Rapid Communication

A new record of the rapidly spreading calanoid copepod *Pseudodiaptomus marinus* (Sato, 1913) in the Levantine Sea using multi-marker metabarcoding

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Abstract

Over the last decade, the calanoid copepod *Pseudodiaptomus marinus*—native to the Indian Ocean—has rapidly spread throughout the European Seas. Here we report its first occurrence in the southern Levantine Sea. Zooplankton samples were collected monthly by vertical net hauls in a coastal monitoring station at the Israeli Mediterranean Sea during 2019–2021. The samples were analyzed using mitochondrial COI and 18S rRNA metabarcoding, revealing the occurrence of *P. marinus* in winter and spring. Following the molecular detection, two individuals of *P. marinus* were observed in the samples and identified morphologically, indicating a low population abundance (0.4 ind. m^{-3}) and confirming its status as widespread but rare, as reported in former colonized areas. Rare species often go undetected in zooplankton assemblages using morphological examination, whereas DNA metabarcoding is a sensitive, rapid, and cost-effective method that can provide valuable presence/absence data of such species. We further show that the use of both mitochondrial and nuclear gene markers provides a robust and comprehensive non-indigenous species (NIS) early-detection system, and stress that combining DNA metabarcoding with morphological examination is necessary for biodiversity monitoring in marine ecosystems that undergo significant transformations due to climate and/or anthropogenic forcing.

Key words: zooplankton, NIS, monitoring, rare species, southeastern Mediterranean Sea, Next Generation Sequencing, COI, 18S rRNA v9

Introduction

Early detection of non-indigenous species (NIS) has been advocated as an important management measure for dealing with biological invasions (Comtet et al. 2015; Simberloff 2014; Simberloff et al. 2005). As a result, many countries have set up regulations including early detection systems to limit the environmental risks related to the introduction of NIS. The European Community has adopted a regulation to prevent, minimize and mitigate the adverse impact on biodiversity of the introduction and spread

of invasive NIS (Regulation 1143/2014, IAS, European Union). Similarly, the US government has maintained a policy to prevent the introduction, establishment, and spread of NIS (Executive Order 13751). These regulations emphasize the priority of rapid identification and detection of NIS (Burgos-Rodríguez and Burgiel 2020; Comtet et al. 2015). To accomplish that, powerful early warning tools were developed to identify species at all developmental stages and detect NIS at even very low concentrations in the introduced geographical range.

Molecular-based approaches have been widely applied in the detection of marine NIS (e.g., Miralles et al. 2018; Rabi et al. 2020). Among these methods, DNA metabarcoding, i.e., high-throughput multispecies identification using the total genomic DNA extracted from an environmental (mixed) sample, enables rapid detection of NIS in a plankton community (Stefanni et al. 2018; Comtet et al. 2015; Suarez-Menendez et al. 2020; Di Capua et al. 2021; Borrell et al. 2017). Although this method is generally qualitative, or semi-quantitative, and strongly limited by the existing reference libraries (e.g., NCBI GenBank, BOLD), it allows a rapid and relatively inexpensive detection of NIS. Furthermore, metabarcoding overcomes the need to isolate or identify individual specimens and thus avoids morphology-based identification problems, e.g., morphological complexities, crypticity, different life stages, and the globally declining taxonomic expertise. These strengths are especially conspicuous in the analysis of complex zooplankton communities (Schroeder et al. 2020; Djurhuus et al. 2018; Bucklin et al. 2016).

The zooplankton communities of the Mediterranean Sea are in particular prone to bioinvasions due to the influx of species through the Suez Canal combined with the acceleration of climate change, facilitating the establishment of thermophilic NIS (Raitsos et al. 2010; Mannino et al. 2017). Among zooplankters, free-living copepods are adept colonizers (Uttieri et al. 2020; Lee 2016). Most of the 61 copepod NIS currently present in the Mediterranean Sea are of Indo-Pacific origin, first introduced into the Levantine Basin via the Suez Canal and by ballast water from shipping and hull fouling (Zenetos et al. 2010, 2012, 2020; Zakaria 2015; Abd El-Rahman 2005). NIS introductions in the Mediterranean Sea are typically first detected in the Eastern Basin. Nevertheless, west-to-east species propagation patterns were also observed and were mostly attributed to shipping (Velasquez et al. 2021). Such propagation pattern is presented by the calanoid copepod *Pseudodiaptomus marinus* (Sato, 1913). Its native distribution is limited to Northern Japan and the coastal and estuarine waters of Eastern Asia (Ohtsuka et al. 2018). Over the second part of the twentieth century, *P. marinus* has been reported from coastal waters of the Indo-Pacific region, and, since 2007, from the European Seas (Mediterranean Sea, Black Sea, European Atlantic coasts, Southern North Sea) (reviewed in Uttieri et al. 2020) (Figure 1).
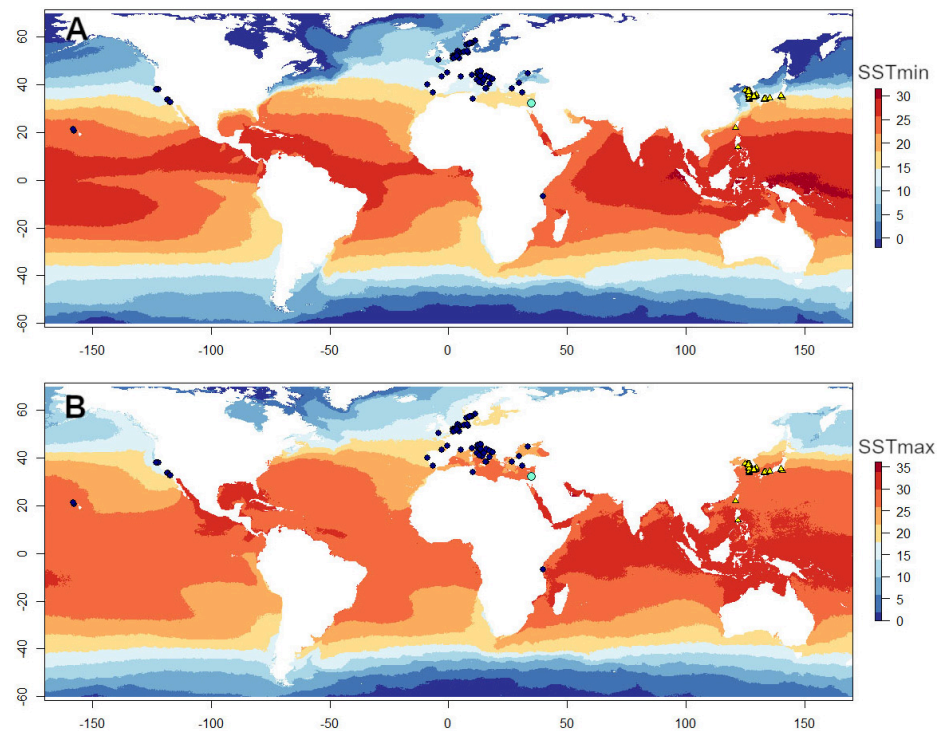
Figure 1. Worldwide distribution of *Pseudodiaptomus marinus*, showing species distribution across native (yellow triangles) and invaded (black circles) ranges, plotted on (A) minimum and (B) maximum sea surface temperatures (SST_{min}, SST_{max}). The new record in the Southeastern Levantine Sea is shown in green. Occurrence data were taken from Uttieri et al. (2020), GBIF.org (10 July 2022, https://doi.org/10.15468/dl.6rwk3c) and OBIS.org (10 July 2022). Temperature layers (SST averages between 2000 and 2014) were obtained from bio-oracle.org.

Due to its rapid and evident spread, *P. marinus* was recognized as one of the 26 top-priority NIS in a horizon-scanning study performed at the European level (Tsiamis et al. 2019). The ICES working group WGEUROBUS (Towards a EURopean OBservatory of the non-indigenous calanoid copepod *Pseudodiaptomus marinUS*) was established in 2018 as a European network of institutions and researchers working on the biology and ecology of *P. marinus*, promoting a better understanding of its introduction and establishment. The expansion and colonization of *P. marinus* in new environments is still ongoing. Recently, the first records of *P. marinus* in the Sea of Marmara (Tiralongo et al. 2022), the mid Tyrrhenian Sea (Di Capua et al. 2022) and in the Adriatic Sea (Schroeder et al. 2020) were reported, identified using integrative (morphological and molecular) taxonomy. Here we report the first record of *P. marinus* in the southern Levantine Sea, detected using multi-marker DNA metabarcoding, and discuss the effectiveness of using this approach.

Materials and methods

Sampling site

Monthly sampling was performed in the Hadera meteo-marine station (MedGLOSS #80), Israel (32.4700°N; 34.6930°E, Figure 1) between September 2019 and December 2021, in the framework of the National Monitoring of

the Israeli Mediterranean Sea by the Israel Oceanographic and Limnological Research (IOLR). The station depth is 26 m, and it is equipped with fixed CTD (Sea-Bird, USA) and ADCP (Teledyne RDI, USA). Environmental data are available online at the Israel Marine Data Center (https://isramar.ocean.org.il). During 2019–2021, the annual water temperature range in the sampling site was 16.2–32.4 °C, salinity ranged 38.3–40.4, and chlorophyll *a* concentration ranged 0.05–0.92 μg L^{-1} (water column integrated values). The temporal variability at the sampling site is described in Velasquez et al. (2021).

Zooplankton sample collection

Mesozooplankton samples were collected monthly by vertical WP2 net hauls from 26 m to the surface (Ø = 57 cm, 200 μm mesh size, Hydro-Bios, Germany). The net was equipped with a mechanical flow meter (Hydro-Bios, Germany) to standardize the samples per filtered water volume. In each sampling event, three consecutive net tows were collected yielding three samples, i.e., a sample for estimating biomass, a second sample for morphological examination and species quantification (fixed with buffered 4% formalin solution), and a third sample for assessing zooplankton molecular biodiversity by DNA metabarcoding. All samples were kept on ice and transported within 1 hour to the laboratory at IOLR. The samples designated for metabarcoding were kept at –20 °C pending analyses.

DNA metabarcoding

Frozen zooplankton samples were thawed, sieved, and homogenized using a tissue homogenizer. The homogenate was centrifuged at 4000 rpm for 30 minutes, resuspended using PCR-grade water (Fisher Scientific, USA), and divided into two sub-samples. Total genomic DNA was extracted from each subsample using the DNEasy Blood and Tissue kit (QIAGEN, Germany) according to the manufacturer's specifications. DNA was amplified with the following primer sets amended with CS1/CS2 tags: i) the 18S rRNA gene V9 region (192 bp) – 1391F, EukBr (Amaral-Zettler et al. 2009), and ii) the mitochondrial cytochrome *c* oxidase subunit I (COI) gene (313 bp) – mlCOIintF, jgHCO2198 (Leray et al. 2013). Library preparation from the PCR products and sequencing of 2x250 bp Illumina MiSeq reads were performed by Hy Laboratories Ltd. (Israel).

Bioinformatics and phylogenetic analyses

Demultiplexed paired-end reads were processed in the QIIME2 V2020.6 environment (Bolyen et al. 2019). Reads were truncated based on quality plots, checked for chimeras, merged, and grouped into amplicon sequence variants (ASVs) with DADA2 (Callahan et al. 2016), as implemented in QIIME2. The 18S rRNA amplicons were classified with a scikit-learn classifier that was trained on the Silva 138 database or BLAST against the Silva 138

and PR2 databases (0.9 minimum identity cutoff, performed best for the analyses of 18S rRNA gene amplicons of microbial zooplankton). COI amplicons were classified with BLAST (0.9 minimum identity cutoff) against the merged NCBI/BOLD (Heller et al. 2018) and MZGdb (Bucklin et al. 2021) databases, which were transformed into QIIME2 format.

COI and 18S rRNA sequences of different *P. marinus* and Pseudodiaptomidae specimens from other regions were retrieved from GenBank and BOLD. *Hemidiaptomus ingens*, a representative of the family Diaptomidae, was chosen as an outgroup in the analyses because this family is closely related to the family Pseudodiaptomidae (Bradford-Grieve et al. 2010). The complete lists of studied sequences and the occurrence localities are reported in Supplementary material Tables S1 and S2. All sequences were aligned using ClustalW in MEGA11 (Tamura et al. 2021). Evolutionary models and parameter estimates were selected using the lowest AICc score obtained with ModelTest to create a phylogenetic and molecular evolutionary analysis, using a Maximum Likelihood method. The phylogenetic trees based on the COI and 18S sequences were generated using the best-fitting models GTR+G+I and K2+G, respectively. The trees were run with 1000 bootstrap replicates. Additionally, genetic pairwise distances (p-distances) using the Maximum Composite Likelihood model were calculated (Tables S3, S4).

Morphological identification

Two *P. marinus* adult female specimens were examined under a stereomicroscope (Olympus SZX16, Olympus, Japan) and a light microscope (Olympus BX50, Olympus, Japan). All measurements were performed on one specimen since the preservation condition of the second specimen was poor. Morphological measurements such as prosome length, (PL), urosome length (UL), caudal ramus length (CRL), caudal ramus width (CRW), total length (PL+ UL +CRL, measured dorsally), P:U ratio, and CRL:CRW ratio, were taken. The identification followed the classification system of Razouls et al. (2005–2022). The specimens were deposited in the National Natural History Collections of the Hebrew University in Jerusalem and assigned with the following voucher numbers: HUJINVKIMOR6010–HUJINVKIMOR6011.

Results

Metabarcoding and phylogenetic inference

Two COI ASVs and one 18S rRNA ASV classified as *P. marinus* (99.98–100% identity, Blastn) were found in the High-Throughput Sequences from the sampling in Hadera on February 2020 and April 2020 (COI ASVs), and in May 2021 (18S rRNA ASV). Both duplicates (subsamples) fully corresponded, thus provided the same indication on the presence/absence of *P. marinus*. Water temperature, salinity, and chlorophyll *a* (integrated for the entire water column) on the dates when *P. marinus* ASVs were detected were 16.9–25.7 °C, 38.8–39.5 and 0.47–0.78 µg L^{-1}, respectively.
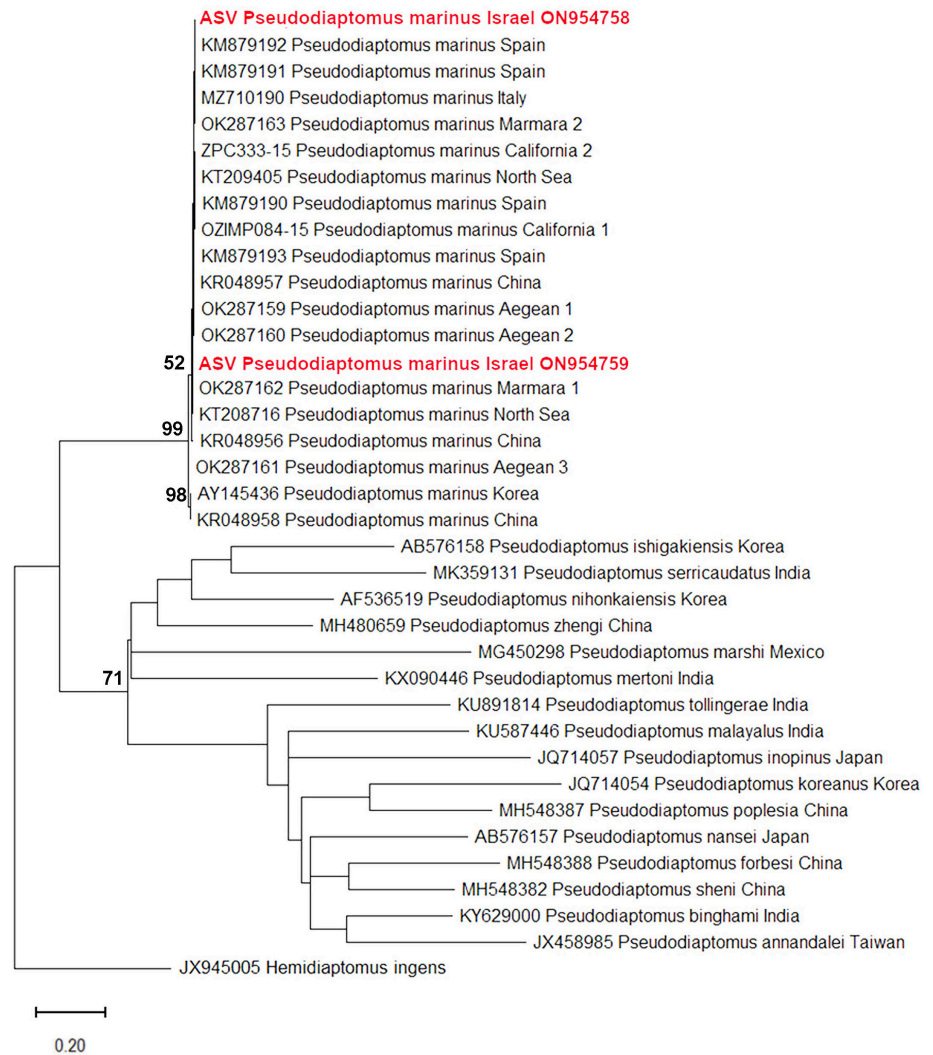
Figure 2. Phylogenetic analysis and molecular identification of *Pseudodiaptomus marinus* based on the mitochondrial COI gene. Sequences obtained in this study are presented in red. The phylogeny was inferred by Maximum Likelihood method based on the best-fitting model (GTR+G+I). The percentage of trees in which the associated taxa clustered together is shown next to the branches (bootstrapping = 1000, only values above 50% are displayed). Sequences obtained from the Levantine Sea are marked in red.

A total of 36 COI sequences of *Pseudodiaptomus* genus were analyzed (Table S1), including 20 sequences of *P. marinus*, of which three were from China, one from Korea, two from San Francisco Bay (USA), two from the North Sea, four from the Northeast Atlantic (Bay of Biscay), one from mid Tyrrhenian Sea (Gulf of Naples), three from the Aegean Sea, two from the Marmara Sea, and two from this study. The analysis using the Maximum Likelihood method produced a phylogenetic tree that showed one *P. marinus* clade (Figure 2) that includes specimens from native (China, Korea) as well as invaded regions (USA, North Sea, Mediterranean Sea, Northeast Atlantic Ocean), presenting congruence with high bootstrap values of 100% and lacking clear evidence for geographical differentiation. A total of 10 18S rRNA sequences of *Pseudodiaptomus* genus were analyzed (Table S2), including four sequences of *P. marinus*, of which one was from Korea, two were from the Northeast Atlantic (Bilbao Estuary), and one from this study.
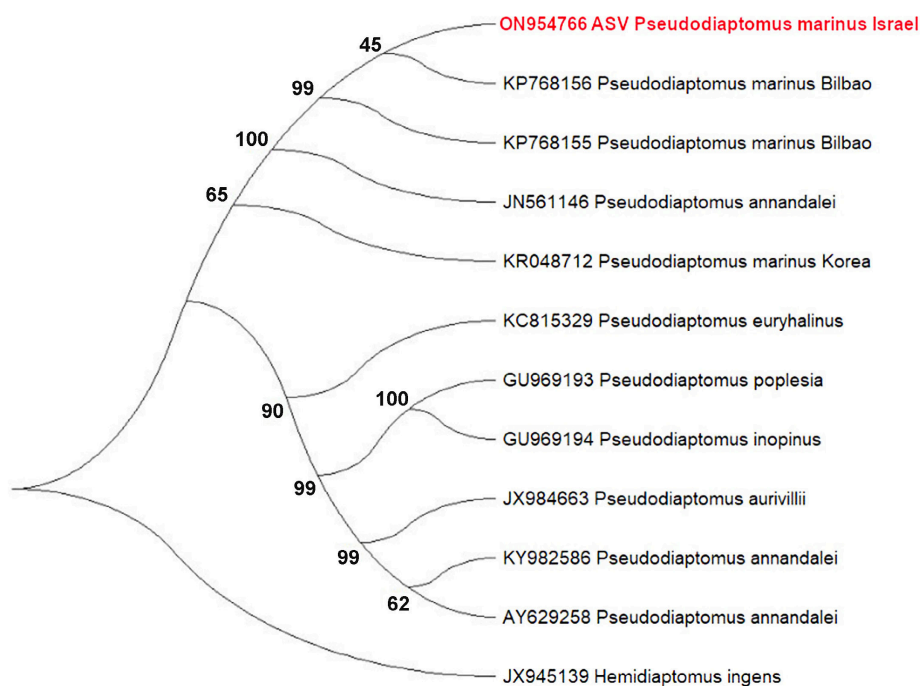
Figure 3. Phylogenetic analysis and molecular identification of *Pseudodiaptomus marinus* based on the 18S ribosomal RNA gene. Sequences obtained in this study are presented in red. The phylogeny was inferred by Maximum Likelihood method based on the best-fitting model (Kimura 2-parameter + G). The percentage of trees in which the associated taxa clustered together is shown next to the branches (bootstrapping = 1000, only values above 50% are displayed). Sequences obtained from the Levantine Sea are marked in red.

The analysis using the Maximum Likelihood method produced a phylogenetic tree that showed one clade shared with *P. marinus* and *P. annandalei* from China (Figure 3).

The two pairwise distance matrixes (Tables S2, S3) confirmed the genetic similarity of the *P. marinus* specimens from the Levantine Sea with the *P. marinus* specimens from the Marmara Sea, Aegean Sea, mid Tyrrhenian Sea, Spain, North Sea, Korea, and China, showing a low intraspecific distance value between 0–0.02. Nevertheless, one 18S rRNA sequence of *P. marinus* collected in South Korea (KR048712.1) showed large dissimilarity to other (native and NIS) *P. marinus* sequences (1.25), likely indicating a misidentification of this specimen, which was more distant to *P. marinus* than *P. annandalei*.

Morphological identification of P. marinus

Following the detection of *P. marinus* in zooplankton samples collected by vertical hauls in Hadera in February and April 2020 and in May 2021, the parallel formalin-fixed samples were inspected. Two *P. marinus* females were found in the sample from May 2021 (Figure 4), indicating a low density of 0.4 ind. m^{-3} (filtered vol. = 5.2 m^3). The total length (TL = PL + UL + CRL, measured dorsally) of the female specimen was 1275 μm. The prosome length (PL) was 815 μm. The urosome length (UL) was 360 μm. The caudal ramus length (CRL) and width (CRW) were 100 μm and 25 μm,
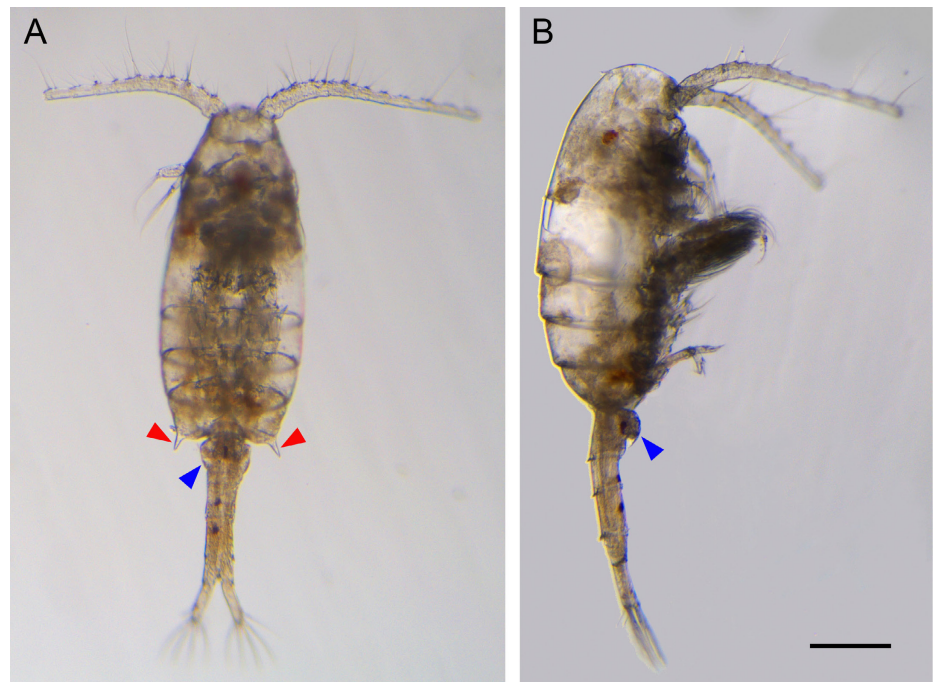
Figure 4. *Pseudodiaptomus marinus*, adult, female. A. dorsal view. B. lateral view. Scale bar = 200 μm. Red arrowheads showing sharp outwardly directed spines on the posterior border of the prosome, blue arrowhead showing the genital segment with slight swellings laterally and a prominent ventral boss. The specimen's antennae (A1) were damaged during the sampling or handling. Photos by X. Velasquez and A.R. Morov.

respectively. The posterior border of the prosome was produced into sharp outwardly directed spines. The genital segment was slightly asymmetrical with slight swellings laterally and a prominent ventral boss. The caudal rami were symmetrical, divergent, and four times as long as wide (CRL:CRW). All morphological characters were in agreement with the original description of *P. marinus* (Sato 1913).

Discussion

Pseudodiaptomus marinus is an epibenthic calanoid copepod found in the coastal, lagoon, and estuarine habitats of tropical and temperate seas, which occurs in the water column during nighttime and over the seabed during daytime (Uttieri et al. 2020; Sabia et al. 2015). The euryhaline and eurythermal nature of *P. marinus*, as well as its behavioral plasticity and resistance to environmental stress, have likely facilitated the acclimatization to regions outside its native range. Within its introduced distribution, the recorded temperature range is 5.2 °C (Sevastopol Bay, Ukraine) – 31.5 °C (Venice Lagoon, Italy), the salinity range is 0.1 (Köprüçay estuary, Turkey) – 38.4 (South Adriatic Sea), and chlorophyll *a* concentrations are > 1 μg L^{-1} (Uttieri et al. 2020). Here, we report the first record of *P. marinus* in the southern Levantine Sea, prevailing under the highest temperature (32.4 °C) and salinity (40.4) levels among those reported so far in its introduced area, confirming the wide thermal and salinity tolerance range of this species, proved by physiological experiments (Svetlichny et al. 2019). Moreover,

although it was previously suggested that *P. marinus* inhabits eutrophic habitats (Sabia et al. 2015), we show here that it can also survive in low-nutrient-low-chlorophyll oligotrophic environments (annual chlorophyll *a* range of 0.05–0.92 µg L^{-1}).

Although the status of *P. marinus* as NIS was defined as established, its reported abundances in the introduced areas are low (comprising < 1% to 8% of the mesozooplankton communities), and hence was defined as widespread but rare (Uttieri et al. 2020). Our finding confirms the rarity of this species. In our study, we detected *P. marinus* using DNA metabarcoding with mitochondrial COI and 18S rRNA v9 region as gene markers prior to its detection using morphological identification. Using morphological identification for detecting rare species in zooplankton assemblages is often hampered by subsampling (Ohman and Lavaniegos 2002). Whereas, DNA metabarcoding is a very sensitive method and thus provides valuable presence/absence data on rare and cryptic species (Bucklin et al. 2016; Lindeque et al. 2013), and can be used as an early warning system for NIS introductions (Comtet et al. 2015). Nonetheless, using metabarcoding for assessing biodiversity was criticized due to primer bias, i.e., the affinity of the primers for the binding site is more conserved in nuclear than in mitochondrial genes, leading to selectivity in the detection of biodiversity (Deagle et al. 2014). The application of multi-marker metabarcoding using mitochondrial and nuclear gene markers for assessing biodiversity alleviates this problem (Stefanni et al. 2018; Günther et al. 2018).

The detection of *P. marinus* in plankton assemblages using DNA metabarcoding was successfully implemented in Bilbao Estuary (Abad et al. 2016), western Adriatic Sea (Stefanni et al. 2018), North Sea (Günther et al. 2018), Venice Lagoon (Schroeder et al. 2020), mid Tyrrhenian Sea (Di Capua et al. 2021), and eastern Adriatic Sea (Lin et al. 2022). Some of these studies have used the combination of 18S rRNA v9 and COI as a multi-marker method (Günther et al. 2018 ; Stefanni et al. 2018), while others used a single marker: 18S rRNA v9 (Abad et al. 2016), 18S rRNA v4 (Di Capua et al. 2021; Lin et al. 2022), or COI (Schroeder et al. 2020). Similar to the studies that used multi-gene markers, we have also found a discrepancy in the detection of *P. marinus* by the two marker genes, likely due to primer bias (Günther et al. 2018). In fact, both gene markers indicated that *P. marinus* is present in the Southeastern Mediterranean Sea from April to late May, but only COI metabarcoding indicated an occurrence of *P. marinus* in late February. This finding is in agreement with the seasonality patterns of *P. marinus* in Berre Lagoon (France), Venice Lagoon (Italy), Bilbao Estuary (Spain), and Lake Faro (Sicily) (Uttieri et al. 2020 and references therein). Nevertheless, in many other introduced regions, *P. marinus* could be found in the summer (July–August). We hypothesize that the high summer temperatures (> 32 ºC) in the Southeastern Mediterranean Sea might trigger metabolically driven quiescence of *P. marinus* facilitating its

survival under harsh conditions. This survival mechanism was found by Svetlichny et al. (2019) in females of *P. marinus* that became torpid in 8 °C. Similar to diapausing resting stages, the ability to induce quiescence can facilitate species survival during its transport across geographical barriers under extreme conditions (Raak-Van den Berg et al. 2013), such as in ballast tanks of ships (Panov et al. 2004), and can thus explain the successful widespread colonization of *P. marinus*. DNA barcoding (Briski et al. 2011) and metabarcoding of sediment samples (Kiemel et al. 2022) in colonized areas where *P. marinus* populations are well established, together with laboratory experiments might provide evidence for this strategy.

The impact of *P. marinus* on the recipient communities in the introduced range is yet to be determined. So far, negative impacts have not been documented (Uttieri et al. 2020). As the spread of this species is evident, future studies should unveil its potential effect on the zooplankton assemblages and food webs in the introduced regions.

Acknowledgements

Authors' contribution

T.G.-H. conceptualized the research, designed the methodology, collected, analyzed and interpreted the data, and wrote the original draft. X.V. collected the data and participated in the data analysis. T.T.K., I.D.C. and M.G.M. assisted with the morphological identification. A.M. collected the data, designed the methodology and analyzed the data. All coauthors reviewed and edited the manuscript.

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Supplementary material

The following supplementary material is available for this article:

Table S1. *Pseudodiaptomus* COI sequences included in the phylogenetic analysis.

Table S2. *Pseudodiaptomus* 18S rRNA sequences included in the phylogenetic analysis.

Table S3. Estimates of evolutionary divergence between Pseudodiaptomidae sequences based on COI sequences.

Table S4. Estimates of evolutionary divergence between Pseudodiaptomidae sequences based on 18S rRNA sequences.

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