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A molecular approach to the reconstruction of the pre-Lessepsian fauna of the Isthmus of Suez: The case of the interstitial flatworm *Monocelis lineata* sensu lato (Platyhelminthes: Proseriata)

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ABSTRACT

Since the opening of the Suez Canal in 1869 the Mediterranean Sea has been affected by a gradually increasing influx of species of Erythraean origin, which significantly modified species composition and ecological interactions of entire communities. Most studies have focused on macrofaunal species. Nevertheless, given the sandy nature of its shores, the Suez Canal is an ideal habitat for many interstitial taxa, whose study has been so far neglected. During a number of sampling campaigns, aimed to appreciate interstitial flatworms fauna in the Suez Canal, specimens of the *Monocelis lineata* (Platyhelminthes: Proseriata) species complex were collected in Lake Timsah.

Here we performed molecular analyses by using the 18S and 28S nuclear rDNA genes. Species delimitation methods agree in considering this population as a distinct entity (*Monocelis* sp. nov.). Molecular phylogeny places *Monocelis* sp. nov. as the sister taxon of the Mediterranean populations of the *Monocelis lineata* species complex. Molecular dating results based on a minimum age calibration point, date the common ancestor of *Monocelis* sp. nov. and Mediterranean populations around the Bramertonian Interglacial optimum (1.8–2.3 myr ago). In that stage, due to high eustatic sea levels, the geographic limit in the Isthmus of Suez shifted southward, with a new biogeographic barrier placed about the southern edge of the Gulf of Suez. It is thus conceivable that the ancestor of present-day *Monocelis* sp. nov. became isolated from its Mediterranean counterparts in the following regression, during the Pre-Pastonian glaciation. Data presented here suggest that populations inhabiting the Isthmus should be carefully screened as they may reveal a long history of persistence in the area and help to reconstruct past evolutionary histories of Mediterranean and Red Sea species. Considering the recent project aimed to widen the Suez Canal, which may significantly modify present ecological conditions, gathering of genetic data from populations of the water bodies of the Isthmus becomes an urgent task, before they are lost forever.

1. Introduction

The opening of the Suez Canal in 1869 connected the Mediterranean and Red Sea, which had been separated since at least the early Pliocene (Por, 1989), and had one of the most devastating impacts on marine communities in historical times. Since then, in fact, the biodiversity of the Mediterranean was affected by a progressively increasing influx of species of Erythraean origin. As a consequence, species composition and ecological interactions of entire communities, particularly in the eastern Mediterranean, were significantly modified (see Por, 1978; Golani, 2000; Wirtz and Debelius, 2003; Azzurro et al., 2013; Sanna et al., 2015 and references therein). The route of the Suez Canal was designed to take advantage of the water bodies already present in the Isthmus of Suez, i.e., Great Bitter Lake, Little Bitter Lake, Lake Timsah and Bardawil lagoon. Before the opening of the Suez Canal they presented different ecological conditions, due to their complex history of connections with the Mediterranean and the Red Sea (Por, 1989). In particular, the long period of isolation of the Great and Little Bitter Lakes resulted in their extremely high salinity values. On the contrary, Lake Timsah, connected to a branch of the Nile, experienced widely fluctuating salinity values in correspondence to the flooding of the Nile (Por, 1978). As extreme as the ecological conditions were, there were anecdotal reports in both water bodies of brackish-water macrofaunal species, such as fish (*Mugil* sp.) and bivalves (*Cerastoderma* sp.), which even sustained some local fishing activity (Por, 1978). After the opening of the Suez Canal, a progressive uniformation and mitigation of

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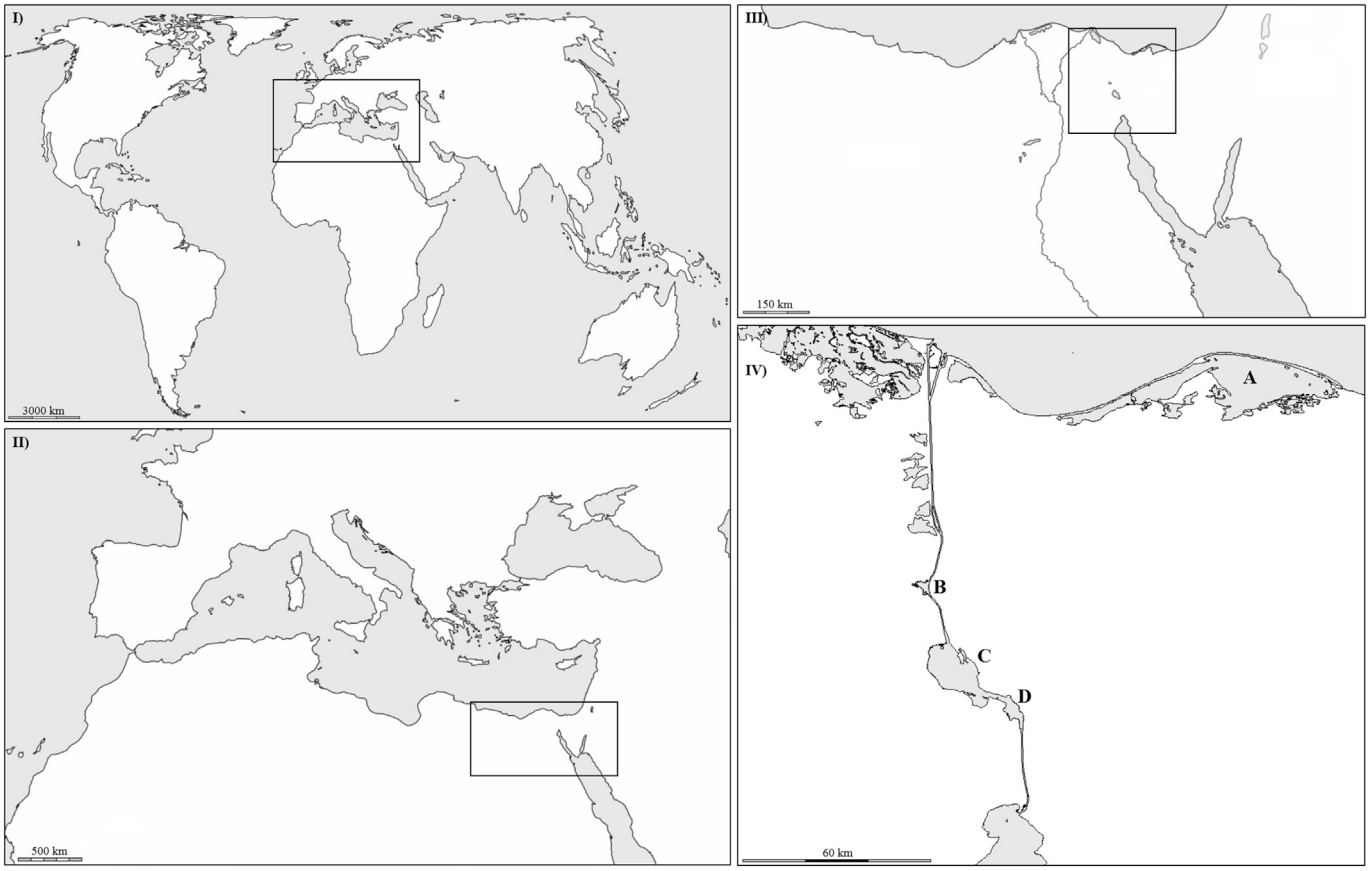

Fig. 1. Map highlighting the position in the world (I) of the Mediterranean Sea (II), the Isthmus of Suez and (III) its water bodies (IV). A: Bardawil lagoon; B: Lake Timsah; C: Great Bitter Lake; D: Little Bitter Lake. Maps upgraded 2015 available at http://www.soest.hawaii.edu/pwessel/gshhg/.
Figure drawn by means of the software GIMP 2.8.18 - available at https://www.gimp.org/downloads/.

the ecological conditions and the connection with the previously separated Mediterranean and Red Sea populations (via vagile adults or larvae) may have affected genetic differentiation, if any, of that isthmian fauna.

This may not be the case for meiofaunal organisms, whose extremely reduced size may have allowed long-term survival in suitable microhabitats and whose quick generation time and lack of dispersal stages may have shaped and preserved distinctive genetic footprints. During a series of sampling campaigns, aimed to study interstitial flatworms in the Suez Canal, a large number of specimens of the *Monocelis lineata* (Platyhelminthes: Proseriata) species complex was collected in Lake Timsah (see Fig. 1). This finding is of particular interest, as it may help to improve the knowledge of the native isthmian fauna, and provides a unique opportunity to better understand the evolutionary pathways of the *M. lineata* species complex. Thus, we sought to achieve a molecular survey of the specimens of *M. lineata* found in Lake Timsah, in order to assess their taxonomic and phylogenetic position. We used the 18S and 28S nuclear rDNA genes, which are the most widely used molecular markers for Proseriata (see e.g., Casu et al., 2009, 2011a, 2014; Scarpa et al., 2017). On these molecular markers the molecular clock was also calibrated and tested (see Scarpa et al., 2015, 2016a), which enabled us to date the separation of Lake Timsah and Mediterranean populations.

2. Materials and methods

2.1. Sampling, DNA extraction, PCR and sequencing

Samples were collected manually by scooping up the superficial layer of sediment. No specific permits were required for the sampling site, and animals in this study are not protected nor endangered. Given the size of the organisms, the collected samples were small (< about 2 l of sediments). Extraction of the animals from the sediment was accomplished by using $MgCl_2$ decantation (see Casu et al., 2012). Each specimen was studied alive by slight squeezing under the cover slip. Specimens collected for molecular analyses were fixed in ethanol 97%. Genomic DNA was extracted using the Macherey-Nagel NucleoSpin Tissue (MACHEREY-NAGEL GmbH & Co. KG), following the supplier's instructions. After extraction, DNA was stored as a solution at 4 °C. Complete 18S and partial 28S (spanning variable domains D1–D6) were analyzed for a total of 53 individuals (for details about specimens and sampling localities see Table 1). The molecular dataset included six specimens belonging to the newly discovered population from Lake Timsah and 45 specimens belonging to the genus *Monocelis*; one specimen of *Archimonocelis staresoi* was included as outgroup (see e.g., Curini-Galletti et al., 2010). PCRs for 18S and 28S regions were performed using the following primers: 18S: A (forward) GCG AAT GGC TCA TTA AAT CAG, and B (reverse) CTT GTT ACG ACT TTT ACT TCC (Littlewood and Olson, 2001); 28S: for (forward) GCG GAG GAA ARG AAA CTA ACA AGG A, and rev (reverse) AAC TCT TCC GGG AAC CAT CGC CGA C (Scarpa et al., 2016b). PCRs were carried out in a total volume of 25 µl containing 1 ng/µl (quantified using NanoDrop™ Lite by Thermo Scientific) of total genomic DNA on average, 1.0 U of Taq DNA Polymerase (Eurotaq, Euroclone), 1 × reaction buffer, 3.5 mM of $MgCl_2$, 0.32 µM of each primer, and 200 µM of each dNTP. PCR amplifications were performed in a MJ PTC 200 Thermal Cycler (Biorad), programmed as follows: 1 cycle of 2 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 54 °C (18S/28S primers' annealing temperature), and 1 min and 30 s at 72 °C. At the end, a post-treatment of 5 min at 72 °C and a final cooling at 4 °C were carried out. Both positive and negative