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Molecular phylogeny of *Bopyrus crangorum*, a parasitic isopod associated with *Palaemon elegans* rock pool shrimp.

by
Angelos Zoupas

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Department of Biology

National and Kapodistrian University of Athens

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Abstract

Bopyrus crangorum belongs to the suborder Epicaridea, which includes obligate isopod parasites feeding on the haemolymph of crustaceans. The parasite attaches on the branchiae of its final host (caridean shrimp) causing sterilization, feminization and reduction in metabolic activity. It has been recorded on seven different species of the genus *Palaemon* including some with commercial value, however, knowledge on its distribution patterns and ecology (i.e. population dynamics of the parasite in relation to its host and environmental parameters) is limited in the eastern Mediterranean. The present study aimed to understand the phylogenetic relationships of the bopyrid parasites, the host's genetic diversity and association of shrimp infection rates to temperature and salinity. Specimens were obtained from two sites at the Saronikos Gulf (Aegean Sea) and both the shrimp and the isopod parasite were identified with the use of morphological methods. Part of the small ribosomal subunit gene (SSU) of *B. crangorum* was employed for phylogenetic analysis, supporting the current taxonomy of the parasite as a member of the Bopyrinae subfamily. Identification of the shrimp was further enhanced by the use of a fragment of the cytochrome oxidase subunit 1 gene (COI) in addition to revealing the presence of type II haplogroup through haplotypic network construction. Finally, a three-year population monitoring (2021-2023) revealed greater infection rates and abundance of the parasite and its host in the months of spring compared to winter. Rock pool - air temperature and salinity followed a similar trend showcasing a positive relationship with infection rates for the same months. This is the first study to investigate the temporal variability of parasitism of *B. crangorum* on *P. elegans* and the possible effects of environmental factors on the percentage of infection in this previously unexplored marine environment.

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Finally, a huge thank you goes to my family and friends for their support throughout my studies for this master’s degree.

1.0 Introduction

Parasitic isopods are typically marine species, infecting fish and crustacean hosts (Rameshkumar and Ravichandran, 2014). They comprise about 2050 species distributed amongst three superfamilies, with Bopyroidea and Cryptoniscoidea, constituting the suborder Epicaridea (Williams and Boyko, 2012). All Epicarideans are obligate parasites of crustaceans that feed on the haemolymph of their host (Boyko and Williams, 2009). Unlike fish parasites which usually cause morbidity and mortality in pisciculture, most native epicarideans affect a variety of commercially important species (crabs and shrimps) without strongly impacting their population(Williams and Boyko, 2012; Kumar *et al.*, 2017). Even though their morphology is quite diverse, their adaptation in the parasitic mode of life with reduced morphological characters, their complex life cycle of multiple larval stages and strong sexual dimorphism, limit an accurate phylogeny based solely on morphology (Boyko *et al.*, 2013; Yu *et al.*, 2018). Recent studies on mitochondrial (Yu *et al.*, 2018; Wu *et al.*, 2022; An *et al.*, 2023) and nuclear genes (Boyko *et al.*, 2013; Boyko and Wolff, 2014; Wu *et al.*, 2022; An *et al.*, 2023) have offered valuable insight into the evolutionary relationships amongst Epicaridea. Families have been re-arranged and Bopyroidea (bopyrids), Rafinesque, 1815 now includes 3 families: Bopyridae, Entoniscidae and Ionidae.

Bopyrus crangorum of the Bopyridae family, is the first bopyrid to be described (Williams and Boyko, 2012; Hadfield, 2019). It is specifically placed in the subfamily Bopyrinae, amongst ectoparasitic isopods which are exclusively found on caridean shrimps (Markham, 1985). Members of the subfamily predominantly attach themselves to the branchial chamber but also to the abdomen of their hosts (Williams and Boyko, 2012; Boyko *et al.*, 2013; An, Boyko and Li, 2015). *B. crangorum* inhabits the shrimps' branchial chamber, with up to five parasites found on one or both sides (de Bhaldraithe, 1972). Similar to other epicarideans, it is heteroxenous, requiring an intermediate calanoid copepod before attaching to its final host (Boyko and Wolff, 2014). The first isopod that attaches to the definite host becomes a female, while the second one becomes a dwarf male (Reinhard, 1956; Anderson, 1990; Boyko and Wolff, 2014). The parasite’s definite host is usually infected as a juvenile, either remaining infected for life (3-5 years) or outliving the parasite (Cash and Bauer, 1993). Effects on the host include sterilization, reduction in the size of secondary sexual characters and gonads, feminization of males and carapace protuberance (Reinhard, 1956; Beck, 1980; Ito and

Watanabe, 1992), as well as reduced shrimp responsiveness and activity (Anderson, 1975; Golin, Moore and O'Dwyer, 2022).

Several *Palaemon* species including *Palaemon serratus* Pennant, 1777, *Palaemon elegans* Rathke, 1837, *Palaemon adspersus* Rathke, 1837, *Palaemon xiphias* Risso, 1816, *Palaemon serrifer* Stimpson, 1860, and *Palaemon pacificus* Stimpson, 1860 are known as hosts of *B. crangorum* (Kim and Yoon, 2020). These cases originate from the Eastern Mediterranean, and the Indo-Pacific, Southwestern Europe and Japan (Burgos and Itani, 2023 and references therein). Caridean shrimps are a crucial link in the transfer of energy from detritus to secondary consumers and play an important role in ecosystem services (Janas and Barańska, 2008). *P. serratus* and *P. adspersus* are commercially valuable species for fisheries or aquaculture production, respectively (Haig, Ryan and Williams, 2014; Yakovenko, Shadrin and Anufriieva, 2022).

Additionally, *P. elegans*, found in intertidal and sub-littoral areas, lagoons and rock pools of shallow sea and brackish waters (Reuschel, Cuesta and Schubart, 2010), has a wide geographical range being native to the east Atlantic coast, the Mediterranean and the Black Sea (d'Acoz, 1999; Grabowski, 2006). The distribution of *P. elegans* has significantly expanded, leading to its invasive status in the Caspian and Aral Seas (Zenkevich, 1963) as well as the Baltic coasts (Grabowski, 2006). In recent years, it has replaced native species in these regions (Grabowski, 2006; Katajisto *et al.*, 2013). *P. elegans* is harvested on a small scale for human consumption or used as fishing bait (Janas and Bruska, 2010). It is also used in small scale aquaculture on the Black Sea coast (Tania, Micu and S., 2006) and constitutes an important food source for commercial fish species (Grabowski, 2006; Yazdani, Taheri and Seyfabadi, 2010).

P. elegans exhibits extensive phenotypic plasticity in rostral characters and coloration (De Man, 1915; González-Ortegón and Cuesta, 2006). Besides morphological diversity, many studies have revealed high genetic variation between populations (Atlantic and Mediterranean), proposing the existence of a cryptic species (Reuschel *et al*, 2010; González-Castellano *et al.*, 2020). Although *P. elegans* is a well-studied species, research on the morphological and/or genetic variation is particularly limited in the area of the eastern Mediterranean and the Aegean Sea in comparison to the western Mediterranean (De Man, 1915; Reuschel *et al.*, 2010; Bilgin *et al.*, 2014; Deli *et al.*, 2018; González-Castellano *et al.*, 2020).

Similarly, the parasitic isopod diversity has been poorly investigated in the eastern Mediterranean, with current research focusing on economically valuable hosts (Leonardos and Trilles, 2003; Paradiso *et al.*, 2004; Kirkim *et al.*, 2008; Castriota *et al.*, 2010; Petrić *et al.*, 2010; Boyko *et al.*, 2018; Deval and Koçanci, 2021). Furthermore, phylogenetic studies on parasitic isopods that incorporate molecular data (Dreyer and Wägele, 2001, 2002) are limited due to inadequate number of available taxa, as well as difficulty in obtaining good quality starting material for sequencing (Dreyer and Wägele, 2002; Wu *et al.*, 2022). Consequently, only 28 species, belonging in the family of Bopyridae have confirmed sequences deposited in Genbank (https://www.ncbi.nlm.nih.gov/nuccore/?term=txid155698[Organism:exp]), with the two species currently placed on the genus *Bopyrus* (including *B. crangorum*) lacking further molecular phylogenetic analysis (Kim and Yoon, 2020).

The objectives of this study were to (1) identify the bopyrid parasite and its host, (2) study the parasite's molecular phylogeny (3) discover the host's genetic diversity and (4) investigate the seasonal variability of infection rates in relation to sea surface temperature (SST) and salinity within two sites over Saronikos Gulf. To achieve this, a comprehensive analysis was conducted utilizing both morphological and molecular methods to identify both species. In addition, the sequencing of a fragment of the parasite's DNA resulted in the first positioning of the parasite on the phylogenetic tree. Concurrently, employing monthly monitoring of shrimp-parasite populations provided a high-resolution temporal seasonal cycle of the parasitism that had never been explored before adding new information on the ecology of the parasite.

2.0 Materials and methods

2.1 Sample collection

Specimens were collected from two locations (Figure 1) of the Saronikos gulf coast, Lagonisi – Glystra (37°77'53.54'' N, 23°89'87.96'' E) and Agia Marina (37°78'82.66'' N, 23°88'37.37'' E) between May of 2021 and December of 2023 with a total of 12 samplings. Rock pool temperature was measured with electronic thermometers and salinity with a refractometer at 10PM. Specimens were captured in the literal zone using 20 cm fish nets. Infected and non-infected shrimps were temporarily collected and counted. Visual identification of infected individuals (inability to collect) was also considered. A small number of parasite baring shrimps was transferred to the laboratory aquariums for further analyses (e.g. morphological identification, observation of the effects of shrimp moulting on parasitism, molecular analysis after sacrificing).

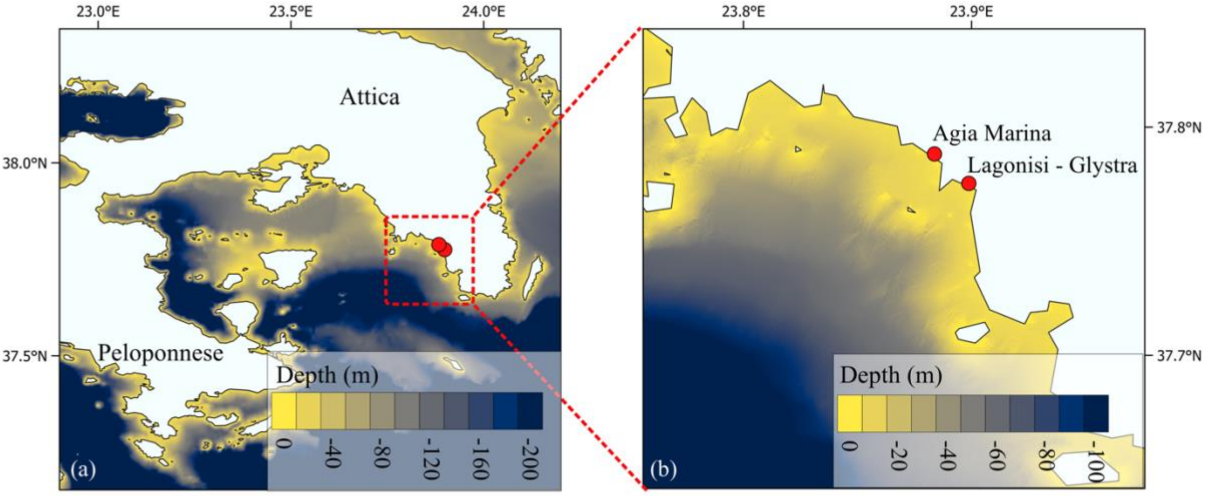

Figure 1. Map of the Saronikos Gulf (a) and part of its north-eastern coast, including the sampling sites of the rock-pool shrimp *P. elegans* and its parasite, *B. crangorum*: Agia Marina and Lagonisi-Glystra (b).

2.2 Morphological identification

Specimens were maintained under the following conditions: Two 10L glass aquariums were kept in a chamber with a fixed temperature of 24°C and 8 hours of daylight provided by LED lamps. Salinity and temperature were checked daily, and freshwater was added accordingly to maintain a 35ppt concentration (due to evaporation). Ammonia, nitrates, nitrites and pH were measured frequently. Water changes were carried out approximately every 3 days. The shrimps were fed brine shrimp flakes (*Artemia*) every 3 days.

A similar experimental procedure was followed for the description and sacrifice of both organisms. Each individual shrimp was photographed in a 5x5 cm plastic container filled with seawater before removing the parasite from the branchial chamber, using sterilized tweezers. Shrimps and parasites were placed in separate tubes and sacrificed by air freezing in -20°C for 4 minutes. Subsequently, their length was measured and the rostrum – antennae – antennule of the shrimps were carefully examined under the stereoscope. Taxonomic identification of the isopod parasites was carried out based on the detailed illustrated description of features published by *Kim & Yoon* (2020). Morphological characters used for the identification include the cephalothorax, abdomen (pleon) and oostegites of the female as well as the anatomy of the male (Figure 2).

All shrimp specimens were observed and photographed directly after removal from the air-freezer storage. Morphological identification was carried out according to the illustrated key for *Palaemon* and *Palaemonetes* by González-Ortegón and Cuesta (2006).

2.3 DNA extraction and molecular analysis

For the molecular identification of the shrimp, a total of 14 specimens were used. The samples were defrosted and approximately 25mg of muscle tissue from the abdomen was excised using a sterile scalpel. Care was taken to prevent contamination from intestinal tissue. Eight parasite specimens were used for the molecular analysis with the exception of a sample that did not yield amplifiable DNA. The whole organism including the eggs and/or embryos, and the attached male were used. Tissues were grinded with a sterile pestle and treated with freshly prepared CTAB buffer. Both organisms' genomic DNA was extracted using the CTAB protocol (adapted from Winnepenninckx, Backeljau and De Wachter, 1993).

A fragment of the small ribosomal subunit gene (SSU) was successfully amplified to study the molecular phylogeny of the species. The universal nSSUB primer (Medlin *et al.*, 1988) modified by Boyko *et al*. (2013) and the 1155F internal primer (Dreyer and Wägele, 2001) were used for that purpose. Other combinations of internal and universal primers modified by *Boyko et al.* (2013) failed to amplify the targeted sequence.

For the identification of the shrimp species, a fragment of the cytochrome c oxidase subunit I (COI) gene was amplified. Testing the LCO1490 and HCO2198 primers (Folmer *et al.*, 1994) didn't yield any PCR products. Consequently, the C1-J-1718 (mt6) and C1-N-2191 (nancy) primers (Simon *et al.*, 1994) were employed for this purpose. Total reaction volume of both the parasite and the shrimp PCR mix was 25μL. PCR reagents and concentrations are listed in Table 1 and thermocycling parameters in Table 2. At the end of each PCR, samples were kept at 4°C for further analysis and were finally stored at -20°C. All samples were tested in three different concentrations (total, 1/50, 1/100).

Table 1. PCR reagents and concentrations for *B. crangorum* and *P. elegans*

	Master mix (parasite)	Master mix (shrimp)
1x Taq buffer	5μL	5μL
$MgCl_2$ (3.5mM)	2-3,5 μL	3.5μL
dNTPs (0.2mM)	0,5μL	0,5μL
primer 1 (0.4μM)	1μL	0,5μL
primer 2 (0.4μM)	1μL	0,5μL
bovine serum albumin (BSA) (1μg/μL)	1.5 μL	2.5μL
Taq polymerase (0.02u/μL).	0.1μL	0.1μL
DNA	1μL	1μL

Table 2. Thermocycling parameters for the parasite and shrimp species.

	parasite	shrimp
pre-incubation	95°C/4 min	95°C/3 min
denaturation	40 cycles/94°C/ 1 min	39 cycles/94°C/ 15 sec
annealing temperature	45°C	40°C/ 1 min
elongation	68°C/ 2 min	72°C/ 1,5 min
final elongation	68°C/ 10 min	72°C/ 10 min

All PCR products were tested for DNA quality using the standard electrophoresis method in a 1% agarose gel (w/v) at 110V for 1 hour. Loaded samples containing 2μL of PCR product were stained with a mixture of orange G (1,5ml) and GelRed® Nucleic Acid Gel Stain (3μl) in a 500:1 ratio, and examined under UV light. PCR products (500bp) were purified using either Macherey Nagel NucleoSpin® Gel and PCR Clean-up kit or the Sera-Mag™ SpeedBeads.

The remaining volume of the parasite's selected samples (23μL) was electrophoresed in 2% agarose gel (w/v) at 90V for 2 hours. Stained DNA bands were examined under UV light, excised with sterile agarose gel band cutters and purified with the Macherey Nagel NucleoSpin® Gel and PCR Clean-up kit. A maximum of 3 separate fragments were amplified from each sample (one sample did not yield any products). All selected samples were (commercially) bidirectionally sequenced at CeMIA SA. laboratories using the Sanger method (Sanger, Nicklen and Coulson, 1977).

2.4 Data analysis

Sequences were edited using CodonCode Aligner version 9.0.2. All generated consensus files were blasted using the standard nucleotide BLAST (Basic Local Alignment Search Tool). The isopod sequences were aligned with additional SSU sequences from isopods retrieved from the GenBank database, and the alignment also included the SSU sequence of

Excorallana quadricornis (serving as an outgroup), resulting in a dataset comprising a total of 21 taxa. Alignment was performed using the MUSCLE algorithm in CodonCode Aligner version 9.0.2. The MUSCLE alignment was 3246bp in length after sequences were trimmed on the 5' (prime) end. Substitution saturation was assessed using DAMBE (Xia, 2018) and evolutionary distances were calculated using MEGA version 11 (Tamura, Stecher and Kumar, 2021).

To investigate the relationships of the studied isopod with other epicarideans, Bayesian inference (Yang and Rannala, 1997) was employed to search for trees based on their posterior probability using MrBayes (Huelsenbeck and Ronquist, 2001). The parameters for the analysis were determined by ModelFinder (Kalyaanamoorthy *et al.*, 2017), using the IQ-Tree version 1.6.12 (Nguyen *et al.*, 2015). The time reversible model (GTR), (Tavaré, 1986) with rate variation set to gamma (with default 4 rate - gamma categories, (Yang, 1994)) and base frequencies set to empirical The program run for 500.000 generations. Results of the phylogenetic study of bopyrids were visualised using the program FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

To compare the population of Saronikos gulf with other Mediterranean and Atlantic populations, a cladistic analysis of gene genealogies associated with *P. elegans,* inferred from COI sequences, was carried out with PopArt (Population Analysis with Reticulate Trees) software (Leigh and Bryant, 2015) using the TCS Network (Clement *et al.*, 2002). Thirteen newly generated individual and consensus COI sequences from the 2 locations of Saronikos gulf as well as reference sequences (Reuschel, Cuesta and Schubart, 2010; Bilgin *et al.*, 2014; González-Castellano *et al.*, 2020) were analysed.

2.5 Sea surface temperature remote sensing observations

Daily (night-time), optimally interpolated (L4), satellite-based estimates of the foundation SST were obtained from the CMEMS reprocessed Mediterranean SST dataset (https://doi.org/10.48670/moi-00173), which has a 0.05° × 0.05° (~ 5.5 km2) spatial resolution, available from 1982—present. The level 4 SST data were averaged over: a pixel nearest to the study site (37.772682, 23.884617). The produced daily, gap-free timeseries was used to compute seasonal (trimester) mean values.

2.6 Air temperature in dry bulb data

Air temperature values for the Hellenic Centre for Marine Research timeseries were retrieved from the POSEIDON Operational Oceanography System (https://poseidon.hcmr.gr). Measurements taken every 10 min by a thermometer attached to a tide gauge positioned at a nearby site (Palaia Fokea - 37.7175, 23.9452), cover the period 2021-05-19 – 2023-12-31. Seasonal (trimester) mean values were computed by considering only quality control flag values =1 (good data).

3.0 Results

The two organisms were morphologically identified up to species level, based on key-features. Further investigation of the parasites' 18S rDNA and subsequent molecular phylogeny indicated a strong affiliation with species within the Bopyrinae subfamily. For the shrimp, species identification was enhanced by molecular studies of the COI gene, facilitating the development of a haplotypic network where all samples were classified under the type II haplogroup. Additionally, an examination of how shrimp infection rates fluctuated with seasonal changes in temperature and salinity during the period of 2021 - 2023 showed that the percentage of parasitism closely follows the seasonal changes of the aforementioned environmental variables when comparing spring and winter .

3.1 Morphological identification and molecular analysis of the parasite

Key features used for the identification of the parasite include the female’s asymmetrical, dorsoventrally compressed body narrowing posteriorly (Figure 2A, B), the partially fused head and first pereon (Figure 2), the oostegites (Figure 2A) and the much smaller first pleonite on the short side (Figure 2C). The dwarf male’s body is symmetrical, narrowing posteriorly, with a semi-circular head separated from pereonite 1 (and pereonites 6 and 7 posteriorly narrower than other pereonites) (Figure 2C).

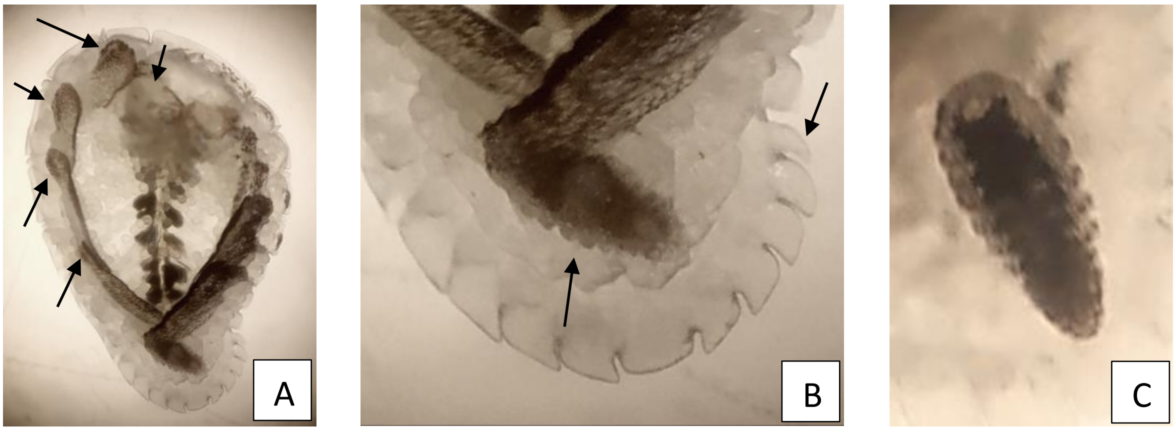

Figure 2. Morphological characters used in the identification of the isopod species. (A) ventral view; arrows indicate oostegites on the right side; (B) posterior end (pleon) with arrows indicating male parasite and small pleonite; (C) male.

During the molecular analysis conducted on the parasites, three distinct fragments were amplified from each sample. The shortest fragment (400bp), which overlapped with the longest one (830bp), belonged to *B. crangorum* and all but one sequences were identical. Using BLAST, it was shown that the consensus sequence (833bp) was closely related to *Probopyrus buitendijki, Probopyrus pacificiensis and Probopyrus pandalicola.* On the contrary, the intermediate fragment (590bp) exhibited a relatively low quality read following sequencing, and subsequent BLAST analysis identified its relation to two families of marine bacteria, *Paracoccaceae* and *Roseobacteraceae*.

Running two independent analyses with MrBayes, 20002 trees were produced with a standard deviation of 0,008 between the two runs. Resulting bootstrap consensus tree is shown in Figure 3. Based on the Bayesian analysis, Bopyroidea forms a monophyletic group (100% posterior probability). Three clades form the three families within Bopyroidea: *Entoniscidae*, *Ionidae* and *Bopyridae*. Likewise, there are three clades inside Bopyridae with the first containing Athelginae, the second the subfamilies Phyllodurinae, Pseudioninae, Bopyrinae, Keponinae and a third one containing Hemiarthrinae, Argeiinae and Orbioninae. According to the phylogenetic analysis, *B. crangorum* is closely related to the genera *Probopyrus*, *Bopyrella* and *Parabopyrella*.

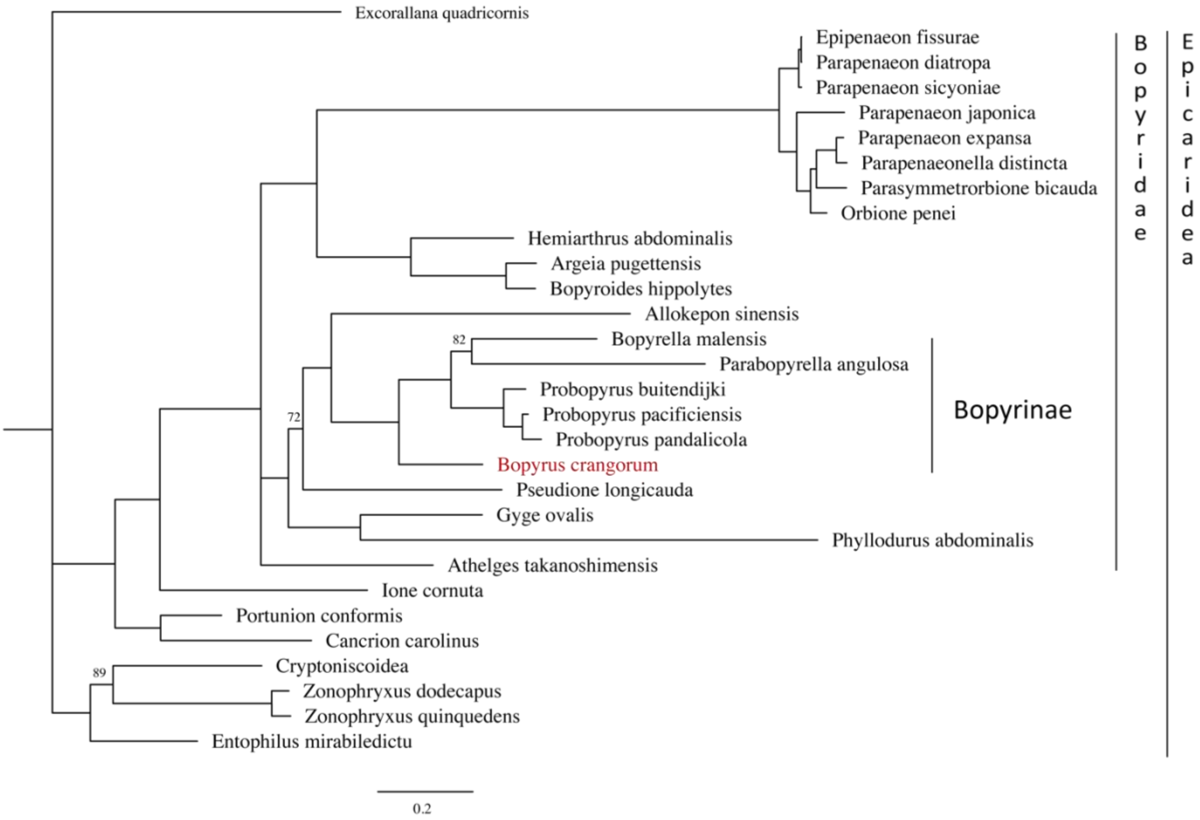

Figure 3. Bayesian best tree of evolutionary relationships of 29 ingroup taxa inferred from an analysis of 18S rDNA data. Posterior probabilities are indicated near nodes. All branches without posterior probabilities were supported by values >95%. *B. crangorum* is the new sequence added from this study.

3.2 Morphological identification and molecular analysis of the shrimp

The antennules, second pereiopod, branchiostegal spine and rostrum teeth were used as the key characters for species identification (Figure 4). The shorter ramus of the outer flagellum of antennule was fused for about half of its length to the longer ramus (Figure 4A). Number of dorsal rostral teeth range from 7 to 10 (most frequent being 8 or 9) whereas ventral rostral teeth range between 2-4 (most common 3) (Figure 4B, Supplementary Figure1).

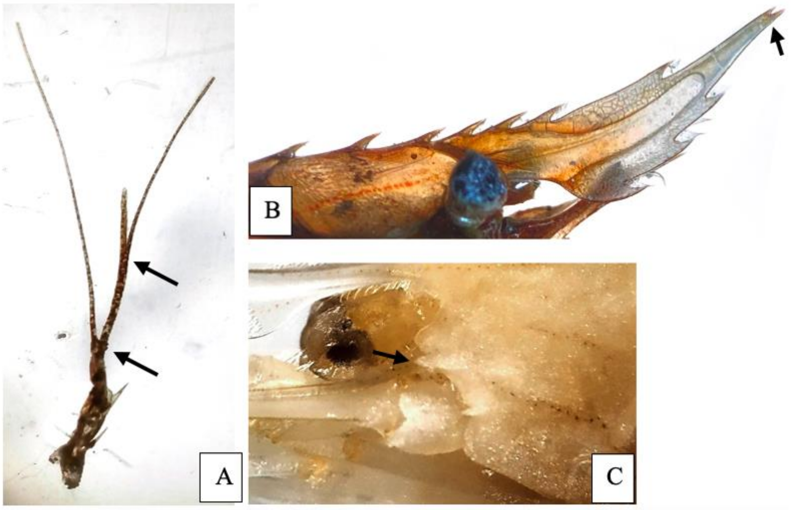

Figure 4. Morphological characters used in the identification of Palaemon species. (A) antennule; arrows indicate fusion area on the outer flagellum; (B) rostrum, arrow pointing at apical cleft; (C) branchiostegal spine.

Using BLAST, the consensus COI sequence (495bp in length after trimmed with CodonCode Aligner (CodonCode Corporation, Centerville, MA, USA)) was matched with that of *Palaemon elegans* (Accession number MT340087.1, Gonzalez-Castellano *et al*. (2020)).

The Saronikos gulf shrimps were grouped in type II haplogroup (Mediterranean populations), along with the single sample (#38) from the Aegean Sea (Bilgin *et al.*, 2014). Haplogroup type II is closely related to the haplogroup I (Atlantic populations) with minimum two mutational steps. Finally, specimens from the Black Sea, the Sea of Marmara and the Levantine coast were grouped in haplogroup type III (cryptic). Eighteen mutational steps differentiated type II from type III haplogroup.

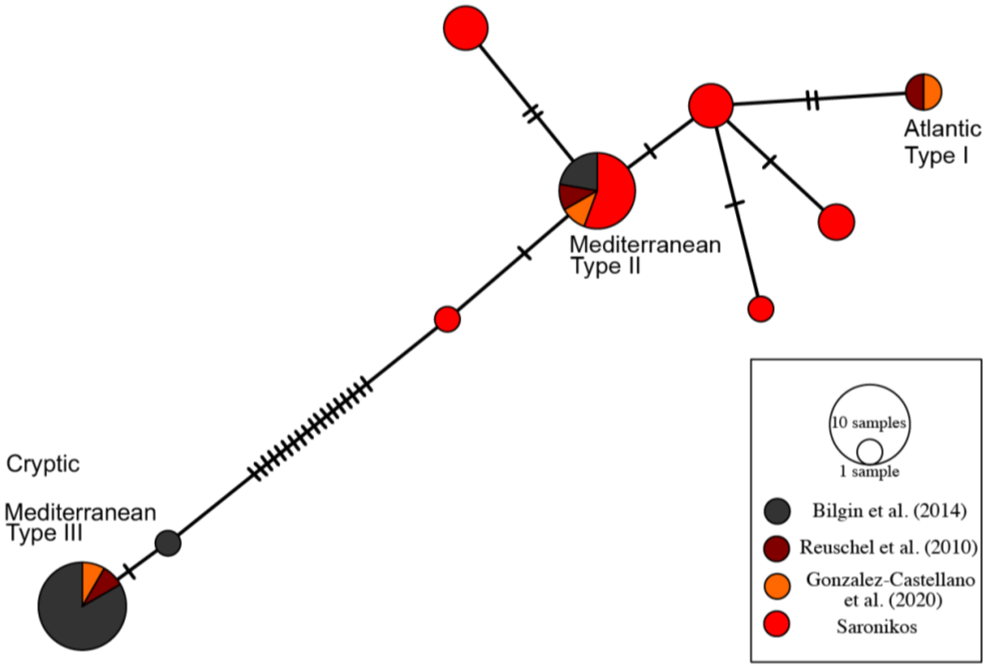

Figure 5. Statistical parsimony network, inferred from COI gene and constructed with PopArt. The network is based on the haplotypes of 32 individual shrimps. Each circle represents a single haplotype, and its diameter is proportional to the frequency of the haplotype, with the smallest circle representing a single individual. Each line represents one substitution; short transverse lines indicate mutations. The shading corresponds to samples from different studies.

3.3 Seasonality of parasitism in relation to environmental factors

The incidence of shrimp infections rates reached its highest during spring, averaging 26.75%, and dropped to its lowest in the winter, averaging 8.25% (Figure7a). Similarly, rock-pool temperatures mirrored this seasonal trend, with an average of 26.5°C in spring and 14.3°C in winter (Figure7b). Salinity levels peaked at 40 PSU in the spring and decreased to 37.5 PSU in the winter (Figure7c). In contrast, SST (Figure 7d) and air temperature (Figure 7e) were significantly lower in spring, at 16.7°C and 16.5°C respectively, compared to in situ temperatures. Conversely, in winter, the air temperature was slightly lower, while the SST was slightly higher. All the comparisons mentioned were statistically significant according to the independent t-test, with p-values of 0.029, 0.036, 0.022, and 0.012 respectively, except for the SST, which had a p-value of 0.677. The number of data points (N) for the spring and winter seasons regarding the percentage of shrimps infected with parasites, rock-pool temperature, and salinity were 3 whereas for SST there were 9 data points and for air temperature there were 7.

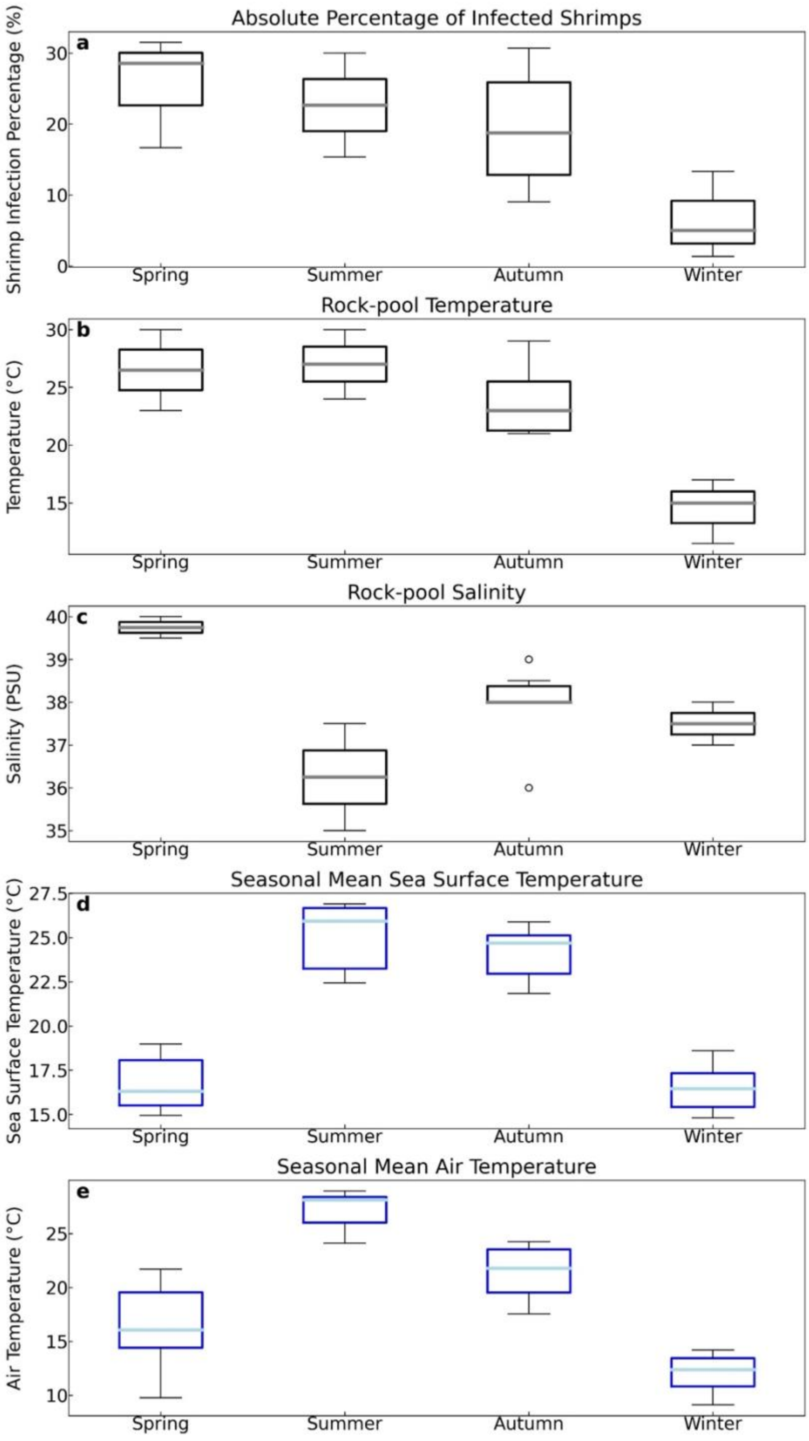

Figure 7. Trimester/seasonal average variability of [a] *P. elegans* infection percentage, [b] rock – pool temperature, [c] salinity, [d] satellite-derived sea surface temperature, [e] air temperature for the years 2021 and 2023. (Winter: December – January – February, Spring: March – April – May, Summer: June – July – August and Autumn: September – October – November).

4.0 Discussion

Molecular phylogeny of bopyrids has been limited (Wu *et al.*, 2022). This is the first study to include the parasite *B. crangorum* in such analysis by sequencing a fragment of the

SSU gene resulting in a reconstructed phylogenetic tree of epicaridean parasites. Integration of morphological and molecular methods provided a holistic view showcasing great morphological variability for the Saronikos gulf *P. elegans* subpopulations, whilst haplotypic network analysis revealed a close relationship between the Saronikos subpopulation (haplogroup type II) and the Atlantic population, offering valuable insights into the genetic diversity of shrimp populations in the studied region. Furthermore, monthly monitoring indicated that the temperature of the rock pools has a significant effect on the populations of the shrimp and the parasite, as higher temperatures were associated with greater infection rates.

In this study, the phylogenetic relationships among various taxa were explored by analysing 18S rDNA sequences from 29 ingroup species, yielding a highly supported phylogenetic tree (Figure 3). The results largely corroborate findings from the most comprehensive related study, with notable alignments and deviations in species clustering (see Figure 1 of Boyko *et al.*, 2013). For instance, *Athelges takanoshimensis* emerged as a distinct clade within the Bopyridae, diverging from previous groupings, while *Hemiarthrus abdominalis* and *Argeia pugettensis* clustered with *Bopyroides hippolytes*, supporting earlier findings (Wu *et al.*, 2022). The consistent clustering of *B. crangorum* with species belonging to the Bopyrinae subfamily validates the current taxonomic understanding (Boyko *et al.*, 2024). Specifically, species of the genus *Probopyrus, Parabopyrella angulosa* and *Bopyrella malensis,* which belong to the Bopyrinae subfamily, form a distinct clade as the sister group of *B. crangorum* lindicating their close relationship. An intriguing aspect outside the main focus of this study involves *Gyge ovalis*, the first parasitic isopod with a fully sequenced mitogenome (Yu *et al.*, 2018). The bopyrid is considered a member of the Pseudioninae subfamily together with isopods infecting the branchial chamber of their hosts. The phylogeny presented in this study shows *Gyge ovalis* to diverge from Pseudioninae and is related closer to *Phyllodurus abdominalis.* Similarly, phylogenetic research utilizing the COI and 18S rRNA genes place the parasite in a separate clade from the species of the Pseudioninae subfamily (Wu *et al.*, 2022; An *et al.*, 2023) suggesting that the taxonomy of *Gyge ovalis* should be re-examined.

Additionally, the intermediate DNA fragment in our PCR product analysis was identified as bacterial, closely related to the families *Paracoccaceae* (genus *Aliiroseovarius*) and *Roseobacteraceae* (genus *Rugeria*). These organisms are associated with coastal ecosystems and microbiota of fish and corals (Sunagawa, Woodley and Medina, 2010; Park and Yoon, 2013; Xing *et al.*, 2013; Tang *et al.*, 2020) and are indicative of potential contamination or symbiotic relationships, highlighting the complex interactions within marine ecosystems.

The haplotypic network analysis positioned the *P. elegans* population within the haplogroup type II which is mostly confined to the south-eastern Mediterranean with many occurrences around Turkey (Deli, Pfaller and Schubart, 2018). Although there were no records of the cryptic species (haplogroup type III) in the investigated sites, both haplotypes have been found to co-occur in the east Aegean (Turkish coast) (Bilgin *et al.*, 2014; Deli, Pfaller and Schubart, 2018), underscoring biogeographic distinctions and the potential impact of historical hydrographic changes on species distribution (Bilgin *et al.*, 2014; Deli, Pfaller and Schubart, 2018; González-Castellano *et al.*, 2020).

Previous research has shown that host abundance is the primary factor influencing infection rates in caridean shrimps and secondarily by environmental variables (Briggs *et al.*, 2017). In this study, there was a notable increase in host abundance during May and June in the first year, whereas in the second year, the increase occurred from August to December. The variation in timing might be linked to the elevated temperatures recorded in the fall of 2023, potentially prolonging the period of high shrimp abundance. These large population numbers can be attributed to the juvenile recruitment period (Bauer, 1989) that also translates to an increased presence of the isopod in the observed shrimp specimens with smaller size. This agrees with the reproductive behaviour (life traits) of *P. elegans* (Abdolmaleki, Emadi and Nezami, 2003; Bilgin, Ozen and Samsun, 2009) and previous studies on other *Palaemon* species showcasing that recently metamorphosed post-larva, young or juvenile individuals are more prone to infection (Beck, 1980; Cash and Bauer, 1993; Pralon *et al.*, 2018).

Seasonal analysis of shrimp-parasite populations demonstrates that both the percentage of infected shrimps and rock pool temperatures were markedly higher in spring than in winter, suggesting a positive relationship between infection rates and temperature. On the contrary, satellite-derived open water SST and broader air temperatures where cooler than the rock pool temperature, highlighting the unique environment of rock pools. Rock pools are characterised by shallow waters with large diurnal and seasonal variations in temperature, a result of direct exposure to sunlight and longer periods of thermic exchange with the atmosphere during isolation events (Martins *et al.*, 2007). The discrepancy between SST and air temperature can be attributed to the water's higher thermal capacity with a more gradual increase in SST. When considering only environmental variables, temperature has been identified as the most critical factor influencing infection and/or mortality rates in caridean shrimps and their bopyrid parasites, with depth and salinity also playing significant roles (Briggs, 2013; Sherman and Curran, 2013; Briggs *et al.*, 2017). Additionally, the elevated salinity values in spring compared to autumn and winter suggest the effects of evaporation and/or precipitation, while the lowest salinity recorded in summer is consistent with prior observations for the study area, as illustrated in Figure S4 of Kalloniati *et al*. (2023). Although the likelihood of parasite presence is associated with higher temperatures (Briggs, 2013), further data collection over additional years is necessary to ascertain any definitive seasonal or annual trends in the study area.

4.1 Possible effects of climate change on epicaridean parasites and their hosts

Even though epicaridean isopods constitute a significant part of the total biomass of parasites on estuaries, it is poorly understood how host castration and behavioural alterations (host manipulation) may affect population dynamics and/ or ecosystem functions (Kuris *et al.*, 2008). Studies focusing on the bopyrids *Probopyrus pandalicola* and *Bopyrus crangorum* have reported decreased metabolic activity, predation, and tolerance to salinity stress for infected shrimps and copepods (Anderson, 1975, 1990; Golin, Moore and O'Dwyer, 2022). In addition, there is evidence of severe host mortality for non-native parasitic isopods that might threaten estuarine ecosystems (Chapman *et al.*, 2012). Specifically, the parasite *Orthione griffenis* has been implicated in the collapse of the mud shrimp, *Upogebia pugettensis*, a species which influences key functions in estuarine ecosystems of north-eastern Pacific (Chapman *et al.*, 2012; Hong, Lee and Min, 2015). On the other hand, the survival of infected hosts is unimpaired when parasites are native and prevalence is low (Anderson, 1990; Williams and Boyko, 2012; Sasaki, Woods and Dam, 2023).

What is more, warming combined with ocean acidification directly affects key physiological processes in aquatic invertebrates such as *P. elegans* (Anderson, 1975; Maia *et* *al.*, 2022) leading to changes in growth, moulting frequency and brood development, which could ultimately translate to a greater parasite fecundity and implications in the food webs (Briggs, 2013; Brinton and Curran, 2015).

The results of this study highlight the importance of considering the possible effects of temperature on parasitism and ultimately the shrimp's population viability under future warmer scenarios. The long term effects of these changes are not yet elucidated, however an increased susceptibility of the shrimp to predation, a reduced performance and a higher vulnerability of juvenile *P. elegans* to extreme temperatures coupled with a reduced number of ovigerous shrimps due to parasitism could potentially lead to a shift in population dynamics of *Palaemon* and other species of caridean shrimps (Sherman and Curran, 2013; Brinton and Curran, 2015; Amsalem and Rilov, 2021).

4.2 Observational remarks

The shrimps kept in the aquariums were observed for the effects of moulting on the mortality of the parasites. In total, five shrimps lost their parasites. The presence of multiple shrimps in the same aquarium resulted in cannibalism thus making it hard to distinguish true mortality. Out of all the shrimps collected, only one exhibited bilateral infection which ultimately led to a "normal" infection of two parasites within one branchia. A possible explanation is that one of the parasites moved to the opposite branchial chamber, as it has been previously reported for *Probopyrus pandalicola* (Anderson, 1990).

Furthermore, our examination of 41 *Palaemon elegans* specimens revealed a prevalent rostrum phenotype with 8 or 9 dorsal and 3 ventral teeth which agrees with literature [33]. Many morphological characters of *Palaemonidae* species exhibit variation, such as the rostrum's shape and relative length, as well as the number, position, and size of the rostral teeth (de Grave, 1999; González-Ortegón and Cuesta, 2006). In this study, the cephalothorax and abdomen *of P. elegans* shrimps exhibited different coloration ranging from black - brownish transverse stripes to yellowish and almost transparent, and variable colouring of the rostrum was also observed. Variability in *P. elegans* body coloration has previously been associated with differences in water turbidity, with the stripes completely fading away in turbid waters (González-Ortegón and Cuesta, 2006). The limited number of data on *Palaemonidae/P. elegans* phenotypic plasticity underscores the need for additional research to comprehensively understand trait variations in this taxonomic group/species.

5.0 Concluding remarks

This comprehensive analysis not only deepens our understanding on phylogenetic relationships of bopyrids and morphological diversity in *P. elegans* but also underscores the intricate ecological dynamics within these species groups. Specifically, *B. crangorum*'s present taxonomic classification is in agreement with the results of this phylogeny, demonstrating that the parasite is closely related to the species of the Bopyrinae subfamily. The three years observations on seasonal variability in the populations of both the parasite and its host showed a positive relationship between the percentage of infection and rock pool - air temperature, with higher temperatures translating to higher infection rates when comparing spring and winter. This is possibly important since oceanic temperatures in the broader study area are constantly increasing (Raitsos *et al.*, 2010; Kalloniati *et al.*, 2023). Furthermore, *B. crangorum* has the potential to infect multiple species of caridean shrimps (and copepods), some of which are of commercial value, thus posing a risk for coastal ecosystems and trophic webs. Considering the fundamental role caridean shrimps play in coastal systems and also as upcoming aquaculture species, future research should focus on investigating bopyrid infection rates over extended periods to more accurately assess how fluctuations in temperature, both seasonal and long-term, influence their distribution and impact on caridean shrimp hosts. An inclusive approach is essential for elucidating infection - distribution patterns, contributing to a holistic comprehension of the ecological dynamics centred around *P. elegans* and its symbionts. Such insights are crucial for informing conservation strategies and sustainable management practices in the context of coastal marine ecosystems.

6.0 Appendix

Morphological assessment of the rostrum features (Figure B) of 41 specimens, showed that the presence of 8 (51%) and 9 (39%) dorsal teeth along with 3 ventral teeth (82.6%) and 2 on the apical cleft (100%) was the most frequently observed trait among the organisms. Besides this common phenotype, shrimps also exhibited variation in both the dorsal (7 - 10) and ventral (2 - 4) rostral teeth (Figure 5).

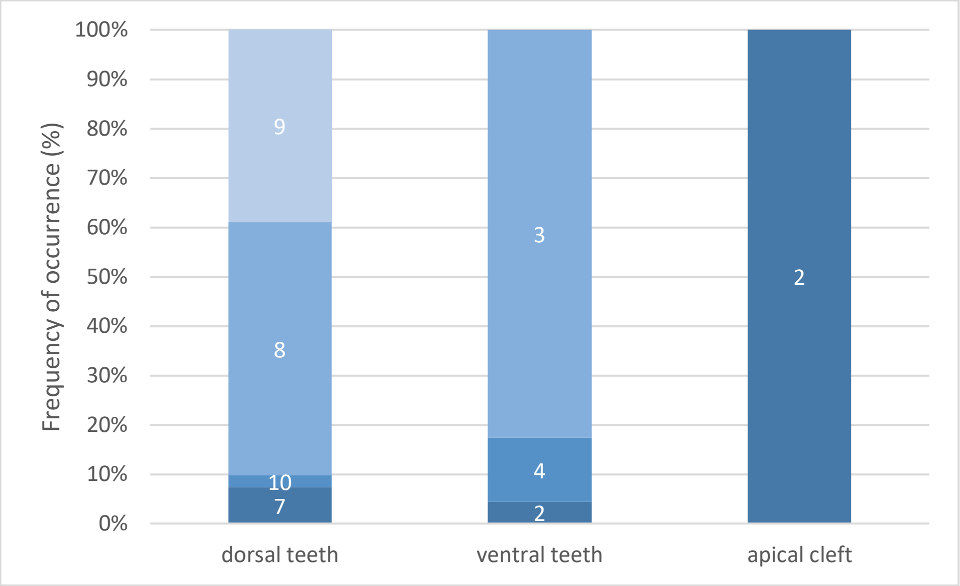

Figure 1. Frequency of variation of the rostrum characters in *P. elegans*: dorsal (including postorbital) teeth, ventral teeth and apical cleft, based on 41 specimens.

Sampling date	Rock pool temperature (°C)
Sep-21	24
Dec-21	15
May-22	30
Jun-22	24
Sep-22	22
Oct-22	21
Nov-22	21
Feb-23	12
Apr-23	23
Jul-23	30
Sep-23	29
Nov-23	26
Dec-23	17

Table 1. Sampling days and mean values for recorded temperatures in the investigated rock pools of Glystra - Lagonisi and Agia Marina.

7.0 References

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