

# *Farfantepenaeus aztecus* (Ives, 1891) (Crustacea: Decapoda: Penaeidae) in N Aegean: first record in Greece by morphological and genetic features

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A multidisciplinary approach based on morphological and genetic characters was applied for the species identification of penaeids sampled from two coastal lagoons of N Aegean Sea. Two species were found: *Melicertus kerathurus* and *Farfantepenaeus aztecus*. The latter is an alien species and it was recorded for the first time in N Aegean Sea; the capture of subadult and adult individuals indicates the establishment of a vivid population in the Eastern Mediterranean Basin.

**Key words:** *Farfantepenaeus aztecus*, *Melicertus kerathurus*, invasive species, genetic analyses, multidisciplinary approach.

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## INTRODUCTION

In the past two decades, ~10 new alien species (on average) are recorded annually in the Mediterranean Sea (Galil, 2009). 573 alien marine metazoan species have been recorded in the Mediterranean Sea, including the description of *Fucus taxiformis* Delile, 1813 – possibly the first alien species recorded from the Mediterranean (Andreakis *et al.*, 2007). Biotas were kept effectively isolated for long periods (in the order of million of years); however, the collapse of biogeographic barriers has been dramatic since the Age of exploration (Mooney & Cleland, 2001). Ricciardi (2007) suggested that the species invasions due to human activities may be several orders of magnitude greater than those of prehistoric invasions.

The invasion of alien species in the Mediterranean affects negatively the marine biodiversity, though no

extinction of native species has been verified until recently (Boudouresque, 2004). The Mediterranean region has a long history of such introductions because of its heavy ship traffic for millennia [Baskin (2002) cited in Bardsley & Edwards-Jones (2007)]. The number of introductions has been rising steadily. Alien species have been entering the Mediterranean through either the Suez Canal (Lessepsian migrants) or the Strait of Gibraltar, mainly by ship transportation (Galil, 2009). One-fifth of the alien species in the Mediterranean have been introduced by vessels (Galil, 2009). The most common taxa of vessel-transported alien species are molluscs (27%), arthropods (19%), rhodophytes (17%) and annelids (14%) (Galil *et al.*, 2008).

Besides the invasion of alien species, marine biodiversity in the Mediterranean Sea has been changed, also, due to the climatic change (Coll *et al.*, 2010), which makes the ecosystems vulnerable to invasion (Ladd *et al.*, 2005). Climate change affects the marine biodiversity through seawater warming and, there-

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fore, affects the distribution and abundance of native and alien species (Coll *et al.*, 2010). Rising temperature enables species (native and alien) to establish in new ecosystems and renders alien species more advantageous compared to the native ones (Coll *et al.*, 2010).

The sudden decline in abundance and/or numerous local extirpations of native species, concurrent with the increase of alien species, dictate that special attention should be paid to monitor invasion incidents. The invasion of alien species may result to the reduction of genetic diversity and lead to biotic homogenization (Galil, 2007)

Numerous studies have shown that the estuaries are considered as “hot spots” for incidents of invasion worldwide (Ruiz *et al.*, 1997; Cohen & Carlton, 1998) and this is valid also for Europe (Paavola *et al.*, 2005). There are four main arguments to explain the high presence of alien species into estuaries (Nehring, 2006): i) intensive shipping that take place in estuaries makes them more vulnerable to invasion than other aquatic zones, ii) brackish water species have a better chance to be transported alive and finally established after release in comparison to other species due to specific characteristics (more salt tolerant when compared with freshwater or euhaline species), iii) brackish waters have limited number of native species (thus, more alien species can potentially establish their populations) and iv) salt-tolerant limnetic alien species could also reach estuaries and can be introduced into inland water.

Estuaries and coastal lagoons have an important role in marine biodiversity and productivity, because of their value as nursery areas for several marine species including commercially important fish and invertebrates (Beck *et al.*, 2001). Among these, the life cycle of shrimp species of the family Penaeidae is connected with estuaries and lagoons; their spawning and larval development take place in the sea, then the post-larvae are settled in estuaries and coastal lagoons where they spend their juvenile stage, and later, as subadults, migrate offshore and join the adult population [Pérez-Castañeda *et al.* (2010) and references therein].

The subphylum of Crustacea includes the most successful invaders among the aquatic alien species (Hänfling *et al.*, 2011). Among the alien species, Crustacea are the most numerous group not only in European freshwater ecosystems (Karatayev *et al.*, 2009) and brackish waters (Ranasinghe *et al.*, 2005) but also in marine coastal environments (Galil, 2008). Greece

is one of the EU countries with the highest number of reported marine alien species. Especially, Saronikos and Thermaikos Gulfs are very important sites for alien arthropods (Katsanevakis *et al.*, 2013).

The aim of this work was to study the shrimp species of the family Penaeidae in an estuary-lagoon ecosystem in Thermaikos Gulf (N Aegean Sea) and screen for possible existence of alien species. Thermaikos Gulf is along the navigation line to the port of Thessaloniki, the second largest port in Greece (more than 3000 ship arrivals per year) (Manousis *et al.*, 2012) and therefore a potential “hotspot” for invasion incidents. The species identification was based both on morphological and genetic characters.

## MATERIALS AND METHODS

### *Sampling*

Specimens of shrimps were collected from Papapouli and Papanika lagoons (SW Thermaikos Gulf, N Aegean – see Fig. 1). The total area of Papapouli lagoon is ~3000 acres and it belongs to an area designated as Wildlife Refuge. Papapouli lagoon is a choked lagoon and has two points of contact with the sea: one large central channel (30 m in length and 15-45 m in width) and one small channel (15-25 m in width). The surface of Papanika lagoon is ~272 acres and it is fed mainly by the waters of Peneios River and sources of Mount Olympus.

Samples of penaeid species were collected using appropriate traps for crustaceans (mesh size 2 cm) or hand net (mesh size 1 cm), during winter and spring in 2012 and were stored at –20 °C. Species identification was based on the taxonomic key of Pérez Farfante (1988).

### *DNA isolation, PCR amplification and DNA sequencing*

Twenty five mg of wet tissue were dissected from each individual (between carapace and 1<sup>st</sup> abdominal segment). DNA was extracted using the protocol of Nucleospin®Tissue «Genomic DNA from Tissue» (Macherey-Nagel, Germany). The 16S fragment was amplified using the primers 16sf-cray (5'-GACCGTGCK-AAGGTAGCATAATC-3') (<http://crandalllab.byu.edu>) and 1472 (5'-AGATAGAAACCAACCTGG-3') (Crandall & Fitzpatrick, 1996). PCR conditions were: 4 min at 92 °C, 35 cycles of 30 sec at 92 °C, 30 sec at 50 °C, 30 sec at 72 °C and a final extension of 5 min at 72 °C. Total reaction volumes (25 µl) consisted of 2.5 µl

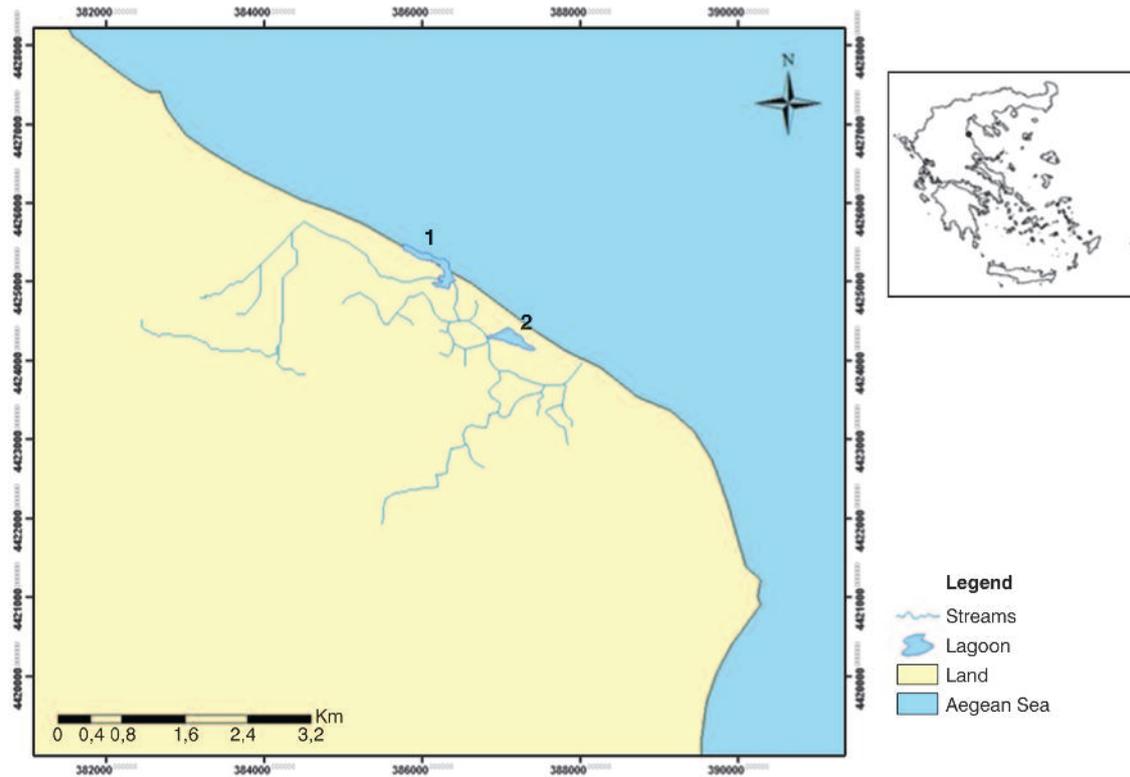


FIG. 1. Map of the sampling area. 1. Papapouli lagoon. 2. Papanika lagoon.

template DNA, 2.5  $\mu$ l  $\times$ 10 PCR buffer, 1 mM  $MgCl_2$ , 0.5 mM dNTPs (New England Biolabs, MA, USA), 0.25 mM of each primer and 0.25 U of Taq DNA polymerase (KAPA Taq, Kapa Biosystems, Cape Town, South Africa). All amplifications were performed on a Techgene (TECHNE, Cambridge, England) thermal cycler. Cycle sequencing products were electrophoresed in agarose gel (SeaKem®, Cambrex BioScience, Rockland Inc., USA) in 1.2% concentration. PCR products were purified using NucleoSpin extract kit (Macherey-Nagel, Germany); purified products were sent to VBC (Wien, Austria) for bidirectional sequencing (primers: 16sf-cray and 1472).

The returned sequences were checked manually (based on the corresponding chromatogram of each sequence) using BioEdit software (Hall, 1999). The identity of the returned sequences was confirmed by applying BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) on the basis of the detected similarities from other Penaeid representatives. The outcome of these searches gave us a hint regarding the species identity of the specimens (data not shown); three out of seven of the 16S rRNA sequences presented great similarities with already deposited sequences of *Melicertus kerathurus* while the rest of them showed great simi-

larities with already deposited sequences of *Farfantepenaeus aztecus*.

#### Data analysis

In total, seven sequences were obtained. These sequences were aligned using ClustalX 2.0.3 (Thompson *et al.*, 1997) with default parameters. The final dataset included 51 sequences of shrimp species (Table 1). Phylogenetic relationships were estimated with PAUP\* 4.0b10 (Swofford, 1998) using maximum parsimony (MP) and maximum likelihood (ML) methods. Also, phylogenetic analysis was implemented in BEAST v1.5.4 (Drummond & Rambaut, 2007) under Bayesian MCMC searches. The best-fit substitution model TVM + I + G (Posada & Crandall, 1998) was determined by Modeltest 3.7 (Posada & Crandall, 1998). The parameters of this model were: unequal base frequencies (A = 33.38%, C = 10.44%, G = 16.72%, T = 39.46%), number of substitution types  $N_{st} = 6$ ,  $p_{inv} = 0.39$ , and  $\alpha = 0.58$ .

For MP, trees were generated using heuristic searches with TBR (tree-bisection-reconnection) branch swapping; nodal support was assessed by 500 bootstrap replicates and gaps were treated as missing da-

TABLE 1. List of the sequences included in the final dataset

Abbreviation	GenBank Accession No	Species	References
faz1	HQ214010.1	<i>Farfantepenaeus aztecus</i>	Unpublished
faz2	HM014401.1	<i>Farfantepenaeus aztecus</i>	Alvarado Bremer et al. (2010)
faz3	AF279811.1	<i>Farfantepenaeus aztecus</i>	Lavery et al. (2004)
faz4	AF192052.1	<i>Farfantepenaeus aztecus</i>	Maggioni et al. (2001)
fbr1	HM014405.1	<i>Farfantepenaeus brasiliensis</i>	Alvarado Bremer et al. (2010)
fbr2	HM014404.1	<i>Farfantepenaeus brasiliensis</i>	Alvarado Bremer et al. (2010)
fbra3	HM014403.1	<i>Farfantepenaeus brasiliensis</i>	Alvarado Bremer et al. (2010)
fbra4	HM014402.1	<i>Farfantepenaeus brasiliensis</i>	Alvarado Bremer et al. (2010)
fd1	JF899810.1	<i>Farfantepenaeus duorarum</i>	Ma et al. (2011)
fd2	HM014406.1	<i>Farfantepenaeus duorarum</i>	Alvarado Bremer et al. (2010)
fd3	AF279812.1	<i>Farfantepenaeus duorarum</i>	Lavery et al. (2004)
fd4	AF192055.1	<i>Farfantepenaeus duorarum</i>	Maggioni et al. (2001)
fsu1	AF192076.1	<i>Farfantepenaeus subtilis</i>	Maggioni et al. (2001)
fsu2	AF192075.1	<i>Farfantepenaeus subtilis</i>	Maggioni et al. (2001)
fsu3	AF192074.1	<i>Farfantepenaeus subtilis</i>	Maggioni et al. (2001)
fsu4	AF192073.1	<i>Farfantepenaeus subtilis</i>	Maggioni et al. (2001)
fpa1	AF192060.1	<i>Farfantepenaeus paulensis</i>	Maggioni et al. (2001)
fpa2	AF192059.1	<i>Farfantepenaeus paulensis</i>	Maggioni et al. (2001)
fc1	NC_012738.1	<i>Farfantepenaeus californiensis</i>	Gutiérrez-Millán et al. (2002)
mke1	AF279826.1	<i>Melicertus kerathurus</i>	Lavery et al. (2004)
mke2	EU430761.1	<i>Melicertus kerathurus</i>	Zitari-Chatti et al. (2009)
mke3	EU430762.1	<i>Melicertus kerathurus</i>	Zitari-Chatti et al. (2009)
mke4	EU430760.1	<i>Melicertus kerathurus</i>	Zitari-Chatti et al. (2009)
plo1	JX196949.1	<i>Melicertus kerathurus</i>	Unpublished
plo2	JX196948.1	<i>Melicertus kerathurus</i>	Unpublished
plo3	JX089984.1	<i>Melicertus kerathurus</i>	Unpublished
pse2	EF589706.1	<i>Melicertus kerathurus</i>	Pascoal et al. (2008)
pse3	EF589705.1	<i>Melicertus kerathurus</i>	Pascoal et al. (2008)
mja1	AY853415.1	<i>Marsupenaeus japonicus</i>	Tsoi et al. (2005)
mja2	AY853414.1	<i>Marsupenaeus japonicus</i>	Tsoi et al. (2005)
mja3	AY853413.1	<i>Marsupenaeus japonicus</i>	Tsoi et al. (2005)
mja4	AY789492.1	<i>Marsupenaeus japonicus</i>	Tsoi et al. (2005)
mno1	JX196954.1	<i>Metapenaeus monoceros</i>	Unpublished
mno2	JX196953.1	<i>Metapenaeus monoceros</i>	Unpublished
mno3	JX196952.1	<i>Metapenaeus monoceros</i>	Unpublished
mno4	JX089983.1	<i>Metapenaeus monoceros</i>	Unpublished
pno1	AF279829.1	<i>Penaeus monodon</i>	Lavery et al. (2004)
pno2	HQ127457.1	<i>Penaeus monodon</i>	Unpublished
pno3	FJ435645.1	<i>Penaeus monodon</i>	Unpublished
pno4	AJ388113.1	<i>Penaeus monodon</i>	Michel-Salzat & Bouchon (2000)
sp1 (Papapouli lagoon)	KF953960	<i>Farfantepenaeus aztecus</i>	Present study
sp2 (Papapouli lagoon)	KF953961	<i>Farfantepenaeus aztecus</i>	Present study
sp3 (Papanika lagoon)	KF953962	<i>Farfantepenaeus aztecus</i>	Present study
sp4 (Papanika lagoon)	KF953963	<i>Farfantepenaeus aztecus</i>	Present study
sp5 (Papapouli lagoon)	KF953964	<i>Melicertus kerathurus</i>	Present study
sp6 (Papapouli lagoon)	KF953965	<i>Melicertus kerathurus</i>	Present study
sp7 (Papanika lagoon)	KF953966	<i>Melicertus kerathurus</i>	Present study
<i>OUTGROUP</i>			
pel1	HE573180.1	<i>Palaemon elegans</i>	Reuschel et al. (2010)
pel2	HE573179.1	<i>Palaemon elegans</i>	Reuschel et al. (2010)
pel3	HE573178.1	<i>Palaemon elegans</i>	Reuschel et al. (2010)
pser	JQ042291.1	<i>Palaemon serratus</i>	Cuesta et al. (2012)

ta. For ML (heuristic search; TBR branch swapping; 500 bootstrap replicates) the best-fit substitution model was employed (TVM + I + G), as determined previously.

For Bayesian analysis, BEAUti software was used in order to prepare the input file for BEAST v1.5.4 (Drummond & Rambaut, 2007). The following parameters were implemented: parameters of the evolutionary model were adjusted according to the values provided by the TVM + I + G model (see above) and an uncorrelated exponential relaxed clock was enforced while assuming a Yule prior on birth rate of new lineages (all other parameters were set to default). Two independent Markov Chain Monte Carlo analyses were run for 10 million generations (sampling every 1000, first 2000000 generations were discarded as burnin). Log parameters from each run were assessed using Tracer v1.5.0 (Drummond & Rambaut, 2007) in order to diagnose convergence and to summarize statistics (for each MCMC analyses separately). The Effective Sample Size (ESS) parameter was applied for checking the adequacy of mixing (values were higher than 200) (Drummond *et al.*, 2006). Data produced by the two independent runs were combined with LogCombiner v1.5.2 and TreeAnnotator v1.5.2 was used to find the best supported tree (2000 trees were treated as burnin). The representation and processing of the selected tree was made in FigTree v1.1.2 program.

## RESULTS AND DISCUSSION

Individuals collected from the two sites (as described in Materials and Methods section) were identified as *Farfantepenaeus aztecus* and *Melicertus kerathurus* (based on morphological characters). In total, 45 individuals were *F. aztecus* and 38 individuals belonged to *M. kerathurus*. It should be noticed that the individuals caught during this research effort were subadults or adults.

Four morphologically characterized *F. aztecus* and three *M. kerathurus* individuals were used for subsequent genetic analysis using 16S rRNA. The length of the aligned sequences in the final dataset was 420 bp. The phylogenetic trees from MP, ML and BI presented almost identical topology (with small differences located mainly in shallow branches). The phylogenetic tree inferred from BI is shown in Figure 2. Apart from the phylogroup comprised from outgroup sequences (pel1, pel2, pel3 and pser), two major clades were evident on the phylogenetic tree. The first one includes

species of the genus *Farfantepenaeus* while the second consists of species belonging to genera *Penaeus*, *Marsupenaeus*, *Melicertus*, *Parapenaeus* and *Metapenaeus*. The sequences sp1 and sp2 from Papapouli lagoon and the sequences sp3 and sp4 from Papanika lagoon were grouped with sequences of *F. aztecus* while the sequences sp5 and sp6 (from Papapouli lagoon) and the sequence sp7 (from Papanika lagoon) were assigned to *M. kerathurus* phylogroup. Genetic analysis confirmed the morphological identification of the species captured in the lagoons under investigation. Multidisciplinary approach (combining morphological and genetic approaches) for species identification was proposed and used by numerous scientists in recent years (e.g. Abatzopoulos *et al.*, 1998; Lucas *et al.*, 2002; Mura *et al.*, 2006; Moschandreu *et al.*, 2012). Especially for *F. aztecus*, which exhibits great morphological similarity with *F. duorarum* (Pérez Farfante, 1988), this approach allowed us to conclude upon the definite taxonomy of the individuals sampled from the two lagoons. It should be noticed that the genetic distance between *F. aztecus* and *F. duorarum* recorded in this study ( $0.58 \pm 0.02$ ) was similar to that reported by Maggioni *et al.* (2001) using the same genetic marker.

Although the presence of *M. kerathurus* individuals in the studied areas was expected, since it has been reported previously from N Aegean Sea (Karani *et al.*, 2005; Kevrekidis & Thessalou-Legaki, 2006), the confirmation of the presence of *F. aztecus* was striking. Until recently, the distribution of this species was constrained to the peninsula of Florida and the Gulf of Mexico, but there are reports of introduction of the species in French Polynesia and New Caledonia (Bartley, 2006). However, a recent invasion of *F. aztecus* into the E Mediterranean Basin has been documented by Deval *et al.* (2010), who reported the presence of *F. aztecus* in Antalya Bay (Eastern Mediterranean Sea) and Marković *et al.* (2014), who documented *F. aztecus* presence in Boka Kotorska Bay (S Adriatic Sea). In both reports, the sampled individuals were caught in the open sea, while in our study, the individuals were captured in lagoons. The presence of *F. aztecus* in the Papapouli and Papanika coastal lagoons was not a surprise, since lagoons are considered as nursery areas for penaeids (needed for completion of their life cycle). Therefore, effective monitoring and sustainable management plans for both lagoons are needed in order to maintain their capacity as nursery fields for selected species.

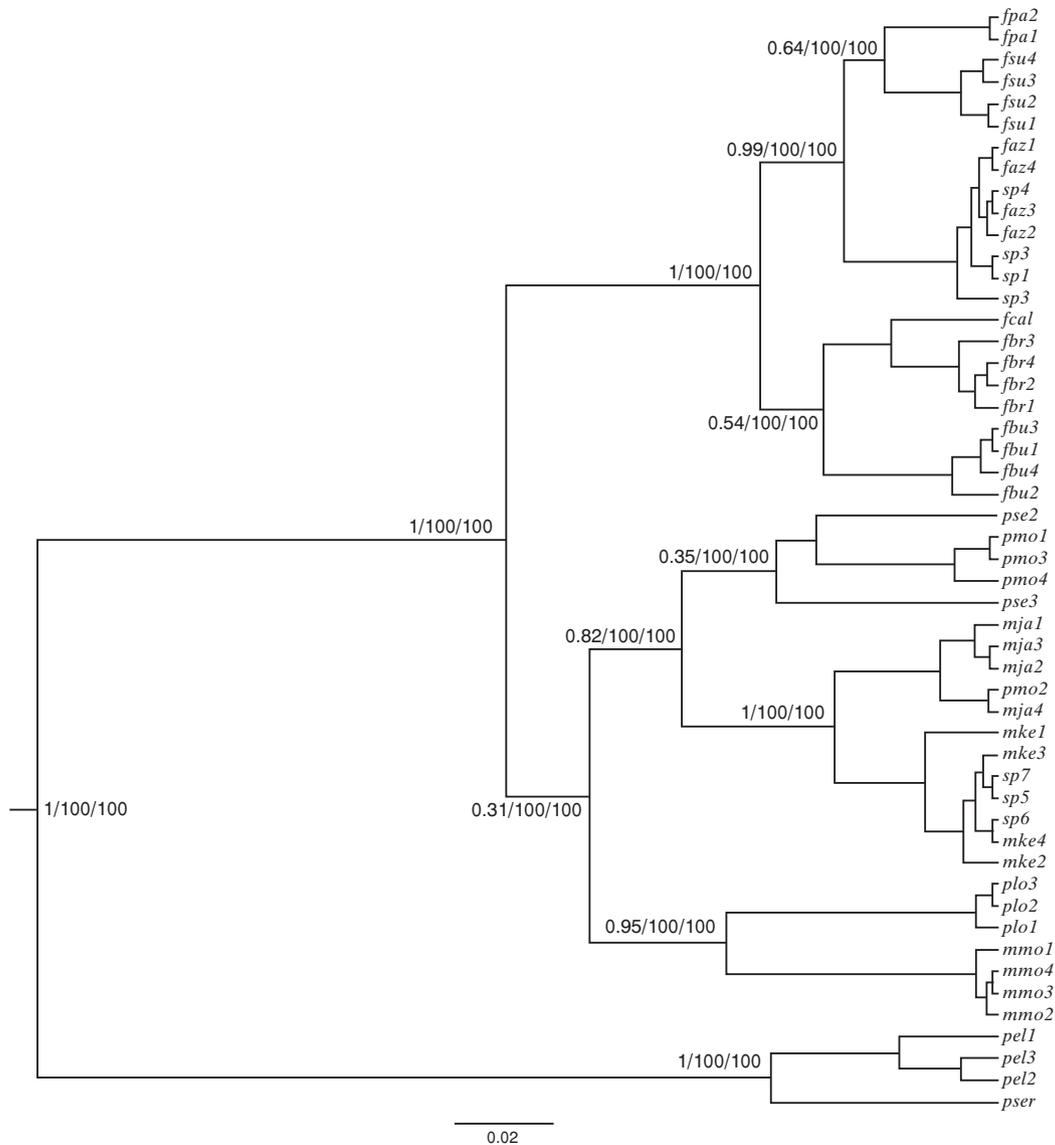


FIG. 2. Bayesian Inference (BI) topology of the 16S rRNA partial sequences. Nodal support (BI/ MP/ ML) was assessed by posterior probabilities (for BI) and bootstrap values (for MP and ML). BI/MP/ML values in shallow branches are not shown for clarity.

As Deval *et al.* (2010) have already pointed out, the most probable vector for *F. aztecus* introduction in the Mediterranean Sea is the ship ballast water, since this species is characterized by slow growth rate and it is not prioritized by aquaculture industry. Another case of a successful invader originated from W Atlantic is the crab *Percnon gibbesi*; this species was introduced via shipping or larval drift in the Mediterranean Basin and succeeded in establishing several populations (spread along the Mediterranean Sea) within almost a decade (Katsanevakis *et al.*, 2012). Zenetos *et al.* (2009) mentioned that from the 202 alien marine species recorded to Greek seas, 7% has come from transport ships. In the inventory of Corsi-

ni-Foka & Pancucci-Papadopoulou (2012) regarding the Crustacea Decapoda from Eastern Mediterranean Sea (Rhodes island), there is no reference of the presence of *F. aztecus*. Based on the above, we may deduce that the two recorded invasion incidents of *F. aztecus* [the first in Antalya Bay (Deval *et al.*, 2010) and the second one in N Aegean Sea (current study)] are the results of two distinct events. The pattern of progressive dispersal (from the southern Turkish coast towards the Greek Aegean coasts), as it was suggested for other alien species by Tzomos *et al.* (2012), should have required longer period of time than the 2-3 years lapsed from the first report of *F. aztecus* in the Eastern Mediterranean and our study.

The current effort is the first multidisciplinary approach (based on genetic and morphological characters) for identifying the invasive species of *F. aztecus* and it confirmed the presence of this species in the Eastern Mediterranean Basin. Also, the capturing of subadults and adults indicates that the species has successfully been established in the area [as it was already proposed by Marković *et al.* (2014)]. In order to evaluate the population dynamic parameters and investigate whether *F. aztecus* population has the potential for commercial exploitation, further experimentation is needed.

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