

**Rapid Communication****The Indo-Pacific nudibranch *Baeolidia moebii* Bergh, 1888 in Greece, with the first documented spawning aggregation in the Mediterranean Sea**Eleni Kytinou<sup>1,2</sup>, Maria Zotou<sup>1</sup>, Riccardo Virgili<sup>3</sup>, Fabio Crocetta<sup>3</sup> and Stelios Katsanevakis<sup>1,\*</sup><sup>1</sup>Department of Marine Sciences, University of the Aegean, Lofos Panepistimiou, 81100 Mytilene, Greece<sup>2</sup>Institute of Oceanography, Hellenic Centre for Marine Research, 46,7 km Athens Sounio ave., 19013 Anavyssos, Greece<sup>3</sup>Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Villa Comunale, I-80121 Napoli, Italy

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**OPEN ACCESS****Abstract**

The nudibranch *Baeolidia moebii* Bergh, 1888 is a sea slug widely distributed in the Indo-Pacific, including the Red Sea, from where it presumably entered the Mediterranean Sea through Lessepsian migration. Although the first record of this species in the invaded area dates back to at least 2007, it is only known so far from the Mediterranean basin based on three scattered records of single individuals, all found in the Levant Sea (Turkey and Cyprus). The present study reports the presence of *B. moebii* in Kalloni Gulf, a semi-enclosed shallow embayment located in Lesvos Island, a record that widens its known distribution to Greece and the Aegean Sea. Field observations of more than 150 individuals encountered in a single sampling event also allowed observation of intraspecific morphological variation and several specimens laying egg masses, thus first reporting of a spawning aggregation and providing concrete confirmation of its establishment success in the entire basin. Monitoring of such spawning events and aggregations may contribute to a better understanding of the population dynamics of the species. The discontinuous known distribution of *B. moebii* in the eastern Mediterranean can be attributed to false absence of the species due to its cryptic behaviour, its under-sampled habitat, or to a truly very patchy distribution because of secondary dispersion by currents or vessels, with establishment only in areas of particularly favourable conditions, such as the Kalloni Gulf.

**Key words:** Aeolidiidae, DNA-barcoding, Kalloni Gulf, Lesvos Island, morphological identification, Non-Indigenous Species (NIS), range expansion

**Introduction**

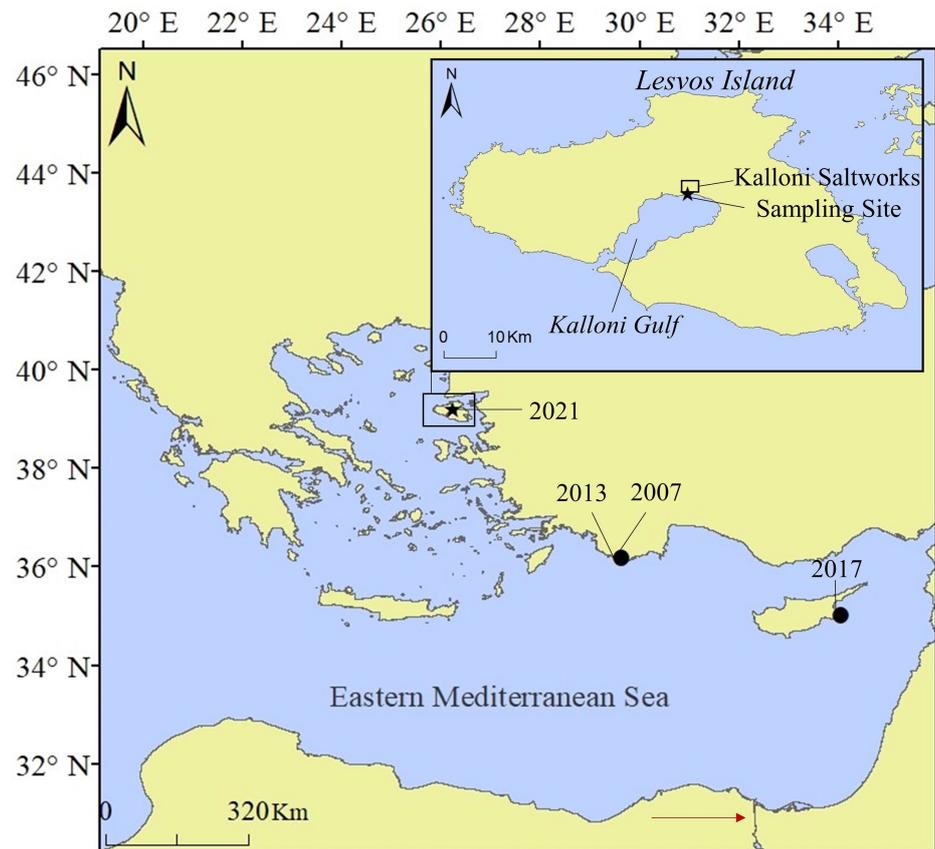
Biological invasions and climate change, along with cumulative human pressures such as fishing, pollution, and habitat alteration, have led to the characterisation of the Mediterranean Sea as one of the most impacted marine ecoregions in the world (Halpern et al. 2019; Micheli et al. 2013). The number of non-indigenous species (NIS) in the Mediterranean Sea is continuously increasing and has already exceeded 1000 marine species, of which more than 600 are established (Zenetos and Galanidi 2020), rendering the Mediterranean as the most invaded sea globally (Costello et al. 2021). Regarding Mollusca, Zenetos and Galanidi (2020) listed 156 non-indigenous

taxa as established in the basin, a number that includes 18 nudibranchs (EASIN 2021).

The family Aeolidiidae Gray, 1827 (Mollusca: Gastropoda: Nudibranchia) is represented in the Mediterranean Sea by 11 taxa (Coll et al. 2010), including a cryptogenic species, namely *Anteaeolidiella lurana* (Ev. Marcus & Er. Marcus, 1967), and an NIS, namely *Baeolidia moebii* Bergh, 1888. The former is a circumtropical species described from Brazil (Marcus and Marcus 1967) and presumably originating in the western Atlantic, that has often been reported under different binomial names (now ascribed to other congeneric taxa) in Spain, Italy, Malta, and Greece (Schmekel 1968; Crocetta et al. 2013; Carmona et al. 2014a; Lipej et al. 2017; Bariche et al. 2020).

The aeolidiid *B. moebii* is native to the Indo-Pacific realm, being described from Mauritius (Bergh 1888) and subsequently recorded from additional localities (Tanzania, Mozambique, Reunion Island, Seychelles, Indonesia, east Australia, the Philippines, South Korea, Japan, the Marshall Islands, Hawaii, California, and west Mexico: Koh 2008; Carmona et al. 2014b and references therein). Recently, *B. moebii* has been also recorded from the northern and central Red Sea from Egypt, Israel (Eilat, Gulf of Aqaba), and Saudi Arabia (Jeddah) (Yonow 2000, 2008), from where it presumably entered the Mediterranean Sea through Lessepsian migration (Paz-Sedano et al. 2019; Çinar et al. 2021). Further confirming this statement, all Mediterranean records of the species come from the Levant Sea (Figure 1), with the first observation made in October 2007 in Kaş (Turkey), where a single individual was photographed at 25 m depth (Turk and Furlan 2011, as *Spurilla major*), the second in October 2013 in the same area, where a single specimen was photographed at 8–10 m depth, and the third in June 2017 in Nissia (Cyprus), where another single specimen was sampled at 20 m depth (Paz-Sedano et al. 2019).

As for *A. lurana*, *B. moebii* also suffered from various nomenclatural and taxonomic issues in the past, and was misidentified with other taxa (Bergh 1888; Gosliner 1979, 1985; Miller 2001). However, the review of the genus carried out by Carmona et al. (2014b), coupled with the integrative taxonomic analysis carried out on the Cypriot specimen (Paz-Sedano et al. 2019), allowed clarification of the above-mentioned mismatches. In particular, the taxa *Baeolidia major* Eliot, 1903 and *Baeolidia major amakusana* Baba, 1937 were considered as junior synonyms of *B. moebii* (see Carmona et al. 2014b), whereas the Red Sea photographic records of *Baeolidia australis* (Rudman, 1982) were confused as they were based on juvenile *B. moebii*, thus suggesting that only *B. moebii* occurs in the Red Sea and that it is the only *Baeolidia* species that has entered the Mediterranean Sea since at least 2007 (Paz-Sedano et al. 2019). Despite the resolution of these taxonomic issues, little is known about the ecology and reproduction of *B. moebii*, both in its native and invaded ranges, apart from the facts that it feeds on different genera of anemones (Gosliner 1979; McDonald and Nybakken 1997) and hosts symbiotic zooxanthellae (Rudman 1982).



**Figure 1.** Records of *Baeolidia moebii* in the Mediterranean Sea, with an enlargement of Kalloni Gulf (Lesvos Island, Greece, NE Aegean Sea). Black circles indicate literature records of the three single individuals of *B. moebii* found so far (i.e., 2007 in Turkey: Turk and Furlan 2011; 2013 in Turkey and 2017 in Cyprus: Paz-Sedano et al. 2019). A black star indicates the site of the present record of > 150 individuals in Kalloni Gulf in 2021. The red arrow highlights the Suez Canal.

Based on recent records of *B. moebii* reported herein from Lesvos Island (NE Aegean Sea), the present study broadens the known distribution of the species in its invaded range, and documents its breeding in the Mediterranean Sea for the first time. Morphological and ecological characteristics of *in situ* observations of multiple individuals are also presented.

## Materials and methods

### Study area

Kalloni Gulf is a semi-enclosed shallow embayment located in Lesvos Island (NE Aegean Sea, Greece; Figure 1) that covers an approximate area of 130 km<sup>2</sup>, with a mean depth of 10 m in the central part of the gulf and a maximum depth that may reach 25 m at the narrow channel connecting the gulf with the Aegean Sea (Kefalas et al. 2016). Along the coasts of Kalloni Gulf there are several small wetlands and two saltworks, the largest of which can be found in the inner part (Kalloni Saltworks, Figure 1; Evagelopoulos and Koutsoubas 2008). The inner part of the gulf is characterised by low flushing and higher nutrient levels compared to the open sea (Panayotidis et al. 1999). During the cold period of the year, the

inner part of the gulf receives high amounts of river water (1632 m<sup>3</sup> per day, Spatharis et al. 2007) and acts as a dilution basin, whereas during the hot period, it acts as an evaporation basin (Panayotidis et al. 1999). The temperature at the surface layer of the inner gulf fluctuates between 6.5 °C and 30.5 °C (2020–2021, *unpublished data*), and the salinity between 36 and 45 psu (Evagelopoulos and Koutsoubas 2008). Kalloni Gulf is a highly productive ecosystem that supports great biodiversity and valuable habitats (Evagelopoulos and Koutsoubas 2008) and is thus included in the Natura 2000 European network of protected areas (Site Code: SCI GR4110004).

### Fieldwork

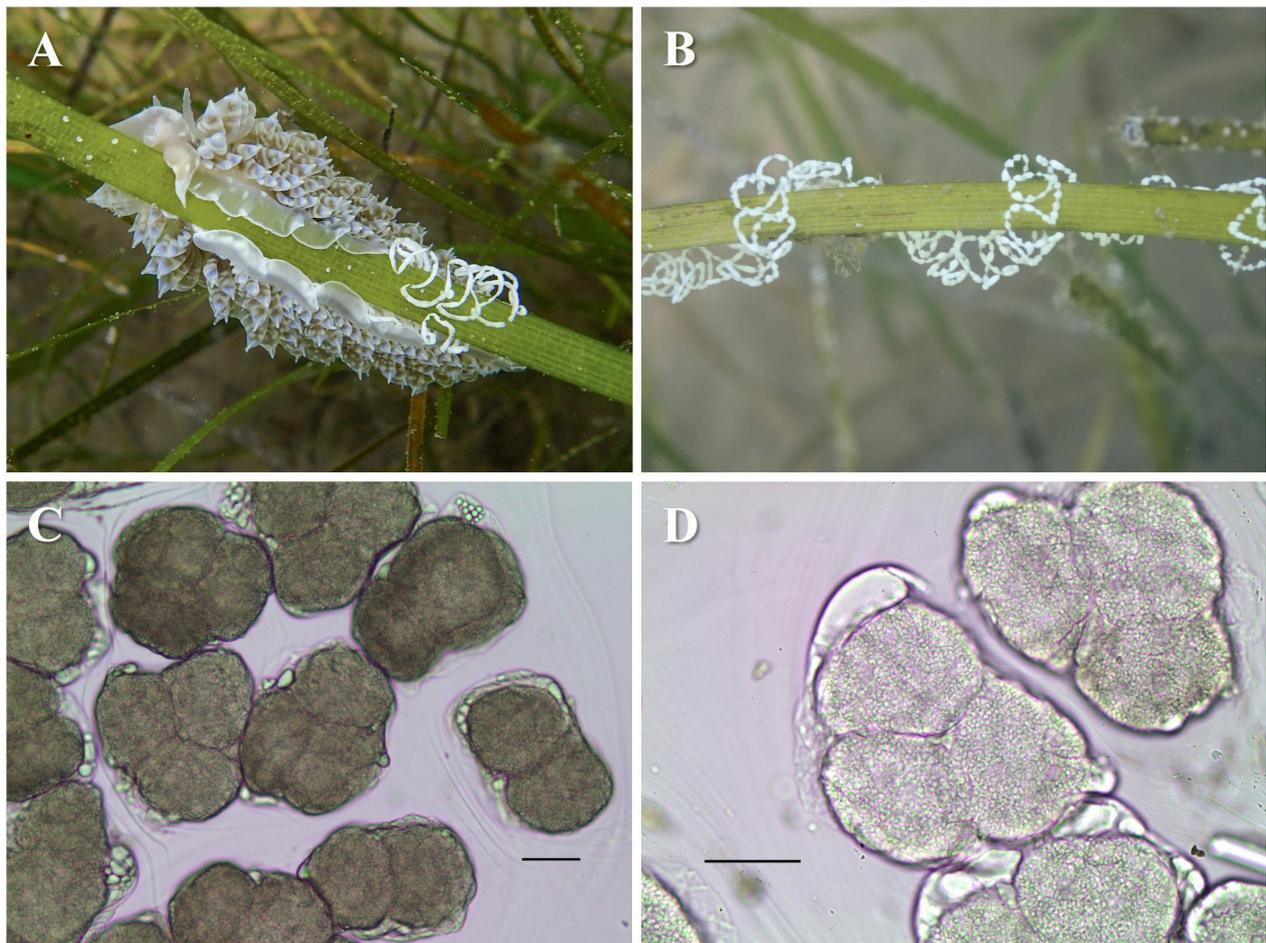
A site (39.206433N; 26.243028E) in the inner part of Kalloni Gulf, near the principal saltworks (Figure 1), was examined through a 60-minute snorkelling survey on soft substrates, on 30 September 2021. The depths surveyed ranged between 0–2 meters, including sandy bottoms, a dense meadow of the seagrass *Cymodocea nodosa* (Ucria) Ascherson, dead leaves of *C. nodosa*, and pebbles. Once a large aggregation of *B. moebii* was spotted, multiple individuals were thoroughly photographed and the species' behaviour observed *in situ*. Other species close to *B. moebii* were also recorded and photographed, together with possible food sources.

The same site was revisited on 7 October 2021, and *B. moebii* individuals were collected for molecular confirmation of the morphological identification. Six living individuals and an egg mass were collected from a depth range of 0.5–1.5 m. Each specimen was placed in a single 50 ml Falcon tube and subsequently fixed in 99.9% ethanol. All the specimens sampled were deposited at the Benthos Laboratory of Stazione Zoologica Anton Dohrn (SZN, Naples, Italy) with the codes SZN-B-2922ML198A–2927ML198F.

### Morphological and molecular identification

Morphological identification of *B. moebii* was based on detailed examination of the external taxonomic characters of the species including morphology, arrangement and colour of cerata, body morphology, and ornamentation and colour of rhinophores. Identification was based on Carmona et al. (2014b) and Paz-Sedano et al. (2019).

To confirm the morphological identification, total genomic DNA was extracted from the foot of three ethanol-fixed specimens using QIAGEN DNeasy® Blood & Tissue kits, following the manufacturer's protocol. Partial sequences of the mitochondrial cytochrome c oxidase I (COI) gene were amplified for each sample through Polymerase Chain Reaction (PCR), using the primer pair provided by Geller et al. (2013): jgLCO1490 (5'- TITCIACI AAYCAYAARGAYATTGG-3') and jgHCO2198 (5'- TAIACYTCIGGRTG ICCRAARAAYCA-3'). PCRs were run in 25 µl total volume, under the following conditions: initial denaturation at 95 °C (5 min), followed by 35



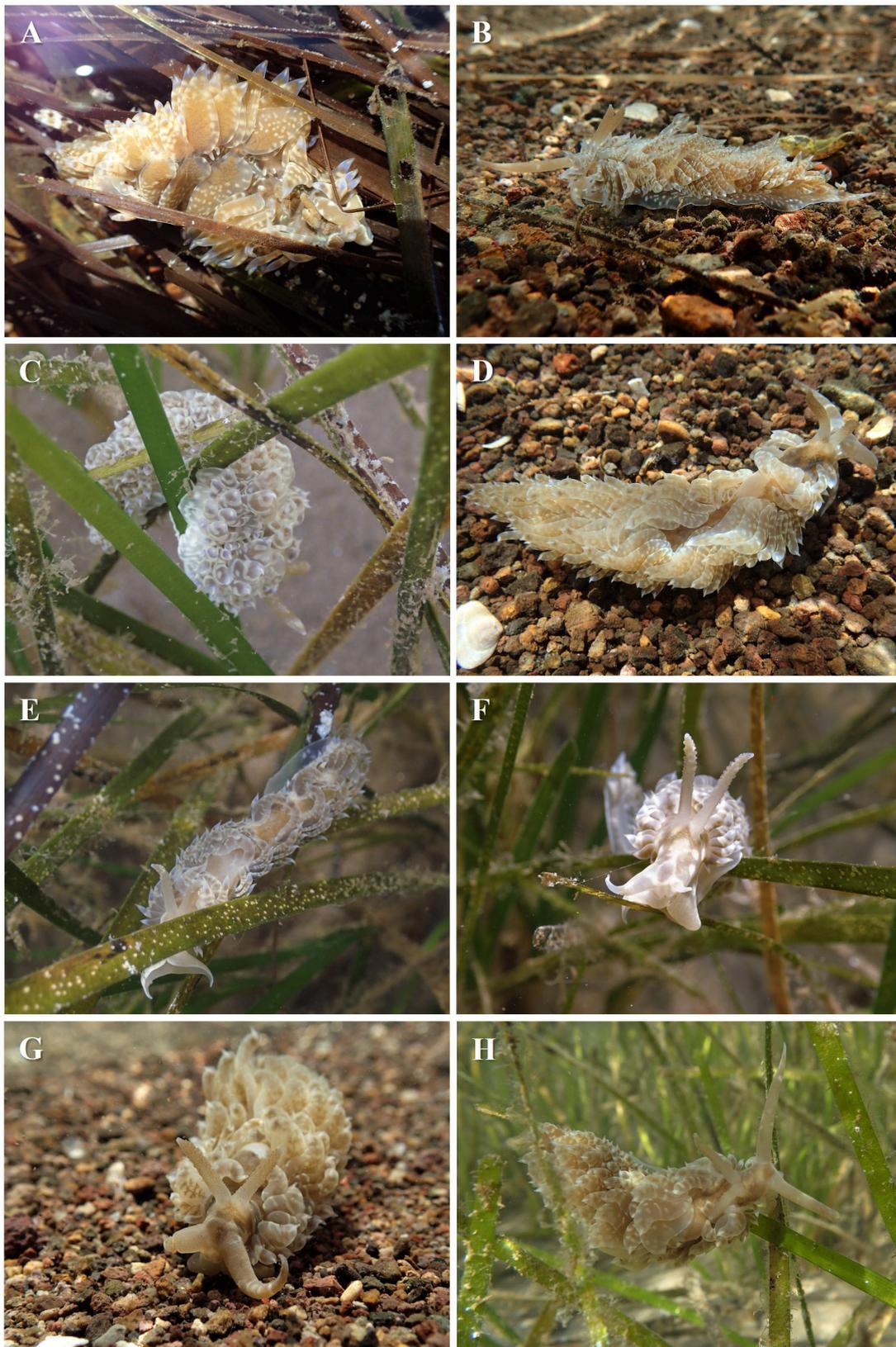
**Figure 2.** *Baeolidia moebii* and its egg masses. A. An individual laying eggs while attached on a leaf of *Cymodocea nodosa*. B. Egg mass of spiral appearance, entangled around a *C. nodosa* leaf. C, D. Microscopic views of eggs. Scale bars: 50 µm. Photographs: E. Kytinou.

cycles of denaturation at 95 °C (1 min), annealing at 49 °C (1 min), extension at 72 °C (1 min), and a final extension at 72 °C (5 min). Amplicons obtained were then purified and Sanger sequenced through an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems), using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Sequences obtained were quality checked, assembled, and edited using Unipro UGene v.39 (Okonechnikov et al. 2012). Basic Local Alignment Search Tool (BLAST: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>; Morgulis et al. 2008) was then used to assess the identity of each sequence against references deposited in NCBI GenBank® (<https://ncbi.nlm.nih.gov>).

## Results

### *Field observations*

During the first survey of the site (30 September 2021), more than 150 *B. moebii* individuals were counted at depths of 0–2 meters. Most of them were located within a *C. nodosa* meadow, attached to seagrass leaves (Figures 2A, 3C, E, F, H), whereas some others were observed outside the meadow towards the shoreline, crawling on dead *C. nodosa* leaves (Figure 3A) and on pebbles (Figure 3B, D, G). A few individuals were photographed



**Figure 3.** Morphological characters of *Baeolidia moebii* from Kalloni Gulf (Lesvos Island). A. Flattened cerata with brownish branches of digestive gland visible through the translucent body wall. Whitish patches on outer side of cerata. Cerata usually with purple subapical band. B. Elongate body. Extended foot with tentaculiform corners and white spots. Oral tentacles longer than rhinophores. C. Body contraction of *B. moebii* when disturbed, with swollen cerata. D. Body narrowing towards posterior end of foot. Body colour pattern with whitish patches. E. Cerata arranged in arches, decreasing in size towards foot. F. Usually, presence of ring on anteriormost edge of head, often encircled by distinct white band. G. Rhinophores studded with minute knobs. Damaged right oral tentacle. H. Body colour commonly pale brown with ochre patterns. Photographs: E. Kytinou.

while laying eggs (Figure 2A), and 18 egg masses were recorded in the area, always entangled around *C. nodosa* leaves (Figure 2B). The maximum total length of *B. moebii* recorded *in situ* was 70 mm. When disturbed (e.g., by a gentle current), *B. moebii* contracted its body, forming a more circular shape with swollen cerata (Figure 3C). Several of the individuals close to the shoreline had a distressed appearance with damaged external body parts and slow movements, and eggs were visible through the translucent foot. Three individuals had one damaged (Figure 3G) or missing oral tentacle and three were missing both oral tentacles. The spawning aggregation was limited to the area of the *C. nodosa* meadow and the adjacent shoreline. No individuals were recorded from the neighbouring sandy substrates. Interestingly, *B. moebii* was often recorded close to the anemones *Bunodeopsis strumosa* Andrès, 1881 and *Anemonia viridis* (Forsskål, 1775) complex. The ragged sea hare *Bursatella leachii* Blainville, 1817 (Mollusca: Gastropoda: Aplysiida) was also concomitantly abundant in the same site, with ~ 50 individuals and one egg mass recorded. This cryptogenic sea slug is established in Kalloni Gulf, with seasonally varying abundances.

During the second visit of the site (7 October 2021), the density of the aggregation was substantially reduced. During a 30-minute snorkelling survey, only 11 individuals were encountered. One individual was found near the shoreline, while the rest were found at 1 m depth on *C. nodosa* leaves. Shortly after collection, individuals of *B. moebii* autotomised some of their cerata.

### *External morphology*

Body large (up to 70 mm in living animals), elongate (Figure 3B), narrowing towards posterior end of foot (Figure 3D). Extended foot with tentaculiform corners (Figure 2A) and white spots (Figure 3B). Body colour variable, commonly pale brown with ochre patterns (Figure 3D, H) or greyish white (Figure 3E). Body colour pattern with scattered whitish patches (Figure 3A, B, D). Usually, presence of a darker ring (bluish, brownish or purplish) on the anteriormost edge of head, often encircled by a distinct white band (Figure 3F). Oral tentacles long, pale brown, usually with white spots (Figure 3B, H), sometimes also with white bands (Figure 3D). Tips of oral tentacles more often white (Figure 3B) and rarely purplish. Rhinophores shorter than oral tentacles (Figure 3B), pale brown, studded with minute knobs (Figure 3F, G); apex white (Figure 3B, F). Brown network pattern of ducts (presumably containing zooxanthellae) usually visible close to rhinophores (Figure 3D). Cerata flattened, almost leaf like, of variable length (Figure 3A, H). Branches of digestive gland brownish, sometimes visible through translucent body wall (Figure 3A, G). Whitish patches on outer side of cerata (Figure 3A). Cerata usually with purple subapical band (Figures 2A, 3A, C, E); apex translucent white and acute (Figure 3A). Cerata arranged in arches and rows, decreasing in size towards foot (Figure 3E). White egg masses of spiral

appearance (Figure 2B), covered by gelatinous membrane (Figure 2C). Eggs  $120 \pm 2 \mu\text{m}$  (SE) in diameter ( $n = 31$ ) (Figure 2C, D).

### *Molecular results*

The amplification of the COI fragments successfully yielded 650–660 base pairs sequences for all the specimens analysed. The three sequences obtained showed high similarity (99.42–100%) with the seven COI sequences of *B. moebii* deposited in GenBank to date [HQ616770–1: Carmona et al. (2011); JQ997060–1, JX087550: Carmona et al. (2013); MK922511: Paz-Sedano et al. (2019); MT884061: *unpublished*], whereas all the other taxa of the family Aeolidiidae (including other species of the genus *Baeolidia* Bergh, 1888) showed a similarity  $\leq 82\%$ , well over the barcoding gap commonly accepted in molluscs ( $\sim 3\%$ ) (Layton et al. 2014). All this confirms molecularly the identification of our specimens as *B. moebii*. Sequences obtained in this work were deposited in GenBank with the following accession numbers: OM658390–1, OM658488.

### **Discussion**

The NIS malacofauna of Greece has been widely investigated over the past few decades, revealing consecutive records of molluscs new to Greece (e.g., Crocetta et al. 2017, 2021; Zenetos et al. 2018, 2020; Ragkousis et al. 2020; Manousis et al. 2020; Kontadakis et al. 2021). The present study contributes to this knowledge with the first record of *B. moebii* in the Aegean Sea and in Greece, the identification of which was determined based on external morphology and confirmed by DNA barcoding. Moreover, the first detection of the species spawning aggregation in the Mediterranean Sea was recorded with the presence of abundant individuals, which provided concrete confirmation of its establishment success in the basin and allowed for *in situ* observations of the intraspecific morphological variation of the species.

Specifically, more than 150 *B. moebii* individuals coexisting in the same environmental conditions in Kalloni Gulf allowed for observations of colour variability, with specimens showing pale colours and others being darker. Presence of some diagnostic characters in all individuals (e.g., body colour pattern with patches, flattened cerata arranged in arches, and rows decreasing in size towards foot, white spots on foot) were confirmed and the occasional presence of additional characters (e.g., ring on anteriormost edge of head, white spots and bands on oral tentacles, visible branches of digestive gland) were highlighted. Most observations are largely in accordance with those reported from the species' native range (Gosliner 1979; Carmona et al. 2014b). Moreover, the three Mediterranean individuals found so far were reported at a depth range of 8–25 m, and their lengths were no more than 40 mm (Turk and Furlan 2011; Paz-Sedano et al. 2019). In the present study, we recorded individuals up to 70 mm, a maximum length which is

more similar to that reported in its native range (100 mm) (Gosliner 1979), in a spawning aggregation at 0–2 m depth, thus extending the bathymetric range that *B. moebii* occupies in the Mediterranean Sea. Observations of individuals while laying eggs allowed for identification of the external morphology of the egg masses (Figure 2A, B).

*Baeolidia moebii* is known to feed on different zooxanthellate metridioidean anemones in its native range, such as members of the genera *Aiptasia* Gosse, 1858 and *Bolocerooides* Carlgren, 1899 (McDonald and Nybakken 1997), without any feeding preference observed between them (Gosliner 1979). In the present study, *B. moebii* individuals were recorded close to metridioidean anemones belonging to *B. strumosa* and to actinioidean anemones of the *A. viridis* complex, both of which are known to host symbiotic zooxanthellae, i.e., dinoflagellates of the family Symbiodiniaceae (Visram et al. 2006; Richier et al. 2006). Interestingly, native Aeolidiidae, such as *Spurilla neapolitana* (Delle Chiaje, 1841), also feed on a variety of anemone species, including *B. strumosa* and *A. viridis* (Wägele and Johnsen 2001). Stable isotope analyses and/or field observations of the feeding preferences of *B. moebii* in its invaded range could investigate if the sea slugs do feed on the encountered zooxanthellate anemones *B. strumosa* and *A. viridis* complex, available around its spawning aggregation in Kalloni Gulf.

Until recently, *B. moebii* was only known on the basis of three scattered sightings of single individuals from the Levant Sea (Turk and Furlan 2011; Paz-Sedano et al. 2019), which led Zenetos and Galanidi (2020) to consider this species as established in the Mediterranean. Paz-Sedano et al. (2019) speculated on a potential establishment of the species, suggesting that lack of further records in the basin may be due to absence of focused surveys and sampling methods, but also discussed the possibility of the three records representing young individuals that did not manage to establish a viable population. Therefore, the present study, with the detection of multiple individuals in a spawning aggregation, definitively removes any doubts on the species' establishment in the Mediterranean Sea.

The discontinuous known distribution of *B. moebii* in the eastern Mediterranean, not being reported from Egypt, Israel, Lebanon, Syria, and the south and central Aegean Sea, can either be attributed to false absences of the species due to its cryptic behaviour (Paz-Sedano et al. 2019), its under-sampled habitat, or to a truly very patchy distribution because of secondary dispersal by currents or vessels, with establishment only in areas of particularly favourable conditions. The species seems to have found a suitable niche for an autumn spawning in the inner part of the productive, shallow, embayment of Kalloni Gulf. Other species of non-indigenous nudibranchs have been reported to gather in large numbers during autumn and spawn inside productive Mediterranean systems with high confinement from marine waters, such as lagoons (Keppel et al. 2012; Yokeş et al. 2018) and semi-enclosed embayments (Mandic et al. 2016). It will be interesting

to monitor how fast *B. moebii* will spread and colonise new areas, and if its spawning aggregations will show any seasonal pattern, habitat preference, and/or dependence on environmental parameters. Such spawning events, if properly monitored, can contribute to better understand the population dynamics of the species, which will be in turn useful in future impact assessments.

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## Authors’ contribution

EK conducted the first underwater survey and photography, morphological analyses, and wrote the original draft. MZ conducted the second underwater survey, prepared the map, and contributed to the draft. RV performed molecular analyses and contributed to relevant parts of the draft. FC confirmed the morphological identification of EK and contributed to relevant parts of the draft. SK conceived and coordinated the study and contributed to the draft. All authors have contributed to manuscript revision and have read and approved the submitted version.

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