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A new genetic lineage of *Asparagopsis taxiformis* (Rhodophyta) in the Mediterranean Sea: As the DNA barcoding indicates a recent Lessepsian introduction

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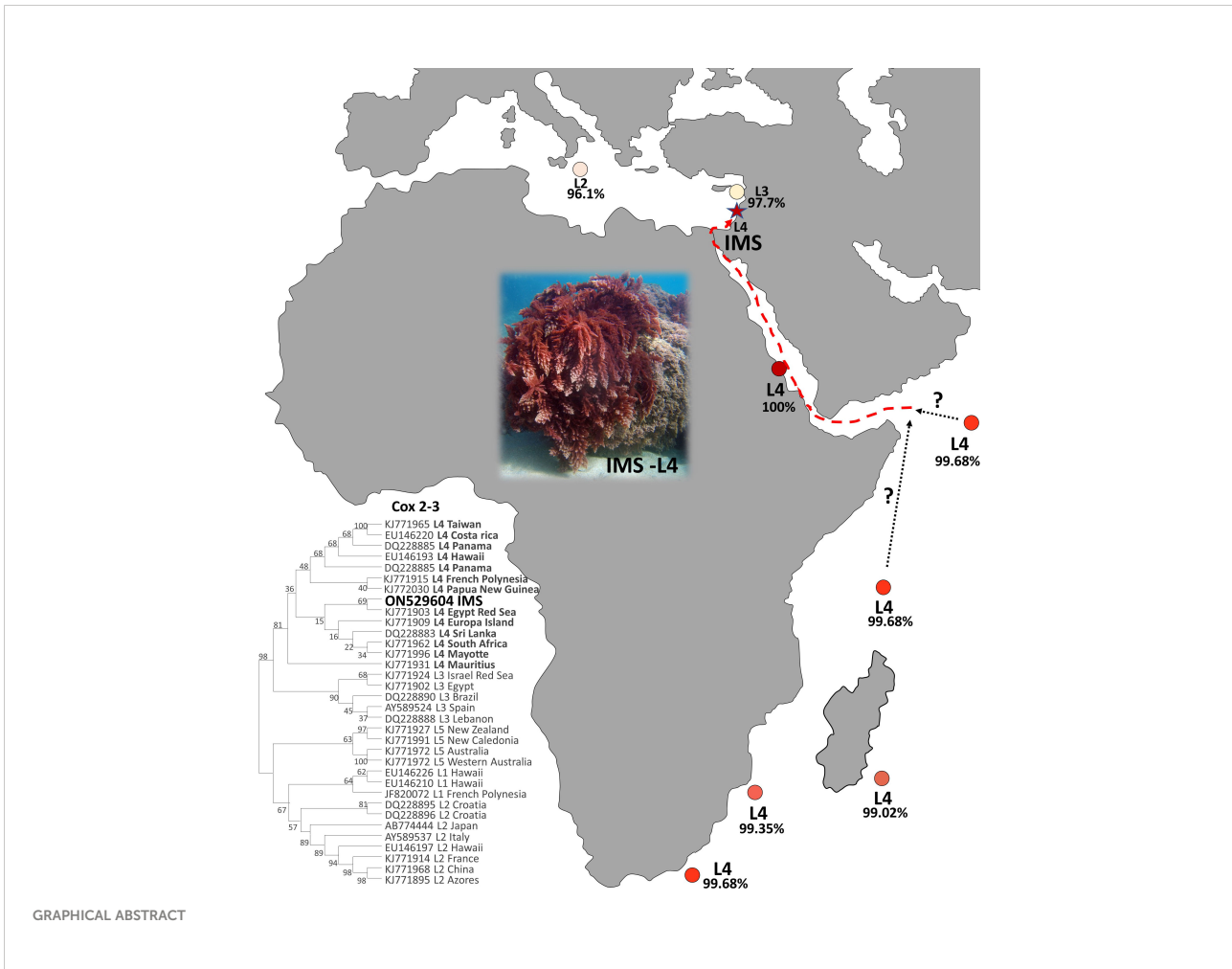
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Asparagopsis taxiformis (Delile) Trevisan is a red marine macroalga (Bonnemaisoniales, Rhodophyta) with high invasive potential and broad worldwide distribution. In the Mediterranean Sea, *A. taxiformis* was reported before the opening of the Suez Canal and is comprised of two different cryptic lineages, named L2 and L3. As for the Israeli Mediterranean Sea (IMS), *A. taxiformis* benthic populations have seemingly expanded with several large seasonal blooms recorded in recent years. However, neither ecology nor molecular substantial studies have been conducted for this particular geographical area. Increasing sampling intensity and geographical coverage may reveal new lineages or indicate human-mediated spread routes not only for *A. taxiformis* but for macroalgae in general. This approach is particularly important in areas such as the eastern Mediterranean Sea, which experiences intense biological invasion on a global scale. In this study, randomly samples specimens ($n = 30$) of *A. taxiformis* and preserved herbarium samples ($n = 4$) collected from the IMS in the past, were all barcoded and taxonomically identified using three molecular genetic markers (LSU, *cox2-3* spacer, and *rbcl*). We found a cryptic lineage 4 (L4) of *A. taxiformis* first reported here for the Mediterranean Sea, and previously described for the western Indo-Pacific and Hawaii. Herbarium samples confirmed the presence of L4 as early as 2013. Comparative assessment of *cox2-3* spacer marker indicates 100% similarity to sequenced L4 samples from Egypt in the Red Sea. The IMS *cox2-3* spacer sequences differed from previously sequenced samples from the Mediterranean Sea by 2.3% and 3.9% bp, compared to L3 and L2 Mediterranean populations, respectively. Morphological inspections indicate monoecious L4

gametophytes which are larger than the L4 population reported previously from Hawaii. Altogether, our results strongly indicate a Lessepsian migration route for *A. taxiformis* L4 with yet unknown consequences for the local marine ecosystems.

KEYWORDS

Asparagopsis taxiformis, *cox2-3* spacer, Lessepsian migration, Mediterranean Sea, seaweed



Introduction

Marine coastal habitats in the Mediterranean Sea are severely biologically invaded ecosystems (Boudouresque and Verlaque, 2002). This is largely due to human-mediated introduction of non-native species through various vectors such as ballast waters of ships, regional aquaculture activities, and by natural dispersal *via* the Suez Canal (Lessepsian invasion) (FD, 1978; Grosholz, 2002; Rilov and Crooks, 2009; Katsanevakis et al., 2014). The total number of non-indigenous seaweed species (NISS) reported for the whole Mediterranean Sea is somewhat variable, which is not

surprising given the complex cryptic nature of some of the species involved. The date of introduction and total numbers are subject to conjecture; nonetheless, agreed estimates may vary from as high as 148 species (as calculated for the year 2022 using the suggested 2–3 new immigrants per year by Zenetos et al. (2012), down to 118 species (Verlaque et al., 2015). The arrival of NISS is more intense in the eastern Mediterranean Sea since there is a consensus that the major vector of the introduction of macroalgae is of Lessepsian origin (Boudouresque and Verlaque, 2005; Zenetos, 2010; Zenetos et al., 2012; Verlaque et al., 2015; Romero et al., 2016).

In the last decades, the Israeli Mediterranean coast has become the host of several NISS, primarily in subtidal areas, and witnessed intense onshore drifts (Israel et al., 2019). Examples are the green alga *Codium parvulum* (Bory de Saint-Vincent) P.C.Silva (Israel et al., 2010), the red alga *Galaxaura rugosa* (J.Ellis & Solander) J.V.Lamouroux (Hoffman et al., 2008), the brown algae *Styopodium schimperi* (Kützinger) Verlaque & Boudouresque (Verlaque and Boudouresque, 1991; Einav and Israel, 2009), *Dictyota* sp. (unpublished observations) and *Lobophora lessepsiana* C.W.Vieira (Vieira et al., 2019), and many others which are unaccounted for. In this context, the red alga *Asparagopsis taxiformis* (Delile) Trevisan, allegedly introduced into the Mediterranean Sea in 1831 (Verlaque et al., 2015) was hardly noticeable in the Israeli shores until about a decade ago. *A. taxiformis* is highly invasive and has therefore been underpinned as one of the 'worst invasive alien species threatening marine biodiversity in Europe' (EEA, 2007), and further listed within the 100 'worst invasive seaweeds in the Mediterranean Sea' (Streftaris and Zenetos, 2006).

A. taxiformis is regarded as a cryptic complex with high diversity and cosmopolitan distribution from warm-temperate to tropical marine environments (Harvey, 1849; Price et al., 1986; Boni and Hawkes, 1987; Silva et al., 1996; Zanolta et al., 2022). The heteromorphic life cycle of *A. taxiformis* includes the erect gametophyte and a filamentous tetrasporophyte (Mairh, 1977; Guiry and Dawes, 1992). The gametophytic stage is highly branched, colored pink to red reaching up to 40 cm tall and commonly occurs on rocky substrates, or as epiphytes (Rojas et al., 1982; Boni and Hawkes, 1987). The tetrasporophytic stage, mistakenly referred in the past as a different species (*Falkenbergia hillebrandii* (Bornet) Felkenberg) (Chihara, 1961), consists of microscopic three-cell row filaments arranged in a pompon morphology up to 2 cm in diameter. Tetrasporophytes can be found free-floating or attached to other algae and are capable of dispersion by flotation. The high vegetative reproduction potential of the tetrasporophyte stage serves as a prolific propagator for expanding population and invasion to new habitats (Chihara, 1961; Mairh, 1977; Zanolta et al., 2022).

To date, worldwide, six mitochondrial lineages have been genetically distinguished in the *A. taxiformis* populations (Andreakis et al., 2007; Sherwood, 2008; Dijoux et al., 2014; Andreakis et al., 2016; Zanolta et al., 2022). The *cox2-3* spacer is considered the ideal marker for this lineage separation differing in 6-23 bp out of the 338 bp of *cox2-3* spacer marker (Dijoux et al., 2014; Andreakis et al., 2016; Kurihara et al., 2016). Based on this molecular tool, lineage 1 (L1) was described for the Pacific region, whereas a L2 additionally described for the Indo-Pacific (Andreakis et al., 2007; Dijoux et al., 2014), the Mediterranean Sea (Andreakis et al., 2007), and North Atlantic regions (Dijoux et al., 2014). L3 is known for the Mediterranean Sea (Andreakis et al., 2007) and also described for the western Atlantic (Andreakis et al., 2007), the Canary Islands and South

Africa (Bolton et al., 2011). L4 can be found in Hawaii and the western Indo-Pacific (Sherwood, 2008) while L5 is found in Western Australia and the southern Indo-Pacific Ocean (Dijoux et al., 2014; Andreakis et al., 2016). Recently, L6, considered endemic to Australia was described by Andreakis et al. (2016). As mentioned before, *A. taxiformis* was first documented in the Mediterranean Sea before the opening of the Suez Canal, and its current distribution is likely the result of several introduction events including possible Lessepsian migration (Andreakis et al., 2004; Ní Chualáin et al., 2004; Andreakis et al., 2007; Dijoux et al., 2014). To date, only two cryptic lineages, L2 and L3, have been described for the Mediterranean Sea (Zanolta et al., 2022). Within the Israeli Mediterranean Sea (IMS), *A. taxiformis* has become abundant at 0-15 m deep, generally attached to hard bottoms. The presence of *A. taxiformis* along the Israeli coasts has been documented only twice (Lipkin, 1962; Einav and Israel, 2008), and no ecological or molecular biodiversity were reported for the local populations in the past. Field collections of specimens preserved at the Seaweed Herbarium of the Israel Oceanographic & Limnological Research, Ltd. (IOLR) (www.seaweedherbarium.com) confirm the expansion of this species during the last decade.

Global human activities are responsible for the spreading of marine organisms and the changes imposed on marine ecosystems on a worldwide scale. To detect and follow those changes, surveys on a local scale are necessary, having significant implications both for marine conservation and coastal ecosystem management (Bickford et al., 2007; Andreakis and Schaffelke, 2012). In this study we investigate for the first time the *A. taxiformis* populations of the IMS, integrating a DNA barcoding approach with morphological tools. In order to gain a better understanding of the distribution and origin of the IMS *A. taxiformis*, three genetic markers, nuclear LSU, mitochondrial *cox2-3* spacer and plastid *rbcL* were used.

Materials and methods

Seaweed collection

Gametophytes (n = 28) and tetrasporophyte (n = 2) of *A. taxiformis* were collected from five sites along the northern coast of the Israeli Mediterranean Sea (IMS) (Bat Galim 32°50'11.1"N 34°58'40.6"E, Tel Shikmona 32°49'33.8"N 34°57'16.8"E, Achziv 33°03'21.9"N 35°06'06.9"E, Rosh Hanikra 33°05'18.0"N 35°06'20.1"E and Sdot Yam 32°29'29.0"N 34°53'02.6"E) (Figure 1) between July 2020 and December 2021. At each site, thalli pieces from three different specimens situated at least three m apart from each other, were placed in 1 ml buffer lysis solution (40 mM EDTA, 50 mM Tris pH 8.3, and 0.75 M sucrose), transported to the laboratory and kept at -80°C until DNA extraction. Occasionally, fewer replicates were collected from



FIGURE 1

Sampling site across the Israeli Mediterranean Sea (IMS). Stars indicate fresh specimens sampling sites and triangles indicate herbarium sampling sites.

sites with low algal density (Table S1). Part of the collected material was kept in a cooler for binocular observations and measurements of morphological parameters, that included: thallus length and maximum width, basal diameter of the main axis, height where branching begins and the length of the branchlets and their basal diameter. Four herbarium samples (www.seaweedherbarium.com) collected between 2013 - 2018 were also analyzed.

DNA extraction and molecular analyses

About 100 mg of fresh macroalgae biomass were placed in 1 ml buffer lysis solution (ISOLATE II Plant DNA Kit, Bioline) and homogenized using a small plastic pestle. DNA extraction from herbarium samples processed similarly as the fresh samples with an additional bead beater step to brake the cells walls. Genomic DNA was extracted as described in the ISOLATE II Plant DNA Kit. Quantity and quality of DNA were examined

using a nanodrop (NANODROP 2000c Spectrophotometer, Thermo Scientific, USA).

Molecular lineages identification

Three barcoding markers, nuclear LSU, mitochondrial *cox2-3* spacer and plastid *rbcL*, were amplified by polymerase chain reaction (PCR) for lineage identification (Zuccarello and Succursale, 1999; Andreakis et al., 2004; Dijoux et al., 2014). Primer information, such as locus names, nucleotide sequences, and references are provided in Table 1. The following PCR conditions were used for both LSU and *rbcL* PCR reaction: initial denaturation at 94°C for 5 min, followed by 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 90 s, with a final elongation step of 72°C for 10 min. For the mitochondrial *cox2-3* spacer the following PCR conditions were used: initial denaturation at 94°C for 4 min, followed by 5 cycles of 93°C 45s, and 72°C for 60 s each, followed

TABLE 1 Molecular markers used in this study.

Target region	Primer	Sequence	Product size	References
28S (LSU)	D1R	5'-ACCCGCTGAATTTAAGCATA-3'	~606 bp	Lenaers et al., 1989 and Orsini et al., 2002
	D3Ca	5'-ACGAACGATTTGCACGTCAG-3'		
cpDNA	<i>rbcL</i> L	5' - TGTGGACCTCTACAAAACAGC-3'	~264 bp	Maggs et al., 1992
	<i>rbcL</i> S	5'-CCCCATAGTTCCCAAT-3'		
mtDNA	Cox2AF	5'-GTA CCT TCG TTA GGT ATT AAG TGT GAT GC-3'	~316 bp	This study, based on Zuccarello et al. (1999)
	Cox3AF	5'-GGA TCA ACT AAA TGA AAT GGA TGA C -3'		

by 30 cycles of 93°C, 50°C, and 72°C for 30 s each, with a final elongation step of 72°C for 10 min as described by Zuccarello and Succursale (1999). All PCR reactions were run in 50 µL containing 1 µL each of forward and reverse primers (10µM), 25 µL of ready Mix (Bioline Meridian Life Science Inc.), 1 µL template DNA, 20 µL of PCR Grade H₂O and 2 µL of bovine serum albumin (BSA). PCR fragments were sequenced by Macrogen Europe (Macrogen; Europe BV, Amsterdam, Netherlands).

Sequence analysis

DNA sequences from this study and sequences deposited from GenBank were aligned using BioEdit v4.8.5 (Hall, 1999). The evolutionary history tree was inferred using the UPGMA method (P.H.A and Sokal, 1973) in MEGA-X software (Kumar et al., 2018). The evolutionary distances were computed using the p-distance method (No Nei and Kumar, 2000).

Results

Overall, 30 specimens of *Asparagopsis taxiformis*, 28 gametophytes and 2 tetrasporophyte were collected in five sites along the northern Israeli coastline, from July 2020 until December 2021. Sampling efforts in southern shores, which are largely

composed of sandy bottoms, indicated the absence of this species in soft bottoms. Morphological features of mature samples collected from the dense Rosh Hanikra site populations are shown in Table 2. IMS samples showed cystocarps (female reproductive structures) and spermatangia (male reproductive structures) in the majority of the samples examined (Figures 2, 3). Specifically, out of the 28 collected gametophytes, 20 were found to be monoecious, and no reproductive organs were observed for the rest.

A total of 27 sequences were successfully generated for the *cox2-3* spacer marker, 29 for the *rbcL*, and 23 for the LSU (Table S1). Phylogenetic trees constructed for the three different genes (Figure 4) revealed that all samples examined clustered within the lineage 4 (L4). All three markers, *cox2-3* spacer, LSU and *rbcL*, show clustering with the IMS samples together with all L4 lineages samples deposited from GenBank. One exception is the LSU marker of the L4 sample (GenBank KJ772105) from the Egyptian Red Sea which clustered alone and may be a result of a single nucleotide polymorphism which is unique from all of the other samples and most likely may represent a sequencing error. Previous sequencing of the *cox2-3* spacer from distant geographic sites such as Hawaii and Panama differ by 0.98% (3 out of 306 bp) from the IMS samples. Closer sequencing can be seen from L4 samples from French Polynesia and Papua New Guinea which have a discrepancy of 0.65% (2 out of 306 bp) from the IMS samples (Andreakis et al., 2007; Sherwood, 2008; Bolton et al., 2011; Dijoux et al., 2014; Andreakis et al., 2016;

TABLE 2 Morphological characters measured in gametophytes of *A. taxiformis* collected from the Rosh Hanikra site in Nov 2021.

		L4 IMS
Thallus	Length (cm)	9.51 ± 2.01
	Maximum width (cm)	1.76 ± 0.47
Main axis	Basal diameter (mm)	1.41 ± 0.24
	Height where branching begins (cm)	1.88 ± 0.81
Branchlets	Length (cm)	1.05 ± 0.32
	Basal diameter (mm)	0.37 ± 0.07

Values are given as mean ± SD (n = 16).

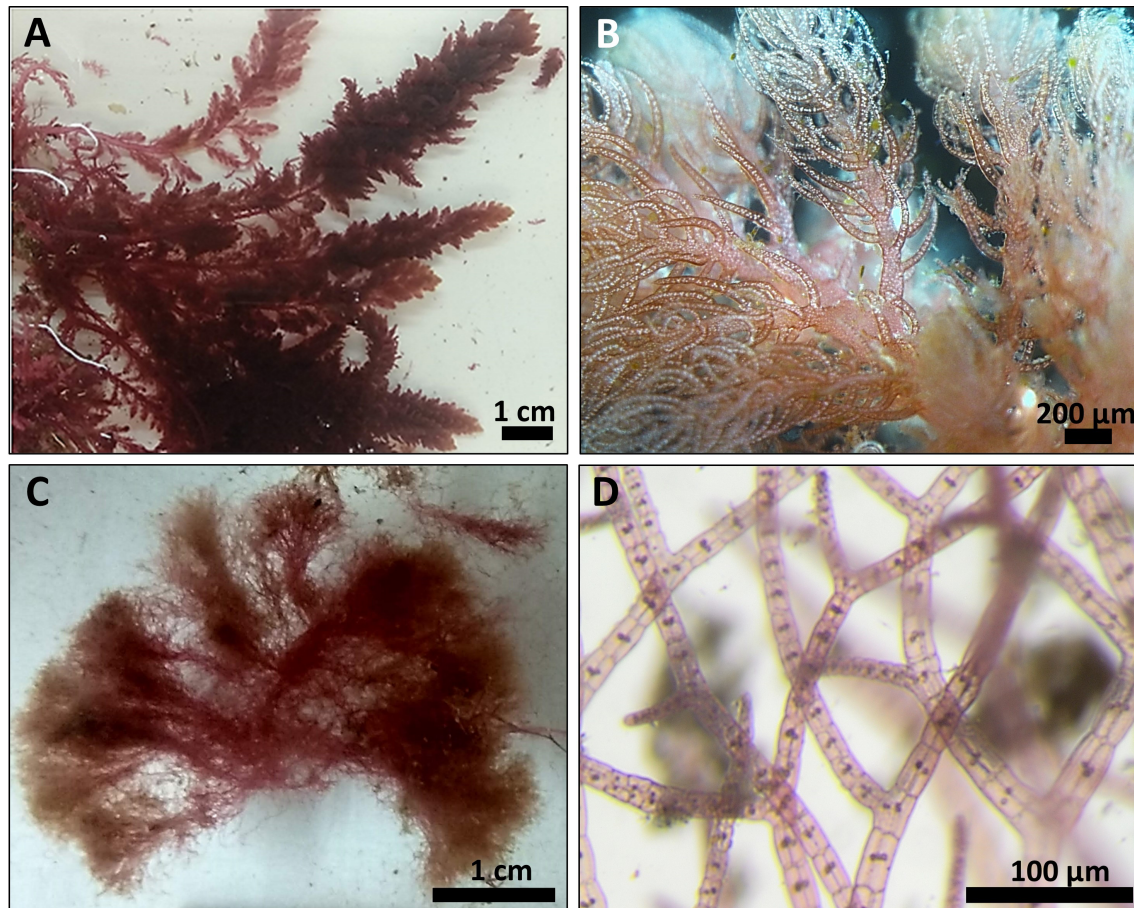


FIGURE 2

Typical *A. taxiformis* gametophyte collected from Tel Shikmona site (A, B) and tetrasporophyte collected from Rosh Hanikra site (C, D).

Kurihara et al., 2016). Samples from South Africa, Europa Island, and Sri Lanka diverged from the IMS samples in only one nucleotide (0.32%). The closest sequencing to the IMS samples is *A. taxiformis* L4 sampled from the Egyptian Red Sea with 100% similarity (Figure 5). In comparison to previously sequenced Mediterranean samples, the IMS *cox2-3* marker differs by 2.3% and 3.9% from L3 and L2 Mediterranean populations, respectively (Andreakis et al., 2004; Andreakis et al., 2007; Dijoux et al., 2014) (Figure 5).

Discussion

The seaweed *Asparagopsis taxiformis* is an iconic example of macroalgal invasion from a global perspective, which has invaded almost all oceans and seas around the world (Zanolla et al., 2022). Until now, two cryptic lineages have been described for the Mediterranean Sea, L2 and L3 (Zanolla et al., 2022). In this study, we present solid evidence of an additional cryptic lineage (so-called L4) of *A. taxiformis* first reported for the

Mediterranean Sea. We regard this lineage as relatively new when compared to the introduction of L2 and L3 genetic lines that were reported in the past. The wide geographic distribution of *A. taxiformis* L4 seems to be the result of long-distance, human-mediated dispersal events (Dijoux et al., 2014). The true native origin of L4 is unknown, however, the pathways of introduction into the Mediterranean Sea can be predicted. A soundly invasion route includes two distinct geographic origins, Sri Lanka or South Africa, both within the Indian Ocean. *A. taxiformis* populations may have established in the eastern Indian Ocean and reached the Red Sea, to eventually penetrate into the eastern Mediterranean Sea via Suez Canal. This possible route is supported by the similarity of the *cox2-3* marker from the IMS sequences and between South Africa, Sri Lanka, and the Red Sea specimens (Figure 6). Whether *A. taxiformis* is a native species or has been introduced into the Mediterranean Sea is still unclear (Boudouresque and Verlaque, 2002; Zanolla et al., 2022). However, based on previous data collected on common Lessepsian migration route (Nunes et al., 2014) taken together

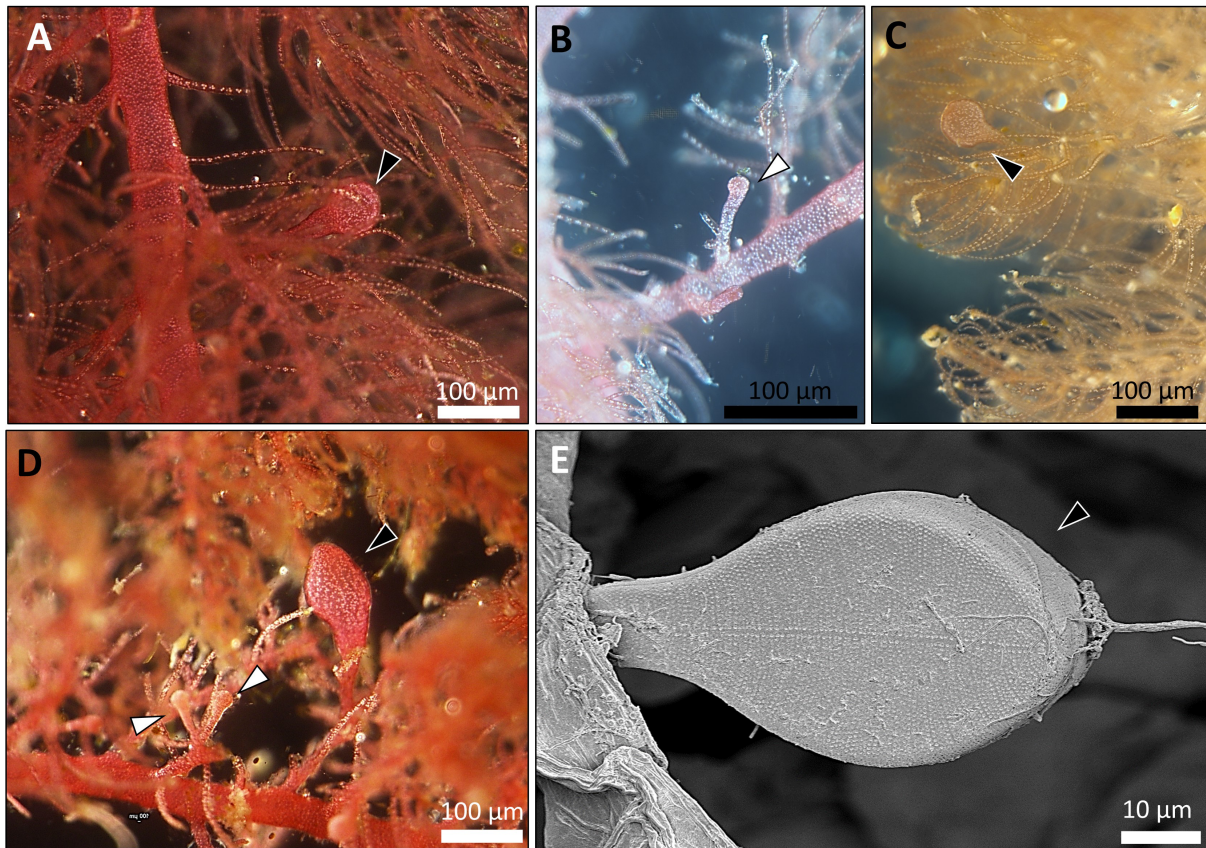


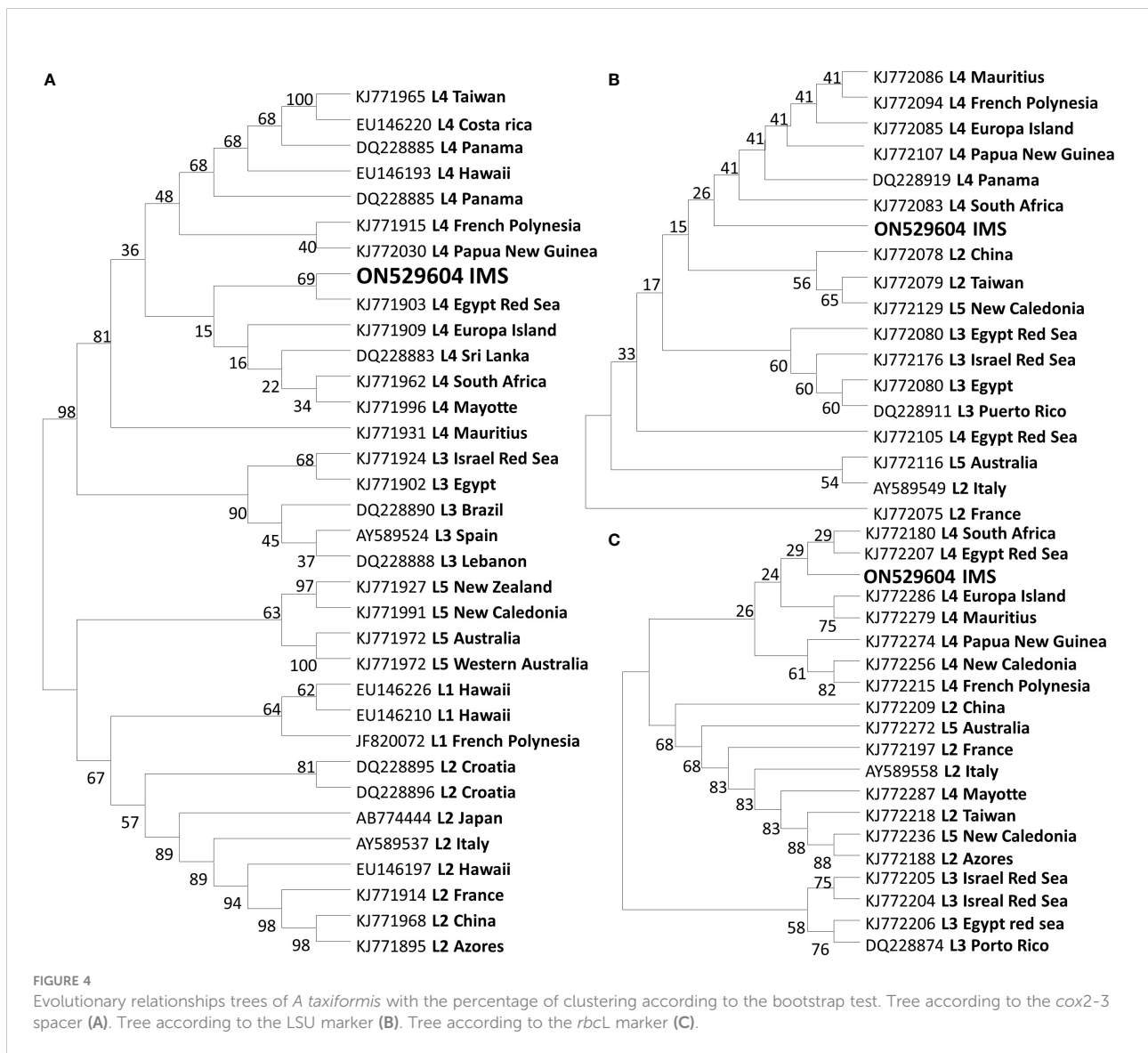
FIGURE 3

Fertile *A. taxiformis* from the IMS (photos of specimens correspond to samples collected during October 2021, from Tel Shikmona and Rosh Hanikra sites). (A–D) light microscope. (E) scanning electron microscope (SEM). Cystocarps (which indicate the development of the gonimoblast, the female reproductive structure) are shown with black arrow heads and spermatangium (male reproductive structure) by white arrow heads.

with the proximity of the IMS to the Suez Canal, as well as the molecular data presented in this study, we hypothesize that *A. taxiformis* L4 is of a Lessepsian origin. From our observations and viewing the lack of ecological and molecular studies in the past, we suggest that *A. taxiformis* L4 introduction into the eastern Mediterranean Sea may have occurred only a few decades ago.

Morphological characters of *A. taxiformis* gametophyte have been used to distinguish between cryptic lineages (Zanolla et al., 2014). Furthermore, gametophytes from the same lineage may show different characters when comparing populations from different geographic regions. For instance, thallus height, basal diameter, and length of the branchlets were all greater in Mediterranean Sea invasive L2 as compared to Hawaiian L2 populations (Zanolla et al., 2014). The trend of increasing thallus morpho-parameters in the Mediterranean Sea populations compared to populations from the same lineage, but from distant geographic sites, was also seen in L4. Specifically, morphological traits of L4 from Rosh Hanikra site, namely

thallus height, main axis basal diameter, and height where branching begins were higher compared to L4 from Hawaii. There is still uncertainty as to whether this tendency is due to the different biotic conditions in the Mediterranean Sea or whether the increased size of some specimens gives them ecological advantages, thus increasing their invasive rates and extent. Recently observed drifts strongly indicate that *A. taxiformis* is widespread in shallow habitats between Rosh Hanikra and Mikhmoret sites. In these sites, *A. taxiformis* is frequently attached to exposed rocky substrates both vertically and horizontally, usually between 0–5 m deep. Nonetheless, *A. taxiformis* is also quite abundant at depths of 20–30 m and significant drifts of fertile gametophytes are carried out to the shore following stormy sea days. We have no quantitative seasonal data for the above observations, yet our frequent field trips indicate that *A. taxiformis* exists all year round with productivity declining during wintertime. Growth capacity together with physiological and reproductive traits related to environmental factors for the IMS L4 *A. taxiformis* are still



unknown. In a previous study which did not include genetic characterization, some populations of *A. taxiformis* were described as dioecious (Barone et al., 2013). As for the L4 lineage from Hawaii, Zanolla et al. did not report the presence of any reproductive structures (Zanolla et al., 2014). In this study, nearly every specimen showed reproductive structures carrying both cystocarps and spermatangia strongly indicating that IMS L4 is monoecious (Figure 3).

Detached gametophytes as seen in large amounts in the IMS could promote further dispersal of the L4 population in two possible ways. First, by vegetative reproduction via fragmentation of the thalli as suggested by Mairh (1977) and second, although the released gametes and tetraspores are not thought to travel very far (Santelices, 1990), detached gametophytes that drift with the currents can carry and release reproductive structures for long distances. The presence of the

gametophyte in a site does not necessarily confirm the presence of the tetrasporophytes stage, and vice versa (Bolton et al., 2011; Orlando-Bonaca et al., 2017). The observation of both reproductive stages in the same site indicates an active reproductive population. Furthermore, the occurrence of tetrasporophytes, which are known as a good dispersal unit, makes this population a potential donor source for future invasive populations

All *A. taxiformis* specimens analyzed from the Mediterranean Sea so far were found to be either L2 or L3 (Zanolla et al., 2022). Studies involving the population dynamics of the Mediterranean L2 suggest the successful expansion of this lineage can be explained by the temperate and tropical preferences of this lineage (Zanolla et al., 2018). Furthermore, rising sea temperatures in some regions due to climate change will also increase the opportunity for this lineage to further spread in those

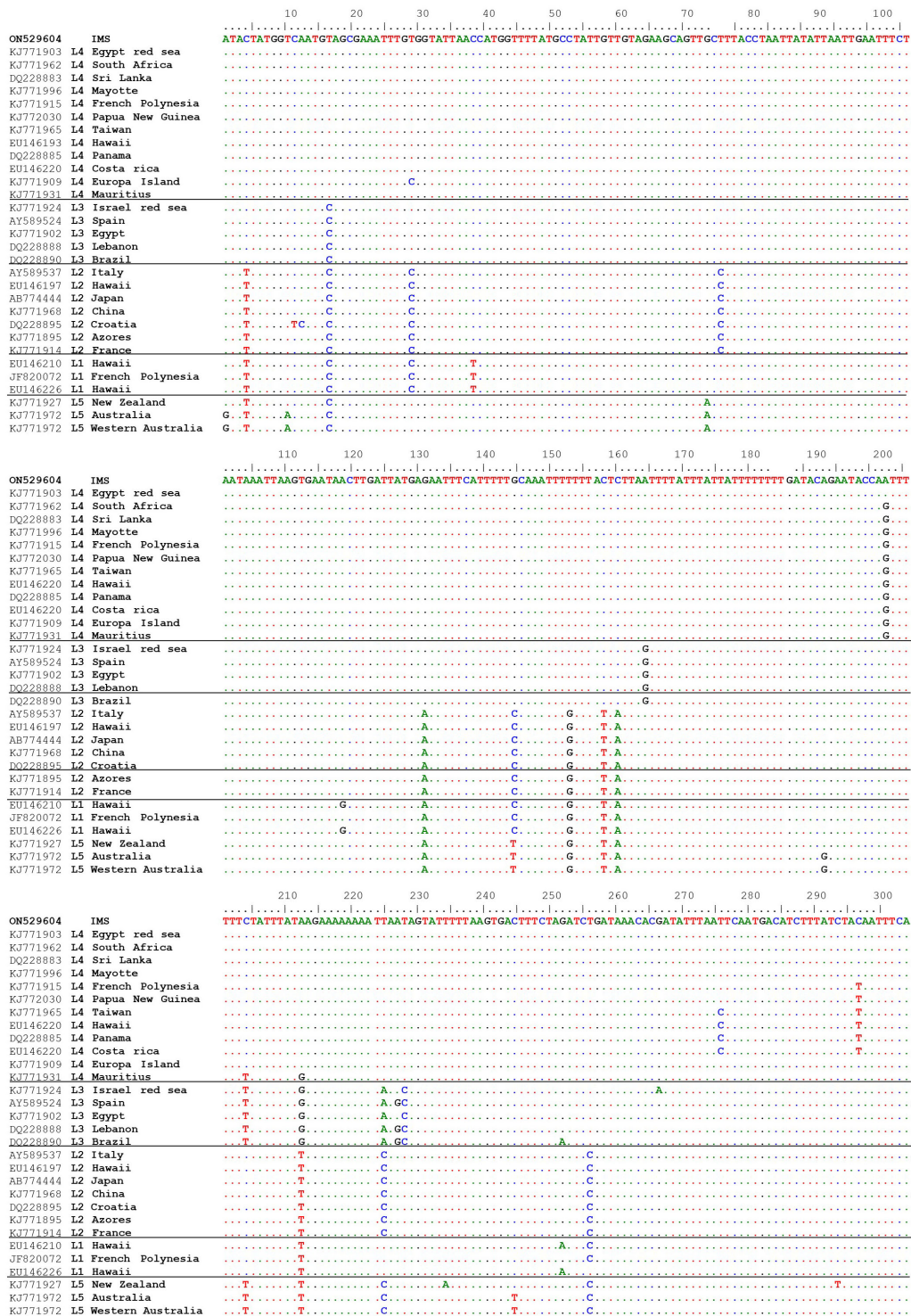
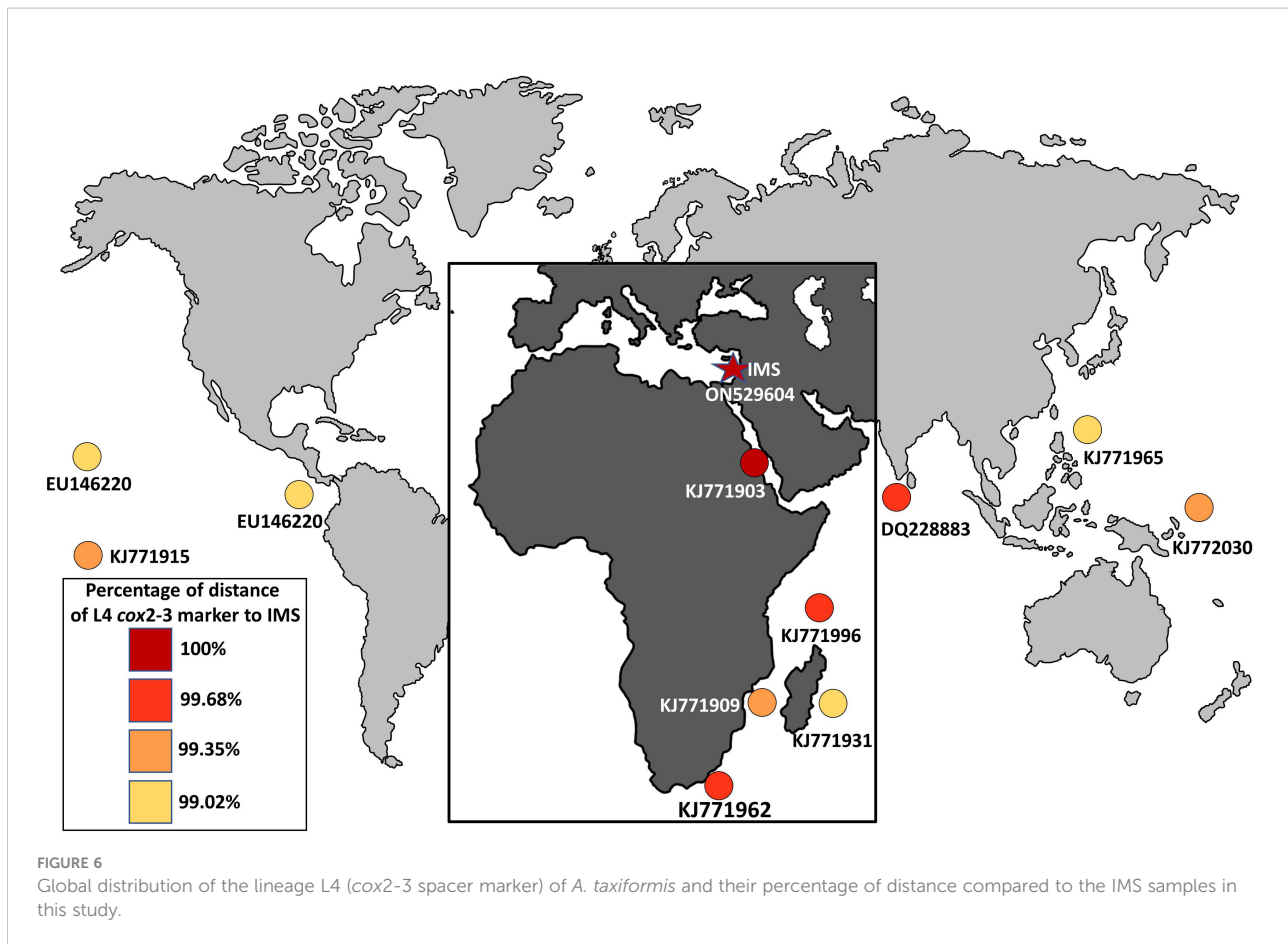


FIGURE 5 Multiple alignments of the *cox2-3* spacer sequence isolated from IMS sample (GenBank accession number ON529604) compared to sequences from GenBank.



areas (Zanolla et al., 2018). Zanolla et al. (2018) also highlight the co-existence of L2 and *A. armata* in some areas, suggesting as the former existence of *A. armata* promotes the L2 expansion in those sites. This conjecture relies on the assumption that communities under stress from an invasive species are more susceptible to additional invaders (Simberloff and Von Holle, 1999). Considering that *A. taxiformis* is already well established in almost all of the Mediterranean Sea, the ability of L4 to flourish in tropical-temperate regions, and the suggested Lessepsian migration route, there is a significant likelihood that L4 populations will spread to other Mediterranean basins. Moreover, considering the classic Lessepsian migration route of NISS we hypothesize Tsiamis and Panayotidis (2007) first description of *A. taxiformis* in Greece (which not include molecular work), might have corresponded to the L4 described in this study. The increasing abundance of *A. taxiformis* L4 in the eastern Mediterranean Sea underlines the need for a comprehensive monitoring strategy for its distribution and lineage description. Lastly, analyses of macroalgae herbaria samples may be crucial to assess their impact on local biodiversity and track their possible route over time.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

All authors design the research and conduct the field collection. ON did the molecular work and data analysis. All authors provided critical feedback and helped shape the research, analysis, and manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.873817/full#supplementary-material>

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