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RESEARCH ARTICLE

First molecular barcoding and record of the Indo-Pacific punctuated flatworm *Maritigrella fuscopunctata* (Newman & Cannon 2000), (Polycladida: Eurylepidae) from the Mediterranean Sea

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Abstract

A first record of the punctuated flatworm *Maritigrella fuscopunctata* (Newman & Cannon 2000) from Maltese waters in the Mediterranean Sea during marine research surveys in summer 2015 is reported in detail. This platyhelminth species is alien to the Mediterranean, as it is native to the Indo-Pacific region. The abundance of this non-indigenous flatworm was observed to be highest in Exiles Bay followed by Spinola Bay (St. Julians) and St. George's Bay with a few others found elsewhere along the North eastern coast of Malta between the Valletta Harbour and St. George's Bay. A sample of these specimens observed were photographed and measured (n=40). A few (n=5) were collected to undertake a first molecular barcode for this euryleptid flatworm. Total body length of the specimens observed in the field varied from 20 to 60mm. Observations in the field indicate that a growing population has been established especially in the presence of the colonial ascidian *Ecteinascidia turbinata*, found among the various fouling organisms. Molecular genetics of euryleptid flatworm species is still scarce and was missing for this species. The present study also contributed toward filling this gap.

Keywords: *Maritigrella fuscopunctata*, alien species, DNA barcoding, Mediterranean, Malta

Introduction

Introduction and movements of alien marine species into the Mediterranean Sea requires long-term monitoring in order to better understand and where possible mitigate and manage for changes these new species may bring upon native communities and habitats (Otero *et al.* 2013). Plathyhelminths have not been featured in the expanding lists of alien species in this region which parallels the scarcity of research work on marine flatworms, especially in the Mediterranean with very few detailed works on this group of organisms (Lang 1884). These

small organisms may hide among boulders and algae as they are reported to be negative photoactive or camouflage through cryptic mimicry in their surrounding environment (Newman and Schupp 2002; Rawlinson 2008; Maghsoudlou and Rahimian 2014). However, their bright colours usually facilitate their sighting during careful visual marine research surveys, especially when the flatworms appear in relatively large numbers in shallow waters. Their very thin and fragile structure is compensated by their ability to regenerate themselves allowing them to survive injuries (Nentwig 1978).

Newman and Schupp (2002) clearly indicate that systematics of the species in the family Euryleptidae is considered problematic due to scarce taxonomic and biological studies on this group. There is also still much that is not known about polyclad larval diversity, development and evolution (Rawlinson 2014). The genus *Maritigrella* is described as being a new genus of euryleptide flatworms (Platyhelminthes, Polycladida) from the Indo-Pacific and reported to be composed of at least eight species (Newman and Cannon 2000). Among these the tiger flatworm, *Maritigrella crozieri* has been the most studied species and was found to associate and feed on the ascidian *Ecteinascidia turbinata*, from which it sequesters chemically active compounds isolated as antitumor compounds (Carte 1996; Newman *et al.* 2000). Due to this fact, *M. crozieri* can be considered to have potentially far reaching economic impact to the pharmaceutical industry. The new species *M. makranica* recently discovered in the Makran area, Gulf of Oman (Maghsoudlou and Rahimian 2014) is the latest addition to this genus and shows some resemblance to *M. fuscopunctata*. Due to the scarce genetic analyses of the species within this genus described so far, molecular genetics research is essential to understand its phylogenetics. For this reason, after a preliminary morphological assessment to identify the species spotted in Maltese waters, molecular genetics was also undertaken to DNA barcode the species, which was reported for the first time as a recognised new alien species for the Mediterranean Sea by Vella (2015a-d).

Materials and Methods

Between 1 July and 30 September 2015, during snorkelling research by the CBRG-UoM, specimens of the euryleptid flatworm *Maritigrella fuscopunctata* were observed. The total body length for 40 specimens encountered was measured in the field. The flatworms were sighted in various bays and inlets including a) Exiles and b) Spinola Bays (both within the St. Julians Bay – location 1 in Figure 1), Dragonara Bay (location 2), St. George's Bay (location 3), Qui si sana Bay (location 4) and Manoel Island (location 5) with respective GPS positions for each of these sighting locations as follows: 1a: at 35.918676N - 14.493055E & 1b: at 35.916574N - 14.497617E; 2: at 35.924827N - 14.494297E; 3: at 35.926671N - 14.488372E; 4: at 35.912364N - 14.507628E; 5: at 35.904433N – 14.507385E). Five small specimens were collected, photographed on a 2X2mm grid scale (refer to Figures 2 to 7) and stored in 96%

ethanol in order to undertake molecular barcode analyses for this species. The diagnostic features for the morphological identification of the specimens were according to Newman and Cannon (2000, 2003 and 2005).

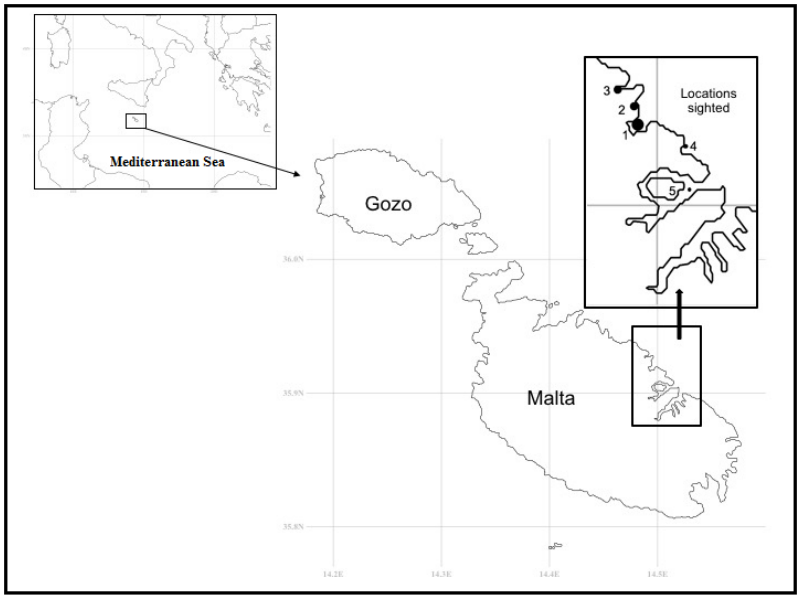

Figure 1. A map of Malta showing the locations and abundance of *Maritigrella fuscopunctata* encountered during ongoing surveys around the Maltese coast. (Locations in order of abundance: 1- a. Exiles and b. Spinola Bays (St. Julians); 2 - Dragonara Bay; 3 - St. George's Bay; 4 - Qui si sana; 5 - Manoel Island).

Genetic analyses:

From each specimen 10mg of tissue samples were digested with Proteinase K and the total genomic DNA was extracted using AccuPrep® Genomic DNA Extraction Kit (Bioneer Inc.). PCR amplifications were carried out for: the partial sequence of the 18S rRNA gene using 18SF2 (Morgan *et al*., 2003) and 1800_mod (Raupach *et al*., 2009); and the partial sequence of the 28S rRNA gene using LSU_D1,D2_fw1 and LSU_D1,D2_rev1, following the amplification protocol by Sonnenberg *et al.* (2007). PCR products were sequenced in both directions using Applied Biosystems 3730xl. The sequences were aligned using Geneious v6.1.6 (http://www.geneious.com, Kearse *et al.* 2012). A 929bp sequence and 992bp sequence representing the smallest homologous 18S rRNA and 28S rRNA sequences were selected for analyses.

Results

Specimens examined and photographed in the laboratory (refer to Figures 2a to 2g) as well as specimens photographed in the field (refer to Figures 2h & 2i),

showed to have an oval body with a cream-white background with large black spots in transverse rows and at times black spots surrounded by purple-violet colour. The body margin also has small black and orange spots which are in a honey comb pattern in the median dorsal region. The ventral surface has the same pattern without orange spots. On the dorsal anterior head part of the body there are two elongated tentacles and two merging patches of cerebral eyes as shown in Figure 2g.

The total body length of the oval shaped flatworms was measured for 40 specimens encountered during research surveys. The body length measurements were found to range from 20 to 60mm in quiet position (mean 40mm; SD 12mm), the maximum length was surpassed by 5 to 10mm when the flatworm extended its body and moved faster increasing the undulating motion of its margins. The flatworms were able to travel upside down by adhering to the water surface while being observed in the laboratory tank. Though *M. fuscopunctata* was sighted in various bays and inlets, Exiles, Spinola and St. Georges Bays were the locations with greater densities which could reach 5 individuals/m^2. Depths at which the specimens were found varied between 5m and 50cm. The presence of these flatworms in large numbers in shallow waters and moving about in broad daylight was found to be rather unique, considering that flatworms of this genus are reported to be negatively photoactive (Newman and Schupp 2002; Rawlinson 2008; Maghsoudlou and Rahimian 2014). In Maltese waters, *M. fuscopunctata* was occasionally found on shallow granular seabed. However, from observations of the habitats specimens were found in, this species appeared to prefer sheltered rocky habitats with boulders and surfaces covered with fouling organisms, including the colonial ascidian *Ecteinascidia turbinata* (mangrove tunicate).

Genetic analyses

The 929bp sequence from the 18S rRNA gene and a 992bp sequence from the 28S rRNA gene were analysed. They were run via BLASTn to identify sequence matches. The 28S rRNA gene confirmed the genus identity with the closest match, among the barcodes available for the genus *Maritigrella*, being *Maritigrella crozieri* [GB: HQ659013] (Rawlinson *et al.* 2011) and *Maritigrella newmanae* [GB: EF514798] (Bolanos *et al.* 2007). Due to the scarcity of data on the different species of this genus, including lack of barcodes, the comparative genetic analyses is limited. The five genetically barcoded specimens had the same genetic sequence for the 18S rRNA, however they exhibited genetic differences on the 28S rRNA sequence leading to the identification of three genotypes. The sequences were deposited in GenBank with accession numbers KU760856 (18S rRNA) and KU674837-9 (28S rRNA).

The 28S rRNA genotypes differed from each other at two base substitutions at position 416 (A/G) and position 832 (T/C) (Table 1). The first two genotypes

exhibited homozygosity, while the third genotype exhibited heterozygozity at both nucleotide positions respectively. As the species is hermaphrodite, thus reproducing sexually, and specimens have been found reproducing on ascidian colonies, this may explain the genetic variation found among these specimens.

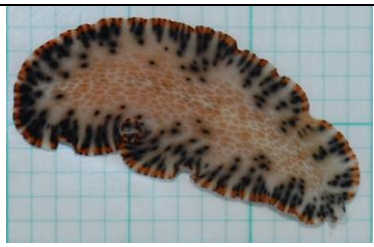

Figure 2a. *M. fuscopunctata* Specimen 1

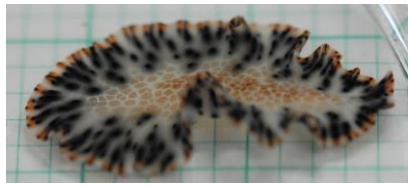

Figure 2b. *M. fuscopunctata* Specimen 2

Figure 2c. *M. fuscopunctata* Specimen 3

Figure 2d. *M. fuscopunctata* Specimen 4

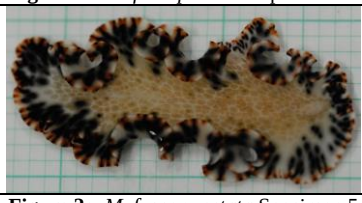

Figure 2e. *M. fuscopunctata* Specimen 5

Figure 2f. *M. fuscopunctata* elongating while swimming

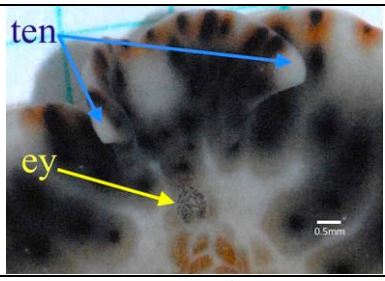

Figure 2g: Dorsal view of the anterior part, with elongated tentacles (ten) and two merging patches of cerebral eyes (ey).

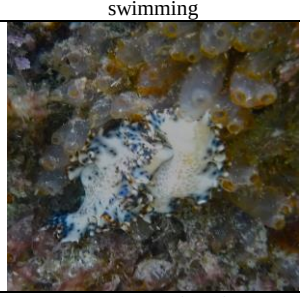

Figure 2h. Specimens of *M. fuscopunctata* closely associated the colonial ascidian *Ecteinascidia turbinata* at Exiles, Malta

Figures 2a-e show the 5 specimens collected for barcoding for this species (all images show the anterior head region on the right hand side and a 2X2mm grid scale). (Barcode sequences for these specimens were deposited in GenBank with accession numbers KU760856 and KU674837-9).

Figure 2i. *M. fuscopunctata* specimen closely associated with the colonial ascidian *Ecteinascidia turbinata* at Exiles Bay, Malta

Table 1. The nucleotide differences between the three genotypes identified in the sampled specimens of *Maritigrella fuscopunctata* in Malta

	416	**832**	**Sample no**
Genotype 1	A/A	T/T	1
Genotype 2	G/G	C/C	2
Genotype 3	A/G	T/C	2

Discussion

This paper documents the first analyses of *M. fuscopunctata* size distributions, ecology and species barcode for the specimens from Maltese waters (Mediterranean Sea). Its body size distribution shows that the species is fairing well and individuals are growing to its maximum reported sizes (40 to 70mm) according to Newman and Cannon (2003). From field observations this species appeared to prefer sheltered rocky habitats with boulders and surfaces covered with fouling organisms, including the colonial ascidian *Ecteinascidia turbinata* where the larger densities were observed. The latter is similarly sought after by the related flatworm species, *M. crozierae* which may find this ascidian species in its tropical mangrove habitats throughout the Caribbean and Bermuda and from South Carolina to Florida. As the colonial ascidian, *E. turbinata* can also be found attached to docks, floating debris, rocks and seagrass blades (Ruppert and Fox 1988; Young and Bingham 1987) it may foul ships' hulls therefore introduce themselves in various part of the globe as well as carry organisms which associate with them, such as flatworms. Flatworm larvae may be transported in ballast water of ships (Carlton and Geller 1993) or naturally spread into the Mediterranean as part of Lessepsian migration of adults or larvae that find suitable conditions for their nourishment, growth to adulthood and reproduction.

The discovery of *Maritigrella fuscopunctata* in Malta was briefly reported for the first time as a recognised new alien species for the Mediterranean Sea by Vella (2015a-d), although it was not referred to by Portelli *et al.* (2015). The current paper represents a detailed presentation of the first scientific study and reporting of this alien species in the Mediterranean Sea, including the first DNA barcode study of *M. fuscopunctata*. The genetic identification of this species is also useful for phylogenetic analysis of the genus *Maritigrella* in the future.

Acknowledgements

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