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Halimeda incrassata (Bryopsidales, Chlorophyta) in Rhodes, Greece, Eastern Mediterranean

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Abstract

The first record of the tropical green seaweed *Halimeda incrassata* (Bryopsidales, Chlorophyta) in the Eastern Mediterranean Sea is presented, based on several thalli found in the stomach of a silver-cheeked toadfish (*Lagocephalus sceleratus*), collected off Plimmiri beach, Rhodes, Greece. Species identification was based on morphological and molecular identification using the tufA gene as a molecular marker. The finding comes 10 years after a report on the species in Mallorca (Western Mediterranean Sea), where *H. incrassata* has spread rapidly. The pathway of its introduction in the Eastern basin is unknown, although shipping or the aquarium trade could be involved in this new introduction. Further studies are necessary for visual documentation of the infested areas around Rhodes and assessment of its possible spread in the following years.

Keywords: Non-Indigenous Species (NIS); Chlorophyta; Levantine; DNA barcoding.

Introduction

During the last decades, intensification of human activities in the Mediterranean Sea has resulted in accelerated phenomenon marine biological invasions (Pancucci-Papadopoulou *et al.*, 2012; Bianchi *et al.,* 2014; Zenetos *et al.*, 2022). The marine waters surrounding the island of Rhodes, especially along the southern part of the island that is constantly influenced by the Asia Minor Current (AMC; Gaines *et al.*, 2006), present subtropical environmental characteristics that are ideal for the colonization of non-indigenous species (NIS) of tropical or subtropical origin (Papaconstantinou, 2014).

Macroalgae consist of an important group of species in terms of their ecosystem services, acting as carbon sinks, storage sites, habitat-engineering and nutrient removers (Granier, 2012). In the Mediterranean Sea, macroalgae is the fourth most successful group of NIS in terms of establishment success, with 77% of the 161 species considered established (Zenetos *et al.*, 2022). NIS macroalgae in Hellenic marine waters (Aegean, Cretan, Ionian and Levantine Seas) include four species of Ochrophyta, 12 Rhodophyta and four Chlorophyta, excluding cryptogenic and questionable taxa, according to the latest published data (Tsiamis, 2012; European Commission *et al.*, 2021). In the southern Aegean Sea, 11 species are classified as established NIS, of which five are invasive, one is casual and two are questionable as regards their establishment status (Zenetos *et al.*, 2020), whereas the potential pathway of introduction of the majority is shipping, followed by angling/fishing, unaided and intentional/unintentional release.

Halimeda incrassata (J. Ellis) J.V. Lamouroux (Bryopsidales, Chlorophyta) is a calcified green macroalgal species, considered as NIS in the Mediterranean Sea, and was first reported in the basin from Mallorca in 2011 (Alos *et al.*, 2016). In Mallorca, *H. incrassata* exhibited invasive behaviour due to its rapid population growth within a few years. The species has also been reported from Madeira, north-eastern Atlantic (Wirtz & Kaufmann, 2005) and the Canary Islands (Sangil *et al.*, 2018). The species has been flagged as top-priority NIS in the EU-scale Horizon Scanning of marine NIS (Tsiamis *et al.*, 2020). Naturally, it is distributed in the tropical western Atlantic, the Indo-Pacific Ocean and the Red Sea (Guiry & Guiry, 2021; Fig. 1).

Within its natural range, the species usually inhabits shallow sandy bottoms, although it has also been found in

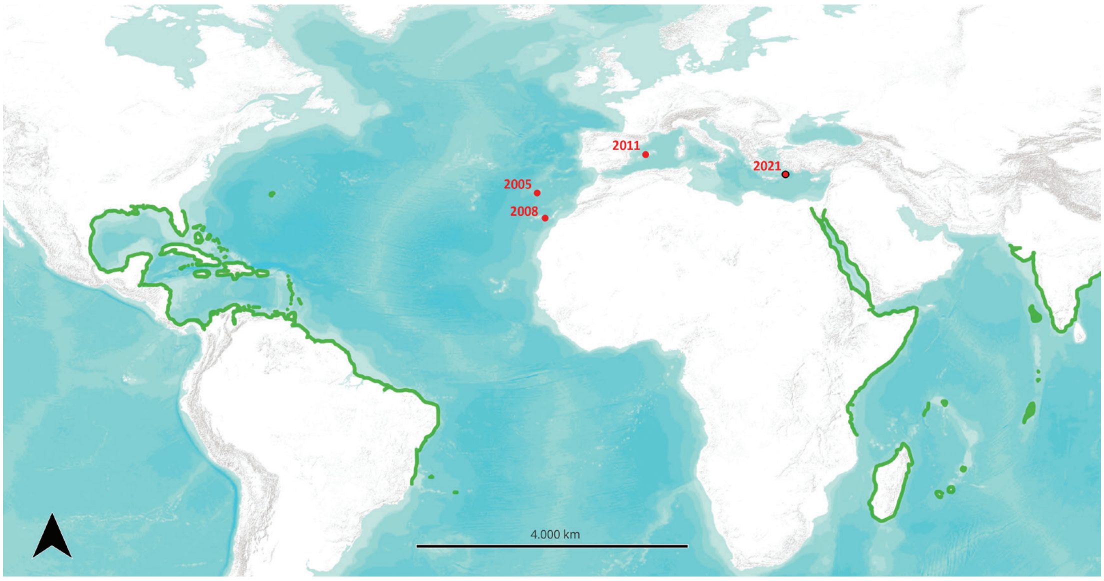

Fig. 1: Distribution of *Halimeda incrassata* in the proximity of the invaded areas. The green line indicates the approximate natural range according to Guiry and Guiry (2021); the red dots indicate the invaded areas.

deeper than 60 m waters (Littler & Littler 2000 in Sangil *et al.*, 2018). In the invaded marine waters of the Canary Islands, it was found to dominate the mid and deep-water habitats, whereas in Mallorca the species was not found below 20 m (Alos *et al.*, 2016; Sangil *et al.*, 2018).

Halimeda incrassata can reproduce both sexually and asexually by rhizoidal extension and fragmentation (van Tussenbroek & Barba Santos, 2011), having considerable growth rates (Multer, 1988; Multer & Clavijo, 2004 and references within). It is characterized as an ecological engineer with a great potential for modifying native habitats (Vivó-Pons *et al.*, 2020), with both positive and negative impacts (Tsirintanis *et al.*, 2022). The species is involved in the formation of carbonate sediments with a considerable annual production of calcium carbonate ($CaCO_3$), depending on the solar cycle (Freile & Hillis, 1997; van Tussenbroek & van Dijk, 2007). Furthermore, the species provides oxygen (e.g. 25.1 mg of O_2 per g of decalcified dry weight per day in Tahiti: Payri, 1988), nourishment (e.g. for the sea-urchin *Diadema antillarum* in Jamaica: Hillis-Colinvaux, 1974; for the bucktooth parrotfish *Sparisoma radians* in Virgin Islands: Lobel & Ogden, 1981 and for the pinfish *Lagodon rhomboides* in the Florida Keys: Ribble, 2019), shelter to other species (Heck & Wetstone 1977) and an ideal surface for the development of epifaunal organisms (Mateo-Ramírez *et al.*, 2022). Although *H. incrassata* is not a significant competitor of the native phanerogam *Posidonia oceanica*, it is known to compete with other macrophytes including *Dasycladus vermicularis* (Sureda *et al.*, 2017) and *Thalassia testudinum* (Davis & Fourqurean, 2001). The species could be favoured by the increasing temperature of the Mediterranean Sea, regardless of acidification, as demonstrated in aquarium experiments (Campbell *et al.*, 2016).

Herein, the presence of the species in the Eastern Mediterranean Sea is reported for the first time from Rhodian Levantine waters, identified by morphological observations and DNA barcoding. Possible pathways of introduction and future challenges are discussed.

Material and Methods

Several thalli of *Halimeda incrassata* (Fig. 2) were found in the stomach content of a silver-cheeked toadfish *Lagocephalus sceleratus* individual (TL 59.5 cm, weight 2350.2 g) caught off Plimmiri beach, SE Rhodes (35.917203°N, 27.860926°E) with bottom long lines (length 600 m, 100 hooks size No 10-12) during experimental fishing with the most commonly used fishing gear, including static nets, longlines, jigs and bottom traps, from April 2021 to March 2022. The fishing gear was deployed by a commercial 106.5 KW fishing vessel at 11-14 m depth, over a sandy-muddy substrate early in the morning of 6 October 2021 and was retrieved one hour later. Along with six more individuals of the species, caught with the same bottom longline, specimens were transported to the Hydrobiological Station of Rhodes (HSR), measured and photographed, while their stomach was removed and analysed for contents. Apart from the chlorophyte, viewing under a stereoscope revealed pieces of a freshly preyed Atlantic horse mackerel *Trachurus trachurus*, an unidentified decapod and a small unidentified pelagic crustacean in the stomach of the aforementioned specimen. Some of the thalli of *H. incrassata* were placed in absolute ethanol for DNA extraction, whereas the remaining were preserved in 70% ethanol and deposited at the HSR collection (catalogue number HSR560).

A Nikon SMZ800 stereoscope and a Nikon AW111 camera were used for morphological observations and photographs of the *H. incrassata* samples.

For the molecular identification of the specimen, the plastid gene tufA was used, following Cremen *et al.* (2016). Total genomic DNA was extracted from a sample of tissue (50mg) using the DNeasy Plant Pro kit (QIAGEN), according to the manufacturer's instructions. The plant tissue was homogenized using TissueLyzer II (two rounds of shaking at 25 Hz). Extracted DNA was PCR-amplified using the primers tufAF and tufAR from Fama *et al.* (2002) and tufGF4 (5'-GGNGCNGCN-

Fig. 2: Thalli of *Halimeda incrassata* from Rhodes, Greece. (Photo credit: G. Kondylatos).

CAAATGGAYGG-3') from Saunders & Kucera (2010), in two different PCR combinations (tufAF-tufAR and tufGF4-tufAR). PCR reactions were performed in a total volume of 12.5μl and consisted of 1μl (~20ng) template DNA, 7.75μL of ddH2O, 2.5μL of MyTaq Red Reaction Buffer (5x), 0.25μl of MyTaq™ Red DNA Polymerase (meridian BIOSCIENCE) and 0.5μL of each primer (10μM). PCR conditions were as in Cremen *et al.* (2016). Sanger sequencing reactions (both forward and reverse) were performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit and were electrophoresed on an ABI 3730xl DNA Analyzer (Applied Biosystems™). The produced sequence was compared to existing GenBank sequences using BLAST and was deposited in GenBank under accession number OQ871581.

Results

Thalli light-green to dark green, calcified, erect, sparsely branched, composed of segments; segments rather flat, becoming barrel-shaped towards the base; they are unlobed or trilobed, obovate–cuneate, broadest at or near their tip rather than at or near their base; segment dimensions ranged between 4.16-5.88 mm in height, 2.19-3.55 mm in width, and 0.67-1.57 mm in thickness; holdfasts were missing.

Anatomical observation revealed a cortex composed of 2-3 (rarely 4) layers of utricles; utricles not inflated, but rather cylindrical; peripheral utricles adhere to one another at their distal end; they measure 70-90 μm in height and 40-60 μm in width; sub-peripheral utricles measure 100-130 μm in height and 50-70 μm in width; peripheral utricles in surface view are polygons with slightly rounded corners, 40-55 μm in diameter. Our specimens are in good match with previous descriptions of the species (Verbruggen *et al.*, 2006; Alos *et al.*, 2016).

The molecular analysis of the specimen produced a sequence of 813 bp in length, which presented 100% similarity with *H. incrassata* sequences deposited in GenBank (accession numbers: FJ624534.1, AM049958.1, KT781884.1), thus confirming the morphological identification. Moreover, the resulting sequence presents less than 98% similarity with all the other deposited sequences of *Halimeda* species. More specifically, our specimen presents 98% similarity with *Halimeda simulans* M.A.Howe, 1907 (accession number: AM049963.1), 97% with *Halimeda monile* (J.Ellis & Solander) J.V.Lamouroux, 1816 (accession number: AM049962.1) and *Halimeda cylindracea* Decaisne, 1842 (accession number: KM820164.1).

Phylogenetic analysis further shows that our specimen (GenBank: OQ871581) is grouped together in a separate clade with *Halimeda incrassata* sequences from GenBank (accession numbers: FJ624534.1, AM049958.1 and KT781884.1). GenBank sequences AM049957.1 and AM049959.1 are deposited in GenBank under species name *Halimeda incrassata*. However, after revision of the paper (Verbruggen *et al.*, 2006), they are identified as *Halimeda heteromorpha* and *Halimeda kanaloana*, respectively, and are represented as such in the phylogenetic tree (Fig. 3).

Discussion

The *L. sceleratus* individual containing the thalli of *H. incrassata* was collected from shallow, 11-14m deep, coastal waters, a depth range where *H. incrassata* can proliferate (Wirtz & Kaufmann, 2005; Alós *et al.*, 2016; Sangil *et al.*, 2018). This information is rather significant because it provides an indicative depth zone where initial underwater observations can begin for the investigation of the current status of *H. incrassata* and its spread in Rhodian waters.

The presence of thalli of *H. incrassata* in the stomach content of the strictly carnivorous *L. sceleratus* is considered a collateral prey item. However, this is a very impor-

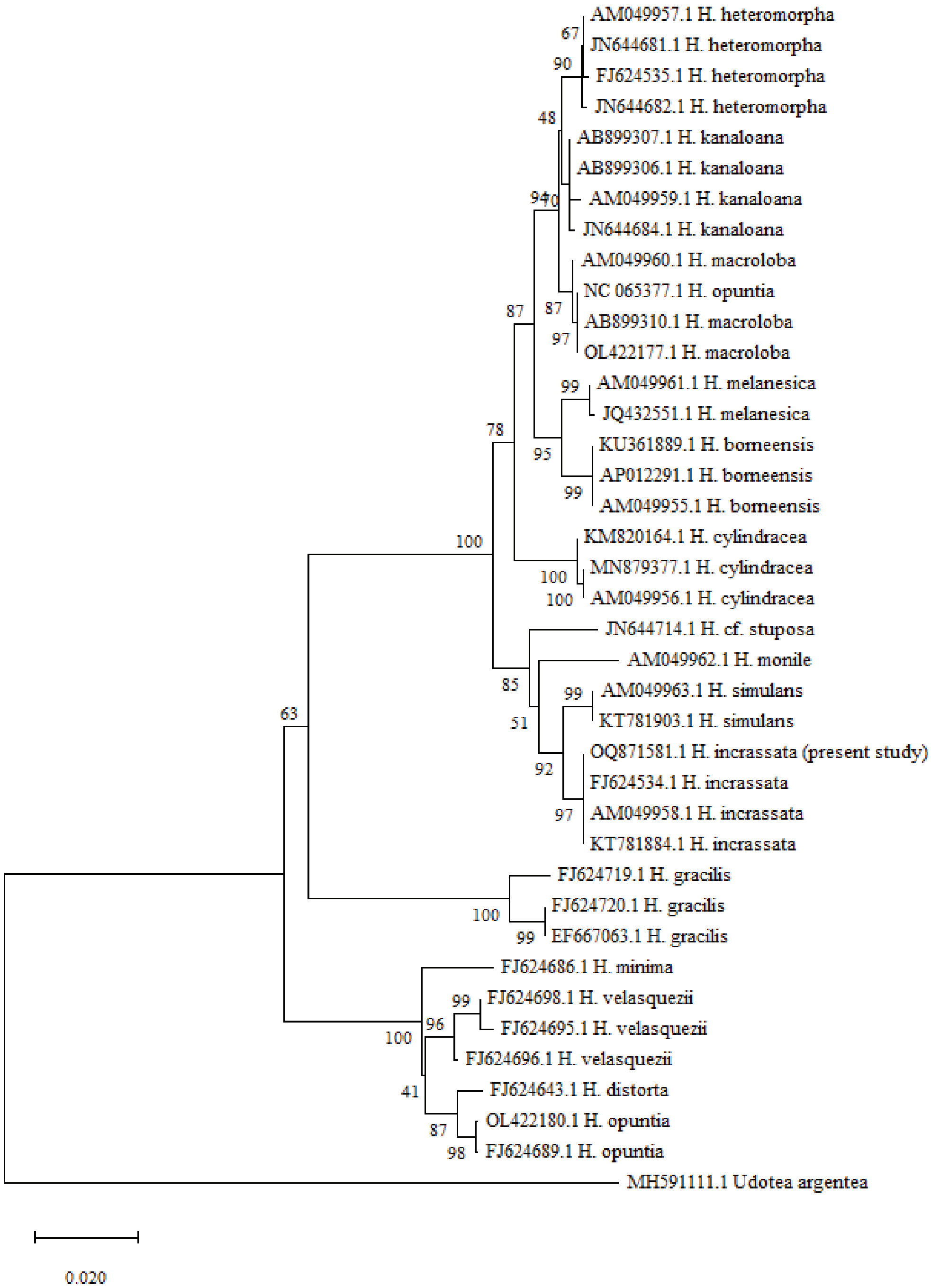

Fig. 3: The phylogenetic position of the *Halimeda incrassata* specimen in relation to other *Halimeda* species with available tufA sequences in GenBank (Neighbour-joining tree using Kimura-2P distances and 1000 bootstrap replicates).

tant finding not only because this is the first record of *H. incrassata* in the Eastern Mediterranean Sea, but also because it reveals another possible pathway for the spread of the species. When the undigested thalli are expelled through the faeces in an area far from the praying site, it is likely that *H. incrassata* will have the opportunity to germinate and start developing a new population.

It is worth mentioning that during the study period (April 2021 – March 2022), a total of 724 individuals of *L. sceleratus* were collected, mainly with longlines and jigs. Analysis of their stomach contents revealed the presence of six specimens of another macrophyte NIS, *Halophila stipulacea* (Forsskål) Ascherson. The species is considered as an established NIS in the south Aegean Sea (Zenetos *et al.*, 2020). The finding of *H. incrassata* in the stomach contents of only one out of the 724 examined *L. sceleratus* individuals, suggests the possibility of an early stage of the colonization process of the species.

Halimeda incrassata is native to the western Atlantic, the Indo-West Pacific and Red Sea waters (Guiry & Guiry, 2021). Two scenarios regarding the pathways of introduction of the species to the Rhodian marine waters are considered as most probable. The first possible scenario is that *H. incrassata* reached the island through shipping, when fragments mixed with soft bottom material were caught on anchors and transported to Rhodes either from the western Mediterranean or the Atlantic via the Strait of Gibraltar or the Red Sea via the Suez Canal. Shipping is a very common vector of introduction of NIS macroalgae in the Mediterranean Sea (Zenetos *et al.*, 2018, 2020) and Rhodes is a favourite destination for cruise ships sailing around the Mediterranean and adjacent Atlantic. During the tourism seasons of 2020 and 2021, more than 200 cruise ships docked at the tourist harbour (36.445572°N, 28.233499°E) of the island (ELIME, 2023). Since our finding comes from coastal water, the anchors of western Mediterranean trawlers fishing in international waters, six nautical miles from the coasts of Rhodes, are unlikely to have been involved. Additionally, ballast waters might also have been involved in the transportation of the species because *H. incrassata* does not have planktonic spores, while its gametes and zygotes are short-lived. The second scenario involves an accidental/intentional release from home/private aquaria, which is a known pathway of NIS introduction (Zenetos *et al.*, 2018), since this macroalga is used for aquarium decoration.

In any case, visual inspection of the soft-bottoms near the ports of the town of Rhodes and along the coastal waters of southern Rhodes is necessary for drawing safer conclusions. Furthermore, relevant underwater observations in the major tourist ports of the Eastern Mediterranean, will further assist in revealing the degree of implication of shipping, mainly cruising, in the dispersion of *H. incrassata* within the basin.

Despite the uncertainty surrounding the pathway of introduction, the colonization of *H. incrassata* in the Western Mediterranean Sea has profound and documented effects on the invaded ecosystem (Tsirintanis *et al.*, 2022). These include the modification of the synthesis of the substrate and of fish communities (Nadal Nebot, 2017) and can be either positive, neutral or negative (Vivó-Pons *et al.*, 2020). For herbivorous organisms, such as herbivorous fish and sea urchins, the presence of *H. incrassata* entails the availability of a new food source. However, *Halimeda* species possess physical and chemical defences in order to avoid predation (Multer & Clavijo, 2004 and references within; Mateo Ramirez *et al.*, 2022 and references within). In that sense, the incorporation of *H. incrassata* in the diet of indigenous and NIS Mediterranean fauna needs further investigation.

Halimeda incrassata is a habitat-engineer (Granier, 2012) and the colonization of the vast sandy and muddy bottoms of the coastal waters of Rhodes could work in two ways. On the one hand, the formation of fields of calcareous algae could significantly contribute to the reduction of the niches of many fish species and other organisms that have been using the substrate for preying, burial and camouflage as pointed out by Vivó-Pons *et al.* (2020), but on the other hand this substitution could create a new niche and favour other species.

Although *H. incrassata* is known to bear epiphytes (Nadal Nebot, 2017), no epiphytes were found on the examined thali of this work, either because of the early colonization phase or the digestion process. Furthermore, the possibility of introduction of exotic epiphytes and alteration of the diversity of epifaunal organisms (Naim, 1988) should be further investigated.

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