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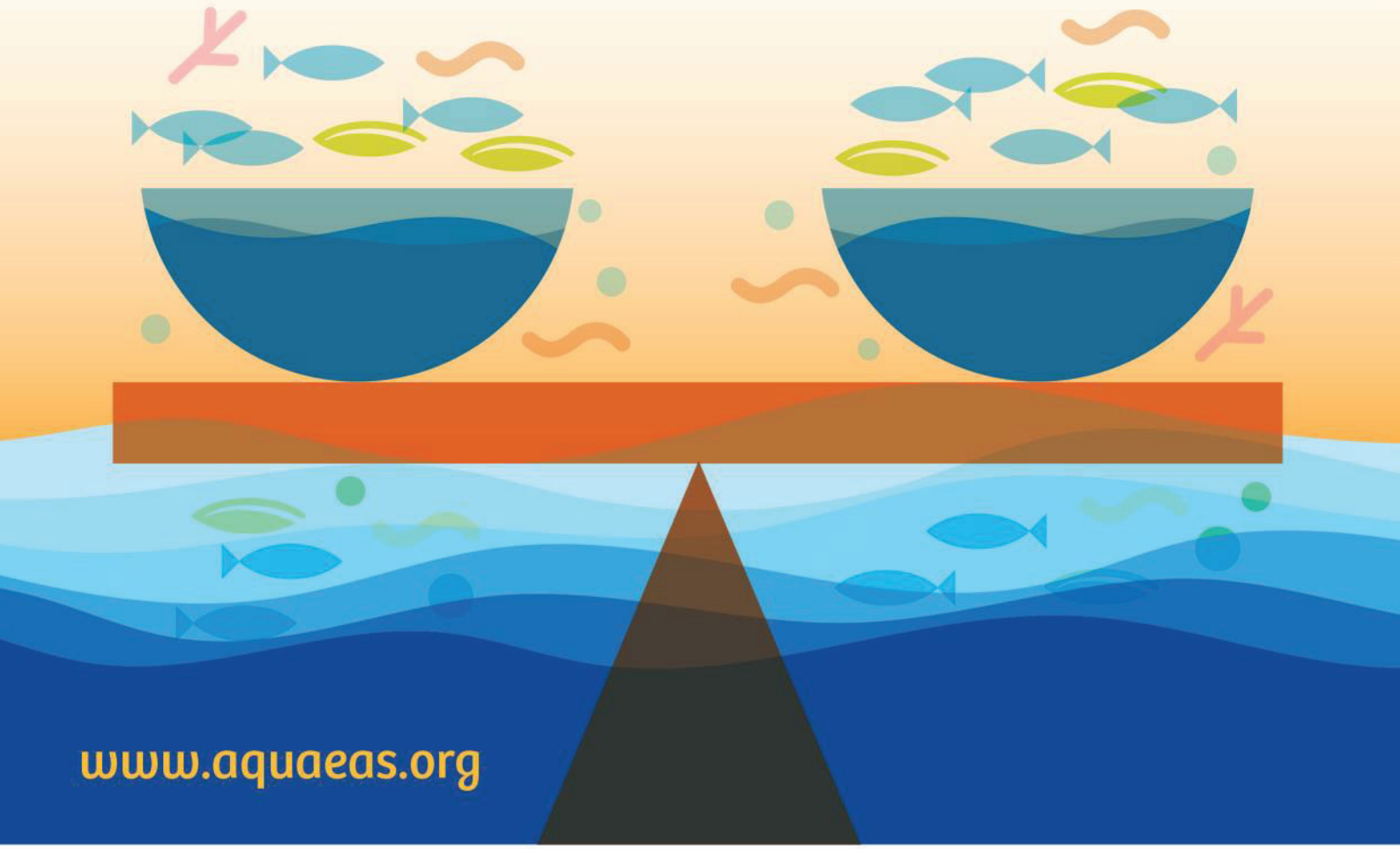

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PHYLOGENETIC RELATIONS OF INVASIVE ASCIDIANS IN GREEK MUSSEL FARMS

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Introduction

Ascidians (Ascidiacea) are marine invertebrate filter feeders with high spatial activity and are ranked among the most important biofoulants (Aldred & Clare 2014). Biofouling (i.e., the dynamic process of attachment, accumulation and development of elements of aquatic flora and fauna on any natural or artificial surface) of ascidians in anthropogenic facilities (e.g., aquaculture), often has detrimental economic and ecological impacts (Tsotsios et al. 2023). Especially in shellfish aquaculture (e.g., in mussel farming), the effects of these invasive species are greater than in other forms of mariculture, as the farmed organism itself acts as a substrate for the biofoulants to settle, creating operational problems in production (Tsotsios et al. 2023). Within Greek marine areas, 75 species of ascidians have been recorded (Antoniadou et al. 2016). Most of them originated from Atlantic, whereas a gradual increase in records of invasive species from the Indian Ocean has been observed (Antoniadou et al. 2016).

Farmers consider ascidian biofouling a major etiological agent of detrimental effects for the viability of Greek shellfish aquaculture (Tsotsios et al. 2023). However, whilst ascidians' genetic structure elucidation is crucial towards biofoulants' proper management, such knowledge is not available for Greek marine areas. The aim of this study is to provide the first insights regarding the identification and phylogeny of biofouling ascidian species from Greek mussel culture facilities, using sequence analysis of both mitochondrial and nuclear DNA barcode markers.

Material and Methods

A total of 77 ascidian specimens were collected from four aquaculture mussel farms located in the Aegean and Ionian Seas. Genomic DNA was extracted using the Nucleospin® Tissue Kit (Macherey-Nagel) according to the manufacturer's protocol. Cytochrome oxidase subunit I (COI) and 18S rDNA gene fragments were amplified using primer sets LCO1490 and HCO2198 (Folmer et al. 1994) and 18S1 and 18S4 (Tsagkogeorga et al. 2009), respectively. Polymerase Chain Reactions (PCRs) contained 1X Kapa Taq buffer, 1.5 – 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μM for COI and 0.4 μM for 18S rDNA of each primer, 1 U Kapa Taq (Kapa Biosystems) and ca. 20 ng of DNA template, in a total volume of 20 μl. PCR cycling conditions consisted of an initial denaturation step at 94 °C for 3 min (COI) or 4 min (18S), followed by 37 cycles of 30 sec (COI) or 40 sec (18S) at 94 °C, 1 min at 48 °C (COI) or 40 sec at 50 °C (18S), and 1 min at 72 °C, with a final elongation step at 72 °C for 10 min. Amplified products were purified using the commercially available NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel) and sequenced on an AB3500 genetic analyzer (Applied Biosystems).

Blast algorithm was employed for species identification of the studied individuals. Manual editing of newly acquired sequences was performed using SeqMan II software (DNASTAR). Clustal Omega was used for the multiple sequence alignment of the COI and 18S rDNA datasets. Individual data sets of COI and 18S rDNA were finally concatenated for a total evidence analysis. For phylogenetic reconstruction of the combined dataset, Bayesian Inference analysis was performed using MrBayes (v. 3.1.2) while the best-fit model according to the Akaike Information Criterion (AIC) was determined by jModelTest software (v. 0.1.1).

Results &Discussion

All individuals were successfully identified at the species level, using both COI and 18S rDNA gene fragments, (Table 1).

Following manual editing and multiple alignment the final dataset comprised 1472 nucleotides (590 for COI and 882 for 18S rDNA). Bayesian Inference (BI) analysis, based on AIC TPM1uf+G model, resulted in a dendrogram with four distinct and well supported clades (1.00), corresponding to the species level (Fig. 1). The species orders formed three robust monophyletic clades following the phylogenetic relationships within the subphylum of Tunicates (Delsuc et al. 2018). Stolidobranchia included the Styelidae (*Styela plicata*) and Pyuridae (*Microcosmus squamiger*) families, Aplousobranchia the Clavelinidae (*Clavelina oblonga*) and Phlebobranchia the Ascidiidae (*Phallusia mammillata*) families (Fig. 1).

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Phylogenetics and population genetic diversity of invasive ascidians have been sporadically evaluated in eastern Mediterranean based on various molecular markers (e.g., Maltagliati et al. 2014). The present study is the first attempt to genetically identify and elucidate the phylogeny of biofouling ascidians invading Greek mussel farming facilities. Investigation of the spread and genetic composition of non-indigenous-cryptogenic ascidians and practices to deal with infestation in mussel farms is necessary to assess the magnitude of the problem in production activities.

Acknowledgments

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