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SHORT COMMUNICATION

Foul play? On the rapid spread of the brown shrimp *Penaeus aztecus* Ives, 1891 (Crustacea, Decapoda, Penaeidae) in the Mediterranean, with new records from the Gulf of Lion and the southern Levant

Bella S. Galil[1] · **Gianna Innocenti**[2] · **Jacob Douek**[1] · **Guy Paz**[1] · **Baruch Rinkevich**[1]

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Abstract Specimens of the penaeid shrimp *Penaeus aztecus*, a West Atlantic species, were collected off Le Grau du Roi, Gulf of Lion, France, and off the Israeli coast, Levant Basin, Mediterranean Sea. This alien species has been previously recorded off Turkey, Greece, Montenegro and the Tyrrhenian coast of Italy. The species identity was confirmed based on morphological characters and by sequencing 406 nucleotides of the 16S RNA gene and 607 nucleotides of the COI. The 16S rRNA sequences of the specimens collected in Israel, France and Italy were identical, and exhibited three different COI haplotypes. The near-concurrent records from distant locations in the Mediterranean put paid to the premise that *P. aztecus* was introduced into the Mediterranean Sea in ballast waters. A more prudent proposition is that many of these populations issue from illegal introductions. Potential risks to native biodiversity and economic value are the likely competition with commercially important native prawns, co-introduction of pathogens and parasites, and risk of infecting penaeid populations elsewhere in the Mediterranean Sea with Erythraean alien disease agents previously limited to the Levant.

Keywords *Penaeus aztecus* · Gulf of Lion · Levant Basin · New records · Illegal introduction · Parasite

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✉ Bella S. Galil
bella@ocean.org.il

[1] Israel Oceanographic & Limnological Research, National Institute of Oceanography, POB 8030, Haifa 31080, Israel

[2] Museo di Storia Naturale, Universita di Firenze, via Romana 17, 50125 Firenze, Italy

Introduction

Penaeus aztecus Ives, 1891, is native to the Western Atlantic, from Massachusetts, USA, to Yucatan, Mexico (Cook and Lindner 1970). Penaeid shrimps are a valuable fishing resource and *P. aztecus* is no exception. In the Gulf of Mexico, the species supports important fisheries both in the USA and in México (Velazquez and Gracia 2000). Landings in 2015 in the USA alone were estimated at 23,000 tons (NOAA 2015). In order to supplement wild catch and reduce fishing pressures on wild populations, in particular to decrease the trawling activity conducted in estuaries, nursery protocols for the rearing of *P. aztecus* were developed (Mays et al. 2006).

The first record of *P. aztecus* in the Mediterranean Sea was collected off Antalya, Turkey, in December 2009 (Deval et al. 2010, as *Farfantepenaeus aztecus*). Soon after, it was collected from trawl catches along the Mediterranean coast of Turkey from Iskenderun to Finike (Gökoğlu and Övzarol 2013; Övzarol and Gökoğlu 2014). Subsequently, it was reported from the Aegean Sea (Nikolopoulou et al. 2013; Kevrekidis 2014; Kondylatos and Corsini-Foka 2015; Minos et al. 2015), off Corfu, Ionian Sea (Kapiris and Apostolidis 2014), Montenegro, Adriatic Sea (Marković et al. 2013), and Tyrrhenian Sea (Cruscanti et al. 2015).

In 2015, additional records of *P. aztecus* were collected at two widely separated locations in the Mediterranean Sea, the Gulf of Lion and the southern Levantine Basin. These records, each collected in the vicinity of fish and shellfish farms, raise the suspicion that *P. aztecus* was introduced and transferred illegally.

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Materials and methods

Sampling

On April 30, 2015, a single male specimen was found in the haul of RV *Shikmona*, trawled at a depth of 36 m, near Palmahim (31°54.973N, 34°38.091E), Israel (Fig. 1a–c). The specimen (total length, rostrum to telson, 142 mm) was photographed, and deposited in the crustacean collection of the Steinhardt National Collections of Natural History, Tel Aviv University, Israel (TAU AR29623). The barcoding pages for this *P. aztecus* specimen were uploaded to the BOLD website (http://v4.boldsystems.org with BOLD ID: BIM-AP 020 and to the IOLR website (israMAR.ocean.org.il/IsraelBarcoding/BarcodingDef.aspx). On October 5, 2015, a single female specimen was found in the haul of M/P *Maline*, off Le Grau du Roi, Gulf of Lion, France. The specimen (total length 165 mm) was photographed, and deposited in the crustacean collection of the Natural History Museum of Florence University (MZUF 4411). Identification followed Pérez-Farfante (1988). A pereiopod was detached from each specimen for molecular analysis.

A pleopod from the single male specimen (total length 135 mm; MZUF 4303) collected in the Follonica Gulf (42°51,146'N 10°39,000'E) on muddy bottom, at depth of 40 m on November 4, 2014 (Cruscanti et al. 2015), was also analysed.

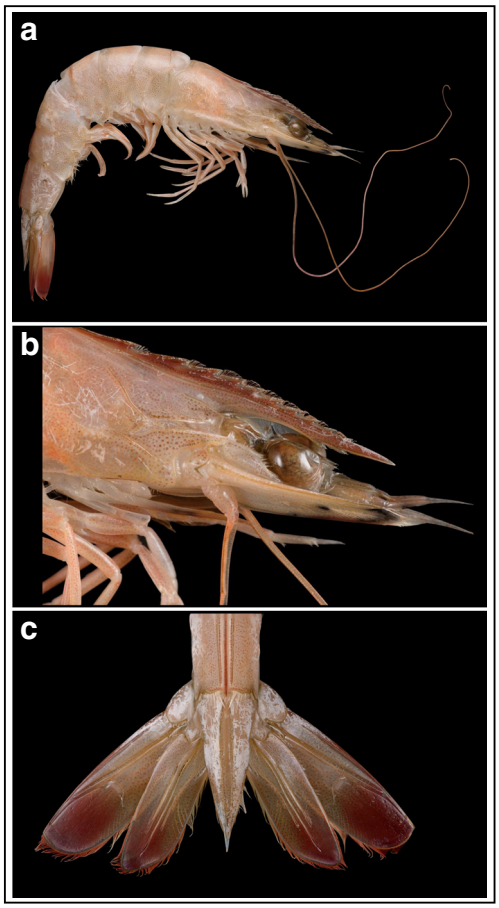

Fig. 1 *Penaeus aztecus* Ives, 1891. Palmahim, Israel, April 30, 2015. Photo: O. Rittner. **a** Lateral view; **b** carapace, lateral view; **c** last abdominal somite and telson, dorsal view

Molecular analysis

DNA extraction

DNA was extracted from the muscles of the pereiopods and the pleopod of 3 specimens collected in Italy (Cruscanti et al. 2015), Israel and France (present article) were placed in 1.5-ml tubes with 300 μl lysis buffer containing 0.25 M Tris-borate pH 8.2, 0.1 M EDTA, 2 % SDS, 0.1 M NaCl and 1 M of $NaClO_4$. An equal volume of phenol/chloroform/isoamyl-alcohol (25:24:1) was added to the tubes, mixed vigorously by vortex for 1–5 min and centrifuged for 10 min at 14,000*g* (4 °C). Then, the aqueous phase was transferred into a new tube and further extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The DNA was precipitated with absolute ethanol, washed twice with 70 % ethanol, dried and resuspended in 50 μl of RNAse/DNAse-free double-distilled water.

PCR amplification

The molecular identification of the *P. aztecus* was based on the amplification of two mitochondrial genes, the cytochrome C oxidase subunit I (COI) and the 3' end of the 16S rRNA mitochondrial gene. For the PCR reactions, 2 μl of diluted DNA (1:50) were added to PCR reaction mixture in a total solution volume of 50 μl containing 25 μl PCR Master Mix (2×; kt201; Tiangen Biotech, Beijing, China) and 0.4 μM of each forward and reveres primers, as follows: COI marine invertebrates' universal primers HCO2198, (TAAACTTCAGGGTGACCAAAAAATCA) and LCO1490, (GGTCAACAAATCATAAAGATATTGG; Folmer et al. 1994) were used for the amplification of the COI. Following Simon et al. (1994), primers 16Sar (CGCCTGTTTATCAAAAACAT) and 16Sbr (CCGGTCTGAACTCAGATCACGT) were used to amplify the 3' end of the 16S rRNA mitochondrial gene. The reaction conditions for both sets of primers were as follows: 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 45 °C or 50 °C (COI or 16S, respectively) for 1 min and 72 °C for 1 min and an additional elongation step of 72 °C for 10 min. The PCR products were screened on 1.2 % agarose gel. Direct sequencing of the PCR products was done using the same PCR primers (Macrogen, South Korea).

Sequence analyses

Forward and reverse sequencing results were aligned, corrected according to the trace file, and trimmed using BioEdit (Hall 1999) and DNA baser 4.12.0 (DNA Baser Sequence Assembler v4 [2013], Heracle BioSoft, www.DnaBaser.com). The COI sequences were compared to the "Barcode of Life Data Systems" (BOLD) Identification System (http://www.boldsystems.org/views/idrequest.php) and to the GeneBank database using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16S ribosomal DNA sequences were compared only to the NCBI database and the most similar sequences of the 16S sequences were selected for further alignment using BioEdit (Hall 1999) and ClustalX (Thompson et al. 1997). The phylogenetic relationships tree of the COI gene was drown by the BOLD system (using Kimura 2 Parameter as distance model). The phylogenetic relationship tree for the 16S sequences was drawn using the Maximum Likelihood method based on the Tamura 3-parameter (T92) model with MEGA6 software (Tamura et al. 2004).

Results

Molecular results

The 16S rRNA sequences (406 bp) of *P. aztecus* specimens collected in Israel, France and Italy were identical. Comparison of the Israeli sequence with the NCBI nucleotide database resulted in 100 % identity to eight 16S rRNA sequences of *P. aztecus* (two sequences archived as *P. duorarum* Burkenroad, 1939 are erroneous identifications of *P. aztecus*, *fide* Sammy De Grave, Oxford University Natural History Museum) (Table 1; Fig. 2a). The *P. aztecus* 16S rRNA sequence revealed 96 % identity to *Penaeus subtilis* (Pérez Farfante, 1967) 16S rRNA, used as an outgroup for the maximum likelihood tree analysis (Fig. 2a).

The COI sequence (607 bp) of the Israeli *P. aztecus* specimen (AP-20) differed by a single nucleotide substitution from either of the specimens collected in France (AZT1) and Italy (AZT3). AZT1 differed from AZT3 by two nucleotides (Fig. 2b), altogether revealing 3 COI haplotypes. The Israeli sample matched (100 % identity) the BOLD database samples of *P. aztecus* from Virginia ($n = 3$) and Maryland (n = 1), United States, and showed 99.84 % identity to *P. aztecus* samples collected at Nuevo Leon, Mexico ($n = 1$) and Maryland ($n = 1$) (available February 5, 2016). The nucleotide sequences of all BOLD records are unavailable to the public at present and the similarity outcomes were created by the neighbor-joining tree based on Kimura 2 Parameter as distance model (Fig. 2c), provided by the BOLD system. Comparisons with the NCBI nucleotide data base revealed no match with *P. aztecus* COI sequences, nor any sequence identity above 91 %.

Accession numbers of 16S and COI are as follows: AP_20_S16 KU958159, AZT_1_S16 KU958160, AZT_3_S16 KU958161, AP_20_COI KU958162, AZT_1_COI KU958163, and AZT_3_COI KU958164.

Discussion

Within an amazingly short period (December 2009–October 2015) *P. aztecus* has been recorded clear across the Mediterranean Sea, from the southeastern Levant to the Gulf of Lion at its northeastern corner (Fig. 3).

Mark–recapture experiments in the northwestern Gulf of Mexico indicated that *P. aztecus* spread parallel to the

Table 1 *P. aztecus* 16S rRNA documentations from the NCBI database which revealed 100 % identity to the Israeli sample and the *P. subtilis* (the outgroup) details

NCBI ID	Species	Location	Reference
AP-20	*P. aztecus*	Israel	This study
AZT1	*P. aztecus*	France	This study
AZT3	*P. aztecus*	Italy	This study
AF192051	*P. aztecus*	Charleston Harbor, SC, USA	Maggioni et al. (2001)
AF279811	*P. aztecus*	Gulf of Mexico, USA	Lavery et al. (2004)
FJ943438	*P. aztecus*	Bocas Province, Panama	Bracken et al. (2009)
HM014401	*P. aztecus*	Galveston Bay, TX, USA	Bremer et al. (2010)
HQ214010	*P. aztecus*	Gulf Breeze, FL, USA	Payne (2010)
JX403846	*P. aztecus*	Bocas Province, Panama	Bracken-Grissom et al. (2012)
KF953960	*P. aztecus*	Thermaikos Gulf, Aegean Sea, Greece	Nikolopoulou et al. (2013)
KF953961			
KF953963			
KF983532	*P. aztecus*	Thermaikos Gulf, Aegean Sea, Greece	Minos et al. (2015)
AF19206	*P. subtilis*	Fortaleza, Brazil	Maggioni et al. (2001)

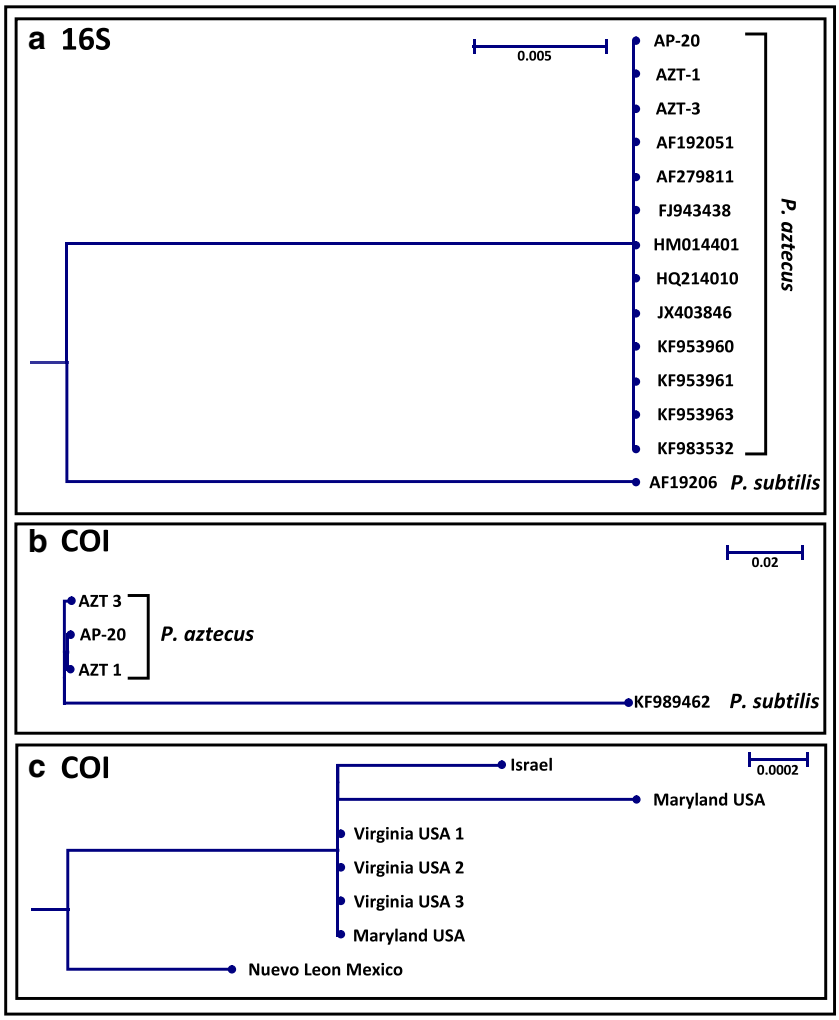

Fig. 2 Phylogenetic relationship trees for the COI and 16S sequences of the three *Penaeus aztecus* samples: **a** comparison of the 16S sequences to the NCBI database including *P. subtilis* as outgroup; **b** COI gene: neighbor-joining comparison between the three haplotypes and *P. subtilis* as outgroups; **c** tree provided by the BOLD system for available COI sequences

coastline at depths between 16 and 30 fms (29–55 m) with minimal offshore movement (Klima 1963). Most of the individuals (90% off Texas, 98 % off Louisiana) remained within 30 miles (48 km) of their release sites. Even individuals collected more than 180 days after their release had traveled only an average of 17 miles (27 km), though the greatest distance recorded was 195 miles (313 km). Of the 6947 *P. aztecus* individuals released in Pamlico Sound, North Carolina, 1061 were captured (McCoy 1968). The average interval between release and recapture was 12 days and the average distance

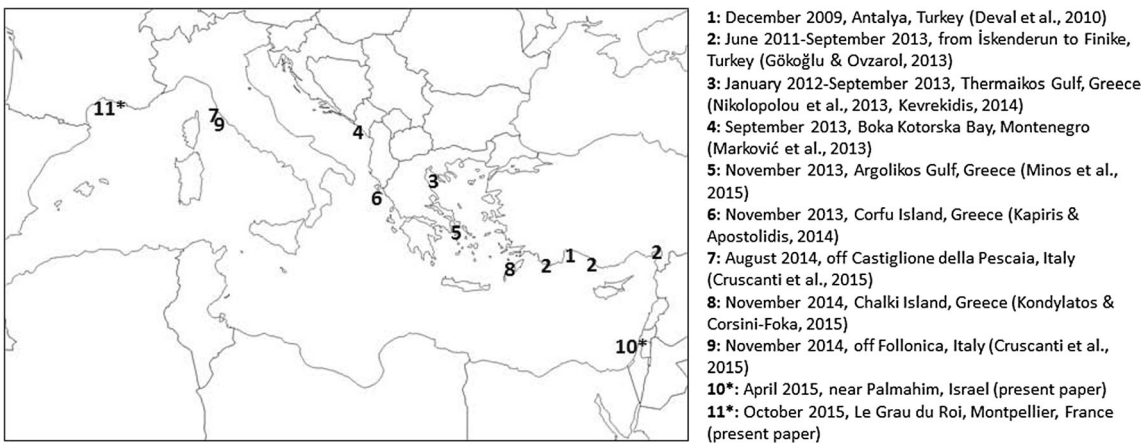

Fig. 3 Records of *Penaeus aztecus* Ives, 1891 in the Mediterranean Sea

traveled was 3 miles (5 km). Only a single individual was recaptured in the adjacent Atlantic Ocean, 47 days and 37 miles (59 km) from its release site.

Several authors (Deval et al. 2010; Nikolopoulou et al. 2013; Kevrekidis 2014; Minos et al. 2015) maintain that ballast waters is the most likely vector for the introduction *P. aztecus* in the Mediterranean Sea. Nikolopoulou et al. (2013) consider that the 'invasion incidents' in Antalya Bay, on the southern coast of Turkey, and in lagoons on the north Aegean coast of Greece, 'are the result of two distinct events'. Yet, the contemporaneous or near contemporaneous records from distant locations in the Mediterranean (see also Minos et al. 2015) strain credulity in this argument. Bearing in mind the limited movement of *P. aztecus* individuals revealed in experiments conducted in its native range (above), it is likely that the newly arrived propagules would need a longer period to disperse progressively along the coastline from the southern Turkish coast to the Ionian, Adriatic, Tyrrhenian and the Gulf of Lion. A more prudent proposition is that many of the Mediterranean populations issue from illegal introductions (Cruscanti et al. 2015). Antalya, Thermaikos and Argolikos gulfs are used for fish and shellfish farming, as well as the area of Castiglione della Pescaia and Follonica, Tyrrhenian coast of Italy, and the entire area surrounding Le Grau du Roi, where the last recorded specimen was collected.

As in previous cases of illegal importation of penaeid prawns–*P. pulchricaudatus* Stebbing, 1914 from Turkey into Italy, *P. merguiensis* de Man, 1888 and *Metapenaeus affinis* (H. Milne Edwards, 1837) into Turkey (Özcan et al. 2006; Aydin et al. 2009)–the introduction of *P. aztecus* had not been reported to the Competent Authorities. The latter species population flourished to the point it is commercially fished by trammel and gill nets in the Bay of Izmir (Aydin et al. 2013). Likewise, shortly after *P. aztecus* was first recorded in the Gulf of Antalya, large quantities were fished there by bottom trawling and trammel nets (Gökoğlu and Özvarol 2013). Kevrekidis (2014) voiced concern over the potential impacts of *P. aztecus* once its population increases to the point where it competes with the commercially important native prawn *Melicertus kerathurus* (Forskål, 1775).

Another potential significant risk to native biodiversity and economic value is the likely co-introduction of pathogens and parasites. The spread of diseases through stock movements has been particularly prevalent in penaeid shrimp and has caused significant declines in production (WB/NACA/WWF/FAO 2001). Indeed, a variety of pathogens were detected in *P. aztecus*, indicating high risk of introduction (LeBlanc et al. 1991; Bortolini-Rosales et al. 2002; Canning et al. 2002; Chávez-Sánchez et al. 2002; Payne 2010). Interestingly, an introduced bopyrid isopod, *Epipenaeon ingens* Nobili, 1906, has infested *P. aztecus* in Antalya Gulf (Korun et al. 2013). The species, native to the Indo-West-Pacific Ocean (Rajkumar et al. 2011), is a promiscuous parasite, infesting 11 penaeid host species (An et al. 2014). *Epipenaeon ingens* is known to infect *P. semisulcatus* De Haan, 1844 and *P. pulchricaudatus* in the Red Sea and the Gulf of Suez (Nobili 1906, as *Penaeus ashiaka* Kishinouye, 1900, Monod 1933, as *P.* aff. *japonicus*, respectively), whence it was introduced with its hosts through the Suez Canal like hundreds of Erythraean aliens (Galil et al. 2016). It was recorded infesting *P. semisulcatus* in the Bay of Mersin, Turkey (Bourdon 1968), as well as *P. semisulcatus* reared from broodstock collected in the Mediterranean coast of Israel in the late 1970s (T. Samocha, personal communication). Since Erythraean alien populations of *P. semisulcatus* are limited to the Levant and the Gulf of Taranto, Italy, addition of a widely spread host in *P. aztecus* increases the risk of infecting penaeid populations elsewhere in the Mediterranean Sea.

The specimens available to us (Israel, Italy, France) exhibited three different COI haplotypes. While COI sequences of *P. aztecus* may be useful for tracing the origin of the Mediterranean populations, to this end additional DNA samples from both its native and introduced range are required.

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