Occurrence and Molecular Characterization of Some Parasitic Copepods (Siphonostomatoida: Pandaridae) on Pelagic Sharks in the Mediterranean Sea

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Copepods of the family Pandaridae are typically ectoparasites of elasmobranch fishes. They display a cosmopolitan distribution and limited host specificity. Published literature on their occurrence on pelagic sharks in the Mediterranean is scarce, often from the past century, or scattered through fish parasite surveys. Moreover, of the 64 valid pandarid species known at present, molecular data from GenBank exists for only 10 species and there are no data from the Mediterranean. In this study, we begin addressing this knowledge gap by exploring the molecular features of some pandarid copepods (i.e., *Dinemoura latifolia*, *Echthrogaleus coleoptratus*, *Pandarus satyrus,* and *Phyllothyreus cornutus*) and their phylogenetic relationships using new material from pelagic sharks (i.e., *Prionace glauca*, *Isurus oxyrinchus*, and *Carcharodon carcharias*) in the Mediterranean. Genetic distances analysis showed intraspecific variation in the mitochondrial DNA cytochrome oxidase *c* subunit 1 (mtDNA *cox*1) sequences and interspecific variations of 0.001–0.081 and 0.196–0.288, respectively, for the small subunit ribosomal DNA (SSU rDNA) and the *cox*1 gene locus. Phylogenetic analyses of pandarid copepods based on sequences available in GenBank plus the sequences generated by our study revealed two major clades: the first, with strong nodal support, included species of *Pandarus*, *Phyllothyreus*, *Pannosus,* and *Pseudopandarus*; the second, with weaker nodal support, included species of *Achtheinus*, *Perissopus*, *Echtrogaleus*, *Nesippus*, and *Dinemoura*. As most pandarid species are missing from the present analyses, we discuss the limitations of our phylogenetic results. Nevertheless, this study represents a first step toward to yielding new information about the phylogeny of parasitic copepods on pelagic sharks in the Mediterranean.

Keywords: parasitic copepods, Pandaridae, *Carcharodon carcharias*, *Isurus oxyrinchus*, *Prionace glauca*, molecular identification, phylogenetic analysis

INTRODUCTION

The Order Siphonostomatoida Thorell, 1859 includes 39 families of copepods and encompasses about 75% of all parasitic copepods on fishes (Gunn and Pitt, 2012). Members of the family Pandaridae Edwards, 1840, are typically parasites of external surfaces of elasmobranchs (Kabata, 1979; Izawa, 2010; Bernot and Boxshall, 2017). Pandaridae consists of 23 valid genera with at least

64 recognized species (Walter and Boxshall, 2021). Pandarid life cycles have been not elucidated, however, a life cycle similar to that of Caligidae Burmeister, 1835 has been proposed based on the close phylogenetic relationships between these taxa (Huys et al., 2007; Dippenaar, 2009). The supposed life cycle of Pandaridae includes two free-living nauplius stages, one infective copepodid stage, four parasitic chalimus stages, two parasitic preadult stages, and the parasitic adult stage (Wilson, 1907).

Pandarid copepods are characterized by attachment organs named adhesion pads (Kabata, 1988). Their adhesive surface is formed by a thick cushion of skin with a ridged outer layer (Wilson, 1907). The site of attachment on the host varies depending on tissue tropism and the fundamental niche of the parasite species; they can colonize fins, gills, the cloacal aperture, the mouth, or nasal passages (Benz, 1981, 1986; Rokicki and Bychawska, 1991). Pandarid species are cosmopolitan in their distribution, occurring in warm and temperate waters with most of the species capable of parasitizing more host species (Alvarez and Winfield, 2001).

Published literature on the occurrence of pandarid copepods on pelagic sharks in the Mediterranean is scarce, often from the past century, or scattered through fish parasite surveys (Brian, 1906; Öktener and Trilles, 2009; Öktener et al., 2020). According to the most recent studies, members of Siphonostomatoida remain largely unexplored in terms of their molecular characterization and phylogenetic relationships (Dippenaar, 2009; Bernot et al., 2021). In particular, of the 64 valid pandarid species listed at present, molecular data from GenBank exists for only 10 species and there are no data from the Mediterranean. The present study aimed to report the occurrence (and characterize using a molecular approach) of pandarid copepods obtained opportunistically on shark species off the coast of Sicily (Italy) and to provide newly generated molecular and phylogenetic data to improve knowledge of the poorly known Pandaridae parasites infecting sharks.

MATERIALS AND METHODS

Sampling and Parasitological Analysis

The material here studied comprised undetermined copepod parasites collected by two co-authors (GI and BZ) under the framework of a project of the Museo Civico di Storia Naturale (MSNC) in Comiso on non-native and rare marine species of the Mediterranean Sea (see Katsanevakis et al., 2020; Deidun et al., 2021). The MSNC is a scientific institution registered at the CITES Secretariat, D.M. 23.03.1994 (Cod. IT030), authorized to take, keep, use and display dead endangered fauna.

The present material encompassed copepod parasites collected from 2003 to 2021 from the coast of Sicily on six pelagic sharks [i.e., three blue sharks, *Prionace glauca* (Linnaeus, 1758), two shortfin mako sharks, *Isurus oxyrinchus* Rafinesque, 1810 and one great white shark, *Carcharodon carcharias* (Linnaeus, 1758)]. The blue sharks were from strandings; the shortfin mako sharks and the great white shark were caught as bycatch (**Table 1**).

The taxonomic identification of sharks followed Compagno (1984). The fishes were weighed, measured (total length) to the nearest 0.1 cm and sexed by visual observation of external characteristics. Copepods from the skin were carefully removed using forceps while gills were removed from carcasses and examined for copepods in Petri dishes under a stereomicroscope. Copepod parasites were counted, washed in physiological saline, and preserved in 70% ethanol (Santoro et al., 2014, 2020). For identification, copepods were sent to the Stazione Zoologica Anton Dohrn in Naples where they were studied using a stereomicroscope and an optical microscope both equipped with the ZEN 3.1 imaging system (Zeiss). Morphological identification of copepods followed the identification keys of Lewis (1966) and Cressey (1967, 1968). After examination, the sharks were prepared and incorporated into the museum collections of the MSNC under inventory numbers as listed in **Table 1**, except the blue shark #2 which was a live individual rescued, rehabilitated, and released back into the wild after the external examination.

Molecular and Phylogenetic Analyses

Following the morphological identification, genomic DNA was extracted from the antennae of six specimens of *D. latifolia*, collected from a shortfin mako shark ($n = 3$) and a great white shark ($n = 3$), and two specimens of *Echthrogaleus coleoptratus* (Guérin-Méneville, 1837), three specimens of *Pandarus satyrus* Leach, 1816 and two specimens of *Phyllothyreus cornutus* (Milne Edwards, 1840), collected from the blue shark. Genomic DNA extraction was performed using a Quick-gDNA Miniprep Kit (ZYMO RESEARCH), following the manufacturer-recommended protocols, with modification of the incubation period with proteinase K to 3 h.

The small subunit ribosomal DNA (SSU rDNA) (∼1,795 bp) was amplified using the primers 18Sf (5′-TACCTGGTTGATCCTGCCAG-3′) and 18Sr (5′-TAATGA TCCTTCCGCAGGTTCAC-3′) (Huys et al., 2007). The partial sequence of the mitochondrial cytochrome *c* oxidase subunit 1 (mtDNA *cox*1) (∼600 bp) was amplified using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994). Both polymerase chain reactions (PCRs) were performed in a 25 μL volume containing 0.6 μL of each primer 10 μM, 2 μL of MgCl2 25 mM (Promega), 5 μL of 5 × buffer (Promega), 0.6 μL of dNTPs 10 mM (Promega), 0.2 μL of Go-Taq Polymerase (5 U/μL) (Promega) and 2 μL of total DNA. PCR temperature conditions for the SSU rDNA were the following: 94°C for 5 min (initial denaturation), followed by 35 cycles at 94°C for 30 s (denaturation), 57°C for 30 s (annealing), 72°C for 30 s (extension) and followed by post-amplification at 72°C for 5 min. PCR cycling parameters for the mtDNA *cox*1 amplifications were: 95°C for 5 min (initial denaturation), followed by 40 cycles at 95°C for 1 min (denaturation), 45°C for 1 min (annealing), 72°C for 1 min (extension) and followed by post-amplification at 72°C for 7 min. PCR amplicons were purified using the AMPure XP kit (Beckman coulter) following the standard manufacturer-recommended protocol and Sanger sequenced from both strands, with the same primers, through an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems), using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies).

TABLE 1 | Available data of shark individuals examined for pandarid copepods from the coast of Sicily.

ID (MSNC*inventory number)	Stranding date	Stranding locality	Total length (cm)	Sex	Weight (Kg)	Parasites (n females/n males)	Site on the host
Great white shark *Carcharodon carcharias* (MSNC 4636)	August 20, 2003	Cava d'Aliga (Ragusa)	122	f	10.8	*Dinemoura latifolia* (6 f/1 m)	Skin around the pelvic fins
Shortfin mako shark *Isurus oxyrinchus* 1 (MSNC 4848)	May 23, 2020	Ognina di Catania (Catania)	318	f	350	*Dinemoura latifolia* (14 f/2 m)	Skin around the pelvic fins
Isurus oxyrinchus 2 (MSNC 4638)	May 2, 2017	Marzamemi (Siracusa)	104	f	10	*Dinemoura latifolia* (1 f)	Skin around the pelvic fins
Blue shark *Prionace glauca* 1 (MSNC 4768)	April 4, 2010	Port of Milazzo (Messina)	310	m	130	*Phyllothyreus cornutus* (5 f/1 m)	Gills
Prionace glauca 2 (released back into the wild)	August 27, 2020	Pozzallo (Ragusa)	312	f	120	*Echthrogaleus coleoptratus* (2 m/2 f); *Pandarus satyrus* (10 f/1 m)	Skin
Prionace glauca 3 (MSNC 4850)	April 4, 2021	Marina di Ragusa (Ragusa)	250	m	63.1	*Pandarus satyrus* (9 f/2 m)	Skin

*MSNC, Museo Civico di Storia Naturale of Comiso.

Contiguous sequences were assembled and edited using MEGAX v. 11 (Kumar et al., 2018). Sequence identity was checked using the Nucleotide Basic Local Alignment Search Tool (BLASTn) (Morgulis et al., 2008). The SSU and *cox*1 data sets were, respectively, aligned with all sequences of Pandaridae available in GenBank (see **Table 2**), using ClustalX v. 2.1 (Larkin et al., 2007). Sequences of both genes (SSU + *cox*1) were concatenated using SequenceMatrix (Vaidya et al., 2011), while the best partition schemes and best-fit models of substitution were identified using Partition Finder (Lanfear et al., 2012) with the Akaike information criterion (AIC; Akaike, 1973). The analyses were performed using the GTR + invgamma substitution model.

Sequences obtained in the present study were deposited in GenBank under the accession numbers as listed in **Table 2**. Genetic distances were computed using the Kimura 2-Parameters (K2P) model (Kimura, 1980) with 1,000 bootstrap re-samplings, by MEGA Software, version 7.0.

The phylogenetic trees were constructed using the maximum likelihood (ML) method by IQ-TREE (Nguyen et al., 2015) with 1,000 ultrafast bootstrap replicates (BS). Clades were considered to have high nodal support if the ML bootstrap resampling ≥ 70%. Due to the phylogenetic position of Pandaridae (see Dippenaar, 2009), the phylogenetic trees were rooted using *Alebion* Krøyer, 1863, as outgroup. The SSU and *cox*1 sequences from GenBank included in the phylogenetic trees are listed in **Table 2**. To corroborate the taxonomic assessment obtained according to the phylogenetic species concept, the species delimitation method on *cox*1 gene locus was also performed using the assemble species by automatic partitioning (ASAP) (Puillandre et al., 2020).

RESULTS

General Data
Available data from shark individuals examined for pandarid copepods, and species, number, and sex of pandarid copepods found are listed in **Table 1**. A total of four species of pandarid copepods were morphologically identified. A single species (i.e., *D. latifolia*) (**Figures 1A,B**) was found on the skin surfaces of the shortfin mako shark and great white shark, and three species (i.e., *E. coleoptratus*, *P. satyrus* and *Ph. cornutus*) (**Figures 1C–H**) were collected on the skin and gills of the blue shark (**Table 1**). Voucher specimens have been deposited in the collection of the Stazione Zoologica Anton Dohrn in Naples with the following accession numbers: SZN-CRUOO2A-2B (*D. latifolia*), SZN-CRU003A-3B (*P. satyrus*), SZN-CRUOO4A-4B (*Ph. cornutus*) and SZN-CRU005A-5B (*E. coleoptratus*).

Molecular and Phylogenetic Analyses
High quality sequences for both SSU and *cox*1 gene loci were successfully obtained for *D. latifolia*, *E. coleoptratus,* and *P. satyrus*. High quality sequences for *Ph. cornutus* were obtained only for SSU rDNA. The six SSU gene locus sequences obtained from *D. latifolia* collected from both the shortfin mako shark and the great white shark were identical to each other, and all sequences showed 100% similarity with the sequence (DQ538501) of *D. latifolia* available in GenBank. The present six *cox*1 sequences of *D. latifolia* showed 83–84% similarity with that (KF483702) of *Caligus robustus* Bassett-Smith, 1898, available in GenBank. Unfortunately, no sequence of *D. latifolia* for the *cox*1 gene locus was retrieved from GenBank for comparison.

The sequence of the SSU rDNA obtained from *Ph. cornutus* showed 100% similarity with the sequence (FJ447449) of *Ph. cornutus* previously deposited in GenBank. Sequences of *E. coleoptratus* and *P. satyrus* were here generated for the first time. The SSU and *cox*1 sequences obtained for *E. coleoptratus* showed 98.38 and 86.71% similarity with the sequences of *Achtheinus oblongus* Wilson, 1908 (FJ447452) and *Caligus mutabilis* Wilson, 1905 (KF483685) available in GenBank, respectively. The present SSU and *cox*1 sequences of *P. satyrus* showed 99.88% and 87% similarity with the sequences of *Pandarus* sp. 2 (FJ447454-FJ447387) available in GenBank, respectively.

Pairwise distances among specimens and species for the obtained SSU and *cox*1 sequences are given in **Table 3**. While

TABLE 2 | Species, host, locality, and accession numbers of *cox*1 and SSU sequences of pandarid copepods included in the phylogenetic analysis shown in **Figure 2**.

Species	Host	Locality	*cox*1	SSU	References
Achtheinus oblongus	*Carcharodon carcharias*	South Africa	FJ447385	FJ447452	Dippenaar, 2009
Dinemoura latifolia	-	-	-	DQ538501	Huys et al., 2007
Dinemoura latifolia	*Isurus oxyrhinchus*	Mediterranean Sea	MZ934715 OL415941-42	MZ935642 OL333874-5	This study
Dinemoura latifolia	*Carcharodon carcharias*	Mediterranean Sea	OL415938-40	MZ935643 OL333872-3	This study
Echtrogaleus coleoptratus	*Prionace glauca*	Mediterranean Sea	OL348230-1	MZ935645 OL333879	This study
Nesippus crypturus	*Sphyrna mokarran*	South Africa	FJ447379	FJ447444	Dippenaar, 2009
Nesippus orientalis	*Carcharodon carcharias*	South Africa	FJ447383	FJ447448	Dippenaar, 2009
Nesippus vespa	*Rhina ancylostoma*	South Africa	FJ447378	FJ447443	Dippenaar, 2009
Pandarus satyrus	*Prionace glauca*	Mediterranean Sea	OL457303-5	OL333876-8	This study
Pandarus smithi	-	-	-	DQ538502	Huys et al., 2007
Pandarus sp. 1	*Carcharias taurus*	South Africa	FJ447390	FJ447457	Dippenaar, 2009
Pandarus sp. 2	*Sphyrna lewini*	South Africa	FJ447387	FJ447454	Dippenaar, 2009
Pandarus sp. 3	*Carcharodon carcharias*	South Africa	FJ447388	FJ447455	Dippenaar, 2009
Pandarus sp. 4	*Isurus oxyrhinchus*	South Africa	FJ447391	FJ447458	Dippenaar, 2009
Pannosus japonicus	*Sphyrna lewini*	South Africa	FJ447384	FJ447450	Dippenaar, 2009
Phyllothyreus cornutus	*Isurus oxyrhinchus*	South Africa	-	FJ447449	Dippenaar, 2009
Phyllothyreus cornutus	*Prionace glauca*	Mediterranean Sea	-	OL333880 MZ935644	This study
Perissopus dentatus	*Carcharhinus obscurus*	South Africa	FJ447386	FJ447453	Dippenaar, 2009
Pseudopandarus longus	*Carcharhinus obscurus*	South Africa	-	FJ447451	Dippenaar, 2009
Alebion sp. (outgroup)	*Carcharhinus obscurus*	South Africa	FJ447377	FJ447442	Dippenaar, 2009

no intraspecific variations were found between SSU sequences, intraspecific variations were found in the *cox*1 sequences of *D. latifolia* (K2P = 0.008 ± 0.003) and *P. satyrus* (0.0032 ± 0.002). SSU sequence divergence among species (i.e., interspecific variation) was found to range from a minimum of 0.001 ± 0.000 between *P. satyrus* and *Pandarus* sp. 4 to a maximum of 0.081 ± 0.007 between *Ph. cornutus* and *Nesippus vespa* Cressey, 1964 (**Table 3**). *Cox*1 sequence divergence among species was found to range from a minimum of 0.196 ± 0.023 between *E. coleoptratus* and *Nesippus crypturus* Heller, 1865 to a maximum of 0.288 ± 0.029 between *P. satyrus* and *Perissopus dentatus* Steenstrup and Lütken, 1861 (**Table 3**).

Phylogenetic analyses were conducted using both separately (**Supplementary Figures 1, 2**) and combined *cox*1 and SSU gene loci (**Figure 2**). The resulting tree for SSU (**Supplementary Figure 1**) showed Pandaridae as a monophyletic group, with high support (BS = 100), and the existence of two main clades. The first clade, with strong nodal support (BS = 100) involved two lineages, that included the genera *Phyllothyreus* Norman, 1903, *Pannosus* Cressey, 1967, *Pseudopandarus* Kirtisinghe, 1950 and the paraphyletic genus *Pandarus* Leach, 1816. At species level, the new generated sequences of *P. satyrus* clustered with that of *Pandarus* sp. 2 previously deposited in GenBank in a well-supported lineage (BS = 98). The obtained sequences of *Ph. cornutus* clustered with high nodal support (BS = 98) with the sequences of *Ph. cornutus* and *Pannosus japonicus* (Shiino, 1960) previously deposited in GenBank.

The second major clade, with weaker nodal support (BS = 71), involved three lineages, that included the species of *Achtheinus* Wilson, 1908, *Perissopus* Steenstrup and Lütken, 1861, *Echtrogaleus* Steenstrup and Lütken, 1861, *Nesippus* Heller, 1865, and *Dinemoura* Latreille, 1829. At species level, the SSU tree topology placed the new sequences of *E. coleoptratus*

within a well-supported lineage (BS = 99) with the sequences of *Achtheinus oblongus* and *Pe. dentatus*, previously deposited in GenBank. The present new generated and the previously deposited sequences of *D. latifolia* clustered in a separate lineage with high nodal support (BS = 99).

In the resulting tree obtained only for *cox*1, Pandaridae was also a monophyletic group (BS = 100). Two major clades were generated, the first well-supported (BS = 100) formed by all sequences of *Pandarus*, *Pa. japonicus*, *A. oblongus*, the new generated sequences of *E. coleoptratus, N. vespa* and *Pe. dentatus,* and the second (BS = 34) formed by the obtained sequences of *D. latifolia* and the sequences of *N. orientalis* and *N. crypturus* previously deposited in GenBank, highlighting the monophyly of *D. latifolia*.

The species delimitation analyses of the *cox*1 gene locus highlighted a total of 10 taxonomic entities, revealing that the sequences of *Pandarus* spp. belonged to two distinct taxonomic entities (as shown in **Supplementary Figure 2**). The sequences of *P. satyrus* obtained in the present study belonged to the same taxonomic entity that included the sequences of *Pandarus* sp. 2, *Pandarus* sp. 3, and *Pandarus* sp. 4 from GenBank (**Supplementary Figure 2**).

The tree inferred by concatenating the SSU and *cox*1 gene loci (**Figure 2**) showed the same topology of the SSU tree (**Supplementary Figure 1**).

DISCUSSION

This study provides the first molecular data on the occurrence of four species of pandarid copepods from the Mediterranean. To our knowledge prior to of the present study only *Ph. cornutus*, *D. latifolia, E. coleoptratus,* and *Pandarus bicolor* have been
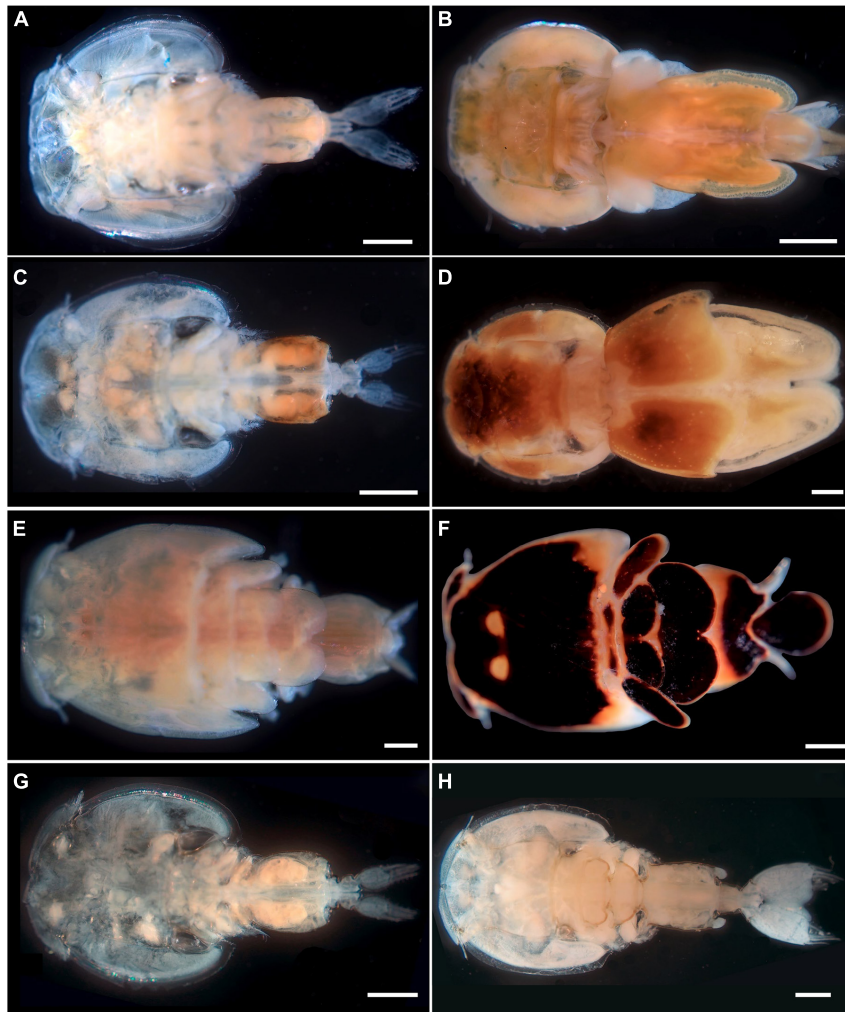
FIGURE 1 | Specimens of pandarid copepods sequenced in the present study. *Dinemoura latifolia* dorsal view of male **(A)** (bar scale: 1,000 µm) and female
(GenBank: OL415938 and MZ935643) **(B)** (bar scale: 2,000 µm) from the great white shark; *Echtrogaleus coleoptratus* dorsal view of male **(C)** (bar scale: 1,000 µm)
and female (GenBank: OL348230 and MZ935645) **(D)** (bar scale: 1,000 µm) from the blue shark; *Pandarus satyrus* dorsal view of male **(E)** (bar scale: 500 µm) and
female (GenBank: OL457303 and OL333876) **(F)** (bar scale: 1,000 µm) from the blue shark; *Phyllothyreus cornutus* dorsal view of male **(G)** (bar scale: 1,000 µm)
and female (GenBank: MZ935644) **(H)** (bar scale: 2,000 µm) from the blue shark.

recorded on shark species in the Mediterranean Sea (Richiardi,
1880; Brian, 1906; Öktener and Trilles, 2009; Öktener et al., 2020).

In general, pandarid copepods are widely distributed
mirroring the movements and distribution of their hosts. In
particular, *Dinemoura* parasitizes the skin of large pelagic sharks.
After Cressey (1967), *Dinemoura* comprises four valid species
including *D. discrepans* Cressey, 1967, *D. ferox* (Krøyer, 1838),
D. latifolia and *D. producta* (Müller, 1785). The only reports of
D. latifolia in the Mediterranean were on blue, shortfin mako,
and thresher sharks *Alopias vulpinus* (Brian, 1906). However,
along with its geographical distribution range *D. latifolia* has
been found at least, on other three shark species (i.e., the
great white shark, the porbeagle *Lamna nasus*, and the school
shark *Galeorhinus galeus*) from North and South Atlantic,
East, and West Pacific, Indian Ocean, and West Indies (see
Williams, 1978).

Phyllothyreus cornutus, the only species in its monotypic
genus, infects the gills of several pelagic sharks (i.e., the blue
shark, the shortfin mako shark, the porbeagle, the smooth
hammerhead *Sphyrna zygaena*, the sandbar shark *Carcharhinus
plumbeus*, and the tiger shark *Galeocerdo cuvier*) from the North
and South Atlantic and North Pacific (Hewitt, 1967; Schaeffner
and Smit, 2019). In the Mediterranean Sea, it has been reported
exclusively on the blue shark (Richiardi, 1880).

The genus *Echthrogaleus* comprises eight species including
E. asiaticus Ho, Liu and Lin, 2012, *E. coleoptratus*, *E. denticulatus*
Smith, 1873, *E. disciarai* Benz and Deets, 1987, *E. mitsukurinae*
Izawa, 2012, *E. pellucidus* Shiino, 1963, *E. spinulus* Morales-Serna,
Crow, Montes and González, 2019 and *E. torpedinis* Wilson,
1907. *Echthrogaleus coleoptratus* has been reported from the
North and South Atlantic, the Pacific and Indian Oceans, and
the Mediterranean Sea (Hewitt, 1967). It parasitizes the skin of

TABLE 3 | K2P genetic distances ± standard error among specimens and species of pandarid copepods.

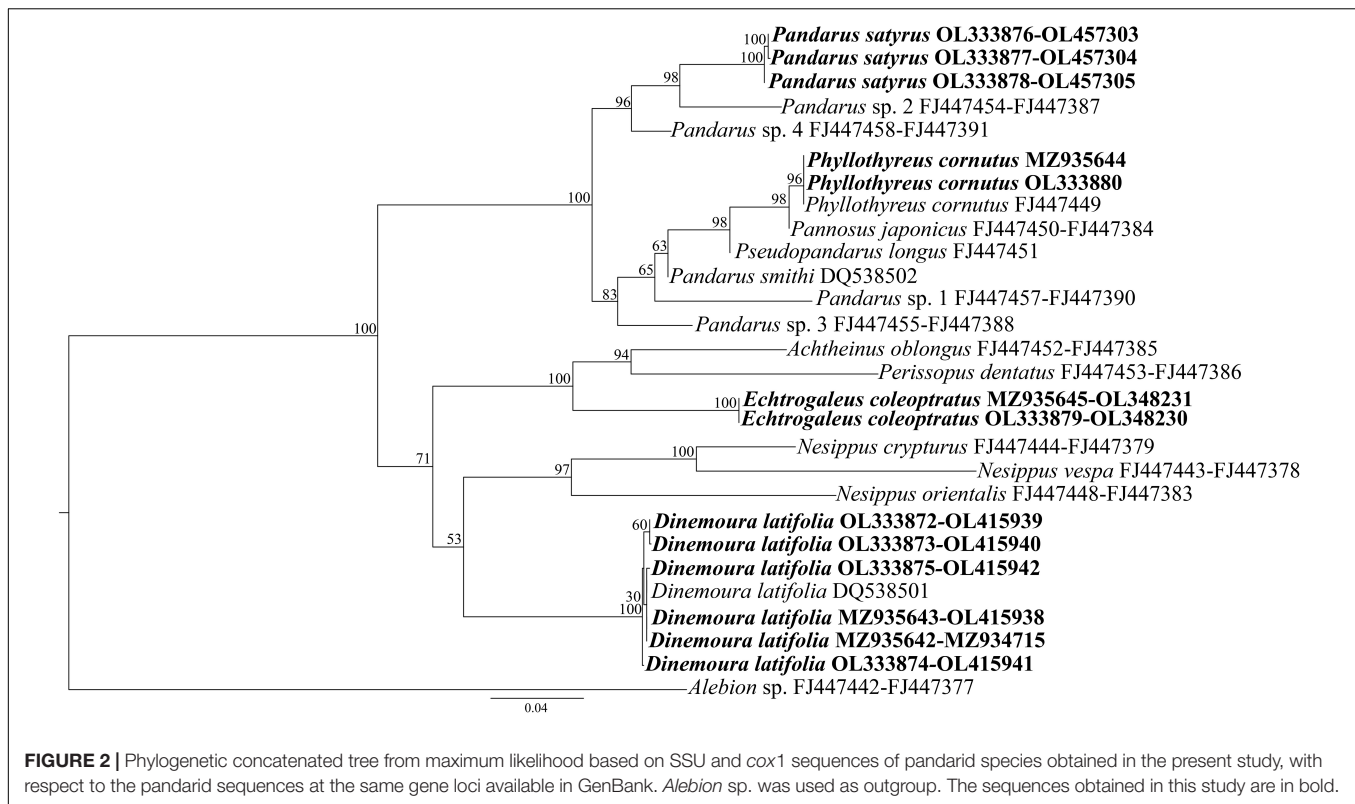|	D. latifolia	E. coleoptratus	P. satyrus	Ph. cornutus
A. oblongus	0.037 ± 0.004	0.015 ± 0.003	0.052 ± 0.005	0.054 ± 0.001
	0.239 ± 0.026	0.209 ± 0.023	0.300 ± 0.030	-
D. latifolia	0.000 ± 0.000	-	0.050 ± 0.005	
	0.008 ± 0.003	-	0.267 ± 0.028	
E. coleoptratus	0.032 ± 0.004	0.000 ± 0.000		0.049 ± 0.005
	0.222 ± 0.025	0.000 ± 0.000		-
N. crypturus	0.049 ± 0.005	0.056 ± 0.006	0.061 ± 0.006	0.062 ± 0.006
	0.216 ± 0.026	0.196 ± 0.023	0.257 ± 0.029	-
N. orientalis	0.045 ± 0.005	0.048 ± 0.005	0.057 ± 0.006	0.059 ± 0.006
	0.221 ± 0.027	0.260 ± 0.029	0.282 ± 0.031	-
N. vespa	0.058 ± 0.006	0.068 ± 0.006	0.078 ± 0.006	0.081 ± 0.007
	0.219 ± 0.025	0.201 ± 0.023	0.280 ± 0.029	-
P. satyrus	0.050 ± 0.005	0.047 ± 0.005	0.000 ± 0.000	
	0.267 ± 0.028	0.302 ± 0.030	0.003 ± 0.002	
P. smithi	0.048 ± 0.005	0.045 ± 0.005	0.006 ± 0.001	0.006 ± 0.001
	-	-	-	-
Pandarus sp. 1	0.048 ± 0.005	0.045 ± 0.005	0.004 ± 0.001	0.005 ± 0.001
	0.252 ± 0.029	0.242 ± 0.027	0.256 ± 0.030	-
Pandarus sp. 2	0.049 ± 0.005	0.047 ± 0.005	0.000 ± 0.000	0.006 ± 0.001
	0.239 ± 0.026	0.218 ± 0.026	0.189 ± 0.023	-
Pandarus sp. 3	0.048 ± 0.005	0.045 ± 0.005	0.006 ± 0.001	0.007 ± 0.002
	0.237 ± 0.026	0.258 ± 0.027	0.222 ± 0.024	-
Pandarus sp. 4	0.045 ± 0.005	0.043 ± 0.005	0.001 ± 0.000	0.004 ± 0.001
	0.214 ± 0.025	0.221 ± 0.026	0.152 ± 0.021	-
Pa. japonicus	0.050 ± 0.005	0.047 ± 0.005	0.007 ± 0.002	0.001 ± 0.001
	0.256 ± 0.029	0.283 ± 0.031	0.205 ± 0.027	-
Ph. cornutus	0.052 ± 0.005	0.049 ± 0.005	0.007 ± 0.001	0.000 ± 0.000
	-	-	-	-
Pe. dentatus	0.040 ± 0.005	0.021 ± 0.003	0.053 ± 0.005	0.055 ± 0.006
	0.261 ± 0.029	0.238 ± 0.027	0.288 ± 0.029	-
Ps. longus	0.048 ± 0.005	0.045 ± 0.005	0.007 ± 0.002	0.004 ± 0.001
	-	-	-	-

The SSU K2P-values are in the upper row, while in the bottom row are reported the cox1 K2P-values (0.000 indicates identity between specimens; - indicates missing data).

about 13 species of sharks; however, it is commonly found on the great white shark, the porbeagle and the blue shark (Hewitt, 1967, 1979; Cressey and Lachner, 1970; Rokicki and Bychawska, 1991; Henderson et al., 2002; Benz et al., 2003; Luque and Tavares, 2007). In the Mediterranean, it has been reported on the blue shark, the gulper shark *Centrophorus granulosus* (Bloch and Schneider, 1801) and the great white shark (Brian, 1906).

The genus *Pandarus* comprises 14 nominal species including *P. ambiguous* (Scott, 1907), *P. bicolor*, *P. brevicaudis* Dana, 1852, *P. carcharhini* Ho, 1963, *P. cranchii* Leach, 1819, *P. floridanus* Cressey, 1967, *P. katoi* Cressey, 1967, *P. niger* Kirtisinghe, 1950, *P. rhincodonicus* Norman, Newbound and Knott, 2000, *P. rouxii* Risso, 1826, *P. satyrus* Dana, 1849, *P. sinuatus* Say, 1818, *P. smithii* and *P. zygaenae* Brady, 1883. *Pandarus satyrus* has a wide geographical distribution including Atlantic, Pacific, and Indian Ocean; however, it has never been reported from the Mediterranean. According to Cressey (1967); Benz (1986), and Rojas et al. (2001), *P. satyrus* has been predominantly found on

the blue shark. It is closely related to *P. cranchii* with which it was synonymized by Shiino (1954) but considered as valid species by Cressey (1967). According to Cressey (1967) the two species are easily separated on the basis of the caudal rami. The rami of *P. cranchii* extend at least to the tip of the abdominal plate (often beyond) whereas the rami of *P. satyrus* extends only about half the length of the abdominal plate. The only other species of *Pandarus* reported from the Mediterranean is *P. bicolor* found on the blue shark, the dusky smooth-hound, the common smooth-hound, the angular rough shark *Oxynotus centrina* Linnaeus, 1758 and the picked dogfish *Squalus acanthias* Linnaeus, 1758 (Richiardi, 1880; Brian, 1906; Öktener and Trilles, 2009; Öktener et al., 2020). *Pandarus bicolor* can be distinguished from *P. satyrus* as the cephalon only occupies 1/3 of the total body length and its caudal rami are small and scarcely visibly dorsally (Cressey, 1967).

Based on specific morphological characters, pandarid copepods have been arranged into two major groups: (i) species with all three thoracic segments provided with dorsal or

FIGURE 2 | Phylogenetic concatenated tree from maximum likelihood based on SSU and *cox*1 sequences of pandarid species obtained in the present study, with respect to the pandarid sequences at the same gene loci available in GenBank. *Alebion* sp. was used as outgroup. The sequences obtained in this study are in bold.

dorsolateral plates (*Pandarus*-group), and (ii) species with the second free thoracic segment without plates (*Dinemoura*-group) (Kabata, 1979). *Pandarus* and *Phyllothyreus* have been included in the first group with *Achtheinus*, *Perissopus*, *Gangliopus* Gerstaecker, 1854, *Pannosus* and *Pseudopandarus*; *Dinemoura* and *Echtrogaleus* have been included in the second group with *Demoleus* Heller, 1865, *Dinemoleus* Cressey and Boyle, 1978, *Nesippus*, *Paranesippus* Shiino, 1955 and *Pagina* Cressey, 1963 (see Kabata, 1979).

In contrast, based on the results of more recent phylogenetic analyses, pandarid copepods have been placed into two major clades: the first clade included the species of *Nesippus*, and the second clade included the species of *Phyllothyreus*, *Pannosus*, *Pandarus*, *Pseudopandarus,* and *Achtheinus* (Dippenaar, 2009). However, Dippenaar (2009) focused mainly on relationships among families of the Siphonostomatoida, while no phylogenetic relationships were deepened among the genera.

Maximum-likelihood analysis inferred by concatenated SSU + *cox*1 data set placed the sequences of pandarid copepods available in GenBank plus the new generated sequences into two major clades, however, some differences were observed when the present results were compared to those of Dippenaar (2009). For example, the present specimens of *P. satyrus* and *Ph. cornutus* were included in a first clade with *Pandarus* spp., *Pa. japonicus*, *Ps. longus*, and *P. smithi* with strong nodal support. Within this clade, *Ph. cornutus*, *Pa. japonicus*, *Ps. longus*, *P. smithi*, *Pandarus* sp. 1, and *Pandarus* sp. 3 were placed in a subclade not supported by the posterior probabilities and bootstrap analysis. Finally, *D. latifolia* and *E. coleoptratus*

were included in a second clade with a weaker nodal support with *Nesippus orientalis* Heller 1865, *N. vespa*, *N. crypterus*, *A. oblongus*, and *Pe. dentatus*.

The phylogenetic pattern for the species here collected was congruent with the morphological characters of the two species groups above mentioned, except for *A. oblongus* and *Pe. dentatus* which were placed into the second major clade with the genera *Echtrogaleus*, *Nesippus* and *Dinemoura*. In contrast, the phylogenetic clustering among the members of the two clades seems to be not related to the host preference. For instance, both clades included parasites capable of infecting shark species belonging to six orders and 11 families, with the second clade that also included parasites capable of infecting five additional families of sharks. Nevertheless, we cannot exclude a coevolutionary hypothesis between copepods and their hosts species. Indeed, little is known regarding the nature of host-copepod association in elasmobranchs (Bernot et al., 2021). Huys et al. (2007) suggested a host switching event in the siphonostomatoid copepods highlighting monostrilloids' alterations in the host utilization, body plan, and life cycle strategy. The scarcity of data regarding the host association and life cycle strategy of pandarid copepods does not help to resolve phylogenetic relationships among species. The present phylogenetic analysis included only a small subset (12) of the 64 valid species of Pandaridae. Therefore, it is possible that the present phylogenetic results may not reflect the true relationships, as a large majority of species is missing from the present analysis. Indeed, as already discussed above, our phylogenetic results were not congruent with those obtained by Dippenar (2009). More thorough sequencing of Pandaridae

species will be needed to better resolve the phylogenetic relationships among the members of this family.

CONCLUSION

In conclusion, we provide additional DNA sequences for *D. latifolia* and *Ph. cornutus*. Furthermore, new molecular data for *E. coleoptratus* and *P. satyrus* are reported, based on, both the nuclear (SSU) and mitochondrial (*cox*1) gene loci. Whilst the single use of the SSU gene locus permitted the molecular identification of the copepod species, the mtDNA *cox*1 could represent a suitable marker to infer population structure of pandarid copepods, and consequently of their hosts (Criscione et al., 2006; Baldwin et al., 2011). In this sense, intraspecific variation of *cox*1 was actually detected for some of the species in the present study. Nonetheless, the scarce reference sequence information, hampered any further understanding on the population structure of these copepod parasites. This study represents the first attempt to yield new molecular and phylogenetic data of pandarid copepods on pelagic sharks in the Mediterranean Sea that could contribute to a better characterization of these poorly known parasites. Future molecular and genetic studies should also provide a more detailed assessment of the host-parasite interactions, ecological data, and life cycle strategy. *Pandarus satyrus* represents a new record for the Mediterranean.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI accession numbers: MZ935642-45, OL333872-80, MZ934715, OL415938-42, OL348230-31, and OL457303-05.

ETHICS STATEMENT

The material here studied has been collected from two co-authors (GI and BZ) of the present paper under the framework of a project of the Museo Civico di Storia Naturale (MSNC) in

Comiso on alien and rare marine species of the Mediterranean Sea. The MSNC is a scientific institution registered at the CITES Secretariat, D.M. 23.03.1994 (Cod. IT030), authorized to take, keep, use and display dead endangered species of wild fauna.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars. 2021.778034/full#supplementary-material

Supplementary Figure 1 | Phylogenetic tree from maximum likelihood based on SSU sequences of pandarid species obtained in the present study, with respect to the pandarid sequences at the same gene locus available in GenBank. *Alebion* sp. was used as outgroup. The sequences obtained in this study are in bold.

Supplementary Figure 2 | Phylogenetic tree from maximum likelihood based on *cox*1 sequences of pandarid species obtained in the present study, with respect to the pandarid sequences at the same gene locus available in GenBank. *Alebion* sp. was used as outgroup. The sequences obtained in this study are in bold. Braces indicate the specimens that according to the species delimitation analyses are part of the same taxonomic entity. Numbers in brackets indicate the 10 taxonomic entities revealed by the species delimitation analyses.

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