P.M., and Li, W.H., (1986). An evolutionary perspective on synonymous codon usage in unicellular organisms. Journal of molecular evolution, 24:28-38.
[37] Felsenstein, J., (1993). Phylogeny Inference Package (PHYLIP). Version 3.5. University of Washington, Seattle, United States of America.
[38] Saitou, N. and Nei, M., (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 4:406-425.
[39] Bandelt, H.J., Forster, P., and Röhl, A., (1999). Median-joining networks for inferring intraspecific phylogenies. Molecular biology and evolution, 16(1):37-48.
[40] Boxshall, G., (2015a). *Mytilicola intestinalis* Steuer, 1902. In: T.C Walter, G. Boxshall, World of Copepods database. http://www.marinespecies.org/aphia.php?p=taxdetailsandid=128900 on 2015-12-07.

[41] Boxshall, G., (2015b). *Mytilicola orientalis*. In: T.C. Walter, G. Boxshall, World of Copepods database. http://marinespecies.org/aphia.php?p=taxdetailsandid=128901 on 2015-12-07.
[42] Tyler, S., (2015). *Urastoma cyprinae* (Graff, 1882). http://www.marinespecies.org/aphia.php?p=taxdetailsandid=142950 on 2015-12-07.
[43] Gibson, D., (2015) *Parvatrema duboisi* (Dollfus, 1923). http://www.marinespecies.org/aphia.php?p=taxdetailsandid=109235 on 2015-12-07.
[44] Bolster, G.C., (1954). The biology and dispersal of *Mytilicola intestinalis* Steuer: a copepod parasite of mussels. Her Maj. Stat. Office.
[45] Korringa, P., (1968). On the ecology and distribution of the parasitic copepod *Mytilicola intestinalis* Steuer. Bijdragen tot de Dierkunde. 38:47-57.
[46] Camacho, A.P., Villalba, A., Beiras, R., and Labarta, U., (1997). Absorption efficiency and condition of cultured mussels (*Mytilus edulis galloprovincialis* Linnaeus) of Galicia (NW Spain) infected by parasites *Marteilia refringens* Grizel et al. and *Mytilicola intestinalis* Steuer. Oceanographic Literature Review. 11: 1351.
[47] Canestri, T.G., Baccarani, E.M., Giannetto, S., Giuffrida, A., and Paesanti, F., (1998) Prevalence of *Mytilicola intestinalis* (Copepoda: Mytilicolidae) and *Urastoma cyprinae* (Turbellaria: Hypotrichinidae) in marketable mussels *Mytilus galloprovincialis* in Italy. Diseases of aquatic organisms. 32:145-149.
[48] Rohde, K., Noury-Sraïri, N., Watson, N., Justine, J.L., and Euzet, L., (1990). Ultrastructure of the flame bulbs of *Urastoma cyprinae* (Platyhelminthes, 'Prolecithophora', Urastomidae). Acta zoologica. 71:211-216.
[49] Cáceres-Martínez, J., Vásquez-Yeomans, R., and Sluys, R., (1996). *Urastoma cyprinae* in natural and cultured mussel (*Mytilus galloprovincialis* LMK) populations in México. Bulletin of the European Association of Fish Pathologists. 16:200-202.
[50] Comps, M. and Tige, G., (1999). Procaryotic infections in the mussel *Mytilus galloprovincialis* and its parasite the turbellarian *Urastoma cyprinae*, France. Diseases of Aquatic organisms. 38: 211-217.
[51] Crespo-Gonzalez, C., Alvarez, R.R., Domínguez, H.R., Búa, M.S., Iglesias, R., Fernández, C.A., and Estévez, J.G., (2005). In vitro reproduction of the turbellarian *Urastoma cyprinae* isolated from *Mytilus galloprovincialis*. Marine Biology. 147:755-760.
[52] Crespo-González, C., Rodríguez-Domínguez, H., Segade, P., Iglesias, R., Arias, C., and García-Estévez, J.M., (2010). Seasonal Dynamics and Microhabitat Distribution of *Urastoma cyprinae* in *Mytilus galloprovincialis*: Implications for Its Life Cycle. Journal of Shellfish Research. 29:187-192. https://doi.org/10.2983/035.029.0113
[53] Holodkovskaya, E.B., (1989). Fauna parazitov i kommensalov midii *Mytilus galloprovincialis* (Lamark, 1819). V severpo-zapadnoi chasti Chernogo mopya (sistematika, ekologia, prakticheskoe znachenie). Moscow State University, PhD Thesis (in Russian).
[54] Gaevskaya, A.V. and Machevsky, V.K., (1995). Impact of man-made coastal structures on formation and function of parasite systems. In: ECOSET'95 Sixth International Conference on Aquatic Habitat Enhancement; 29 Oct.-02 Nov. 1995; Tokyo. 531-536.

[55] Holodkovskaya, E.B., (2002) Biological diversity of parasites of *Mytilus galloprovincialis* in the Black Sea. In: Minicheva GG, editor. The Black Sea Ecological Problems. Odessa, Ukraine: SCSEIO. 265-269.
[56] Holodkovskaya, E.B., (2003). Study on symbiont fauna of commercial Black Sea bivalve molluscs. Ecol Morya. 63:66–72.
[57] Popov, M.A., Kovrigina, N.P., Machevsky, V.K., Lozovsky, V.L., and Kozintsev, A.F., (2010). Influence of the anthropogenic factor on hydrochemical parameters, mussel *Mytilus galloprovincialis* LAM., and its endosymbionts in a Balaklava Bay. Ternopol нац. Nat пед. Ped ун-ту. University Сер. AvgБіол. 44:205-208.
[58] Zrncic, S., Le Roux, F., Oraic, D., Sostaric, B., and Berthe, F., (2001). First record of *Marteilia* sp.in mussels *Mytilus galloprovincialis* in Croatia. Diseases of aquatic organisms. 44: 143-148. https://doi.org/10.3354/dao044143
[59] Balseiro, P., Montes, A., Ceschia, G., Gestal, C., Novoa, B., and Figueras A., (2007). Molecular epizootiology of the European *Marteilia* spp., infecting mussels (*Mytilus galloprovincialis* and *Mytilus edulis*) and oysters (*Ostrea edulis*): an update. Bulletin of the European Association of Fish Pathologists. 27:148-156.